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TESI DI LAUREA

INNOVATIVE MANAGEMENT OF END-STAGE HEART FAILURE IN INFANTS: UNDERSTANDING THE MECHANISM OF MYOCARDIAL REHABILITATION AFTER PULMONARY ARTERY BANDING IN AN EXPERIMENTAL RODENT MODEL OF INDUCED DILATED CARDIOMYOPATHY

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1. RIASSUNTO

Presupposti dello studio: Lo scompenso cardiaco pediatrico rappresenta una delle cause più importanti di morbidità e mortalità nel periodo infantile, causato, in primo luogo, dalle cardiopatie congenite, seguite dalle cardiomiopatie, in particolare quella dilatativa. La gestione è impegnativa, in quanto i pazienti spesso necessitano di un supporto meccanico cardiocircolatorio, con lunghi periodi di ospedalizzazione e aumentato rischio di infezioni e trombosi, in attesa del trattamento definitivo che, laddove possibile, è rappresentato dal trapianto cardiaco. Tuttavia, la sopravvivenza a lungo termine limitata, la scarsa disponibilità di donatori, i costi e il trattamento immunosoppressivo (con rischio di infezioni e neoplasie) rendono quest'opzione subottimale per un lattante o bambino nella prima infanzia. Sulla base dell'ipotesi che l'interazione ventricolo-ventricolare possa migliorare la condizione del ventricolo sinistro dilatato, seguendo il protocollo di Giessen ideato da Schranz et al., dal 2015 si è usata nel nostro centro la strategia del bendaggio dell'arteria polmonare (PAB) per trattare lo scompenso cardiaco terminale in pazienti con funzione ventricolare destra conservata, come "bridge" al trapianto o al recupero funzionale.

Scopo dello studio: tale studio si articola in due parti: 1. clinica; 2. sperimentale. 1. Ci si propone di valutare gli attuali risultati di questa strategia innovativa in uno studio che ha coinvolto 4 centri europei (Padova, Italia; Varsavia, Polonia; Ghent e Leuven, Belgio) e 1 centro asiatico (Manila, Filippine).

2. Lo scopo è quello di indagare l'effetto del PAB su un modello animale (Ratto Sprague-Dawley), attraverso la valutazione funzionale ecocardiografica, e l'analisi istologica e molecolare dei campioni tissutali, per comprendere i meccanismi molecolari di riabilitazione ventricolare e verificare se vi sia o meno rigenerazione miocardica indotta dal PAB, con proliferazione dei cardiomiociti.

Materiali e metodi: La parte clinica (1) consiste in uno studio multicentrico retrospettivo internazionale che ha reclutato pazienti in età pediatrica, con scompenso cardiaco terminale esitante in una cardiomiopatia dilatativa, non rispondente alla terapia medica convenzionale. I criteri di esclusione sono stati la presenza di scompenso biventricolare, età > 5 anni, mancanza del consenso genitoriale, concomitante cardiopatia congenita (eccetto per l'ALCAPA corretta), insufficienza tricuspidale moderata-severa, ipertensione polmonare sproporzionata con cardiomiopatia in stadio terminale. I dati clinici, ecocardiografici e di risonanza magnetica sono stati raccolti in un database comune (REDCap). Si sono valutate le complicanze precoci e tardive al Follow-up clinico. Si è definito l'indice del successo terapeutico del PAB (PAB-Efficacy-Index) come la libertà globale da morte/VAD/trapianto.

Per la parte sperimentale (2), per la prima volta in letteratura, si è valutato, su 46 ratti Sprague-Dawley "giovani" e "adulti", l'effetto del PAB in due modelli di cardiomiopatia dilatativa: il primo indotto dalla somministrazione intraperitoneale della doxorubicina (DOX, chemioterapico cardiotossico), il secondo dall'ischemia provocata dalla legatura temporanea chirurgica dell'arteria discendente anteriore sinistra (LAD), attraverso valutazioni ecocardiografiche, istologiche e genetiche. In parallelo, in collaborazione con l'ICGEB di Trieste, su 6 ratti Sprague-Dawley giovani e sani, si è valutata la proliferazione dei cardiomiociti indotta dal PAB attraverso l'iniezione di sistemi reporter fluorescenti basati su vettori virali adeno-associati (AAV) e un analogo nucleosidico (EdU, 5-ethynyl-29-deoxyuridine), incorporato in molecole di DNA neosintetizzate.

Risultati: Parte 1. 31 pazienti (18 maschi) sono andati incontro a PAB ad un'età mediana di 210 giorni (IQR: 131-357), con frazione di eiezione preopoperatoria <30% in 21 pazienti (68%), ≥30 in 5 (16%) e non specificata in 5 (16%). Tre pazienti hanno subito interventi associati: chiusura dotto arterioso pervio, correzione ALCAPA, ed ECMO+atriosettostomia. Dopo la procedura, complicanze si sono verificate in 14 pazienti (47%), tra cui sindrome da bassa portata in 5, infezioni in 4 e AKI in 2. I reinterventi precoci hanno compreso: impianto di ECMO in 1 paziente e di VAD in 3, chiusura ritardata del torace in 7

e restringimento chirurgico del PAB in 4. Le morti precoci sono state 4 (13%): 3 per insufficienza cardiaca congestizia, 1 per sepsi e MOF dopo VAD. Ad un follow up mediano di 2,9 anni (IQR: 1,20-4,85) si è avuto un decesso a distanza, e 3 pazienti sono andati incontro al trapianto cardiaco, preceduto da VAD in due casi, rescue ECMO+VAD in un caso. La dilatazione del bendaggio è stata necessaria in 14 (60%) dei 23 sopravvisuti col cuore nativo. La terapia pre e post PAB include: ACE-inibitori o ARBs, beta-bloccanti, diuretici, nonché l'uso di cicli di Levosimendan iv. All'ultimo follow-up ecocardiografico (mediana di 2.7 anni, IQR: 1.25-4.6), l'FE è aumentata fino al valore mediano di 60% (IQR: 53.75-63.25) da un valore mediano di 25% (IQR: 17.5-27.75) all'ammissione ospedaliera ($p_{holm\text{-}adj.}= 2.69e^{-09}$). Lo Z-score del diametro telediastolico del ventricolo sinistro si è ridotto da un valore mediano al momento del ricovero ospedaliero di 9.72 (IQR: 6.76-12.48) a uno all'ultimo follow-up di 2.52 (IQR: 0.07-3.07) ($p_{holm\text{-}adj.}= 1.91e^{-05}$). Il PAB Efficacy INDEX si attesta al 74.2%, mentre la sopravvivenza globale (inclusi i trapiantati) all'84%. Il genere femminile ha mostrato una maggiore libertà da morte/VAD/trapianto cardiaco rispetto al maschile (p=0-045). Infine, il rischio di andare incontro precocemente a VAD è significativamente inferiore nei pazienti con età < 12 mesi rispetto a quelli con età > 12 mesi (p=0.012).

Parte 2. I risultati preliminari della nostra sperimentazione animale dimostrano che il modello roditore di cardiomiopatia dilatativa tramite iniezione di DOX è solo parzialmente adeguato. Il danno funzionale di contrattilità è stato ben evidenziato tramite ecocardiografia bidimensionale, mentre non si sono avute chiare evidenze tramite istologia o analisi molecolari. Tuttavia, si è notato che nei ratti sottoposti a PAB dopo danno indotto da DOX vi è stato un certo grado di miglioramento contrattile. Non si è stabilito, invece, un modello adeguato tramite legatura temporanea della LAD. I risultati preliminari dello studio attraverso l'iniezione di sistemi reporter fluorescenti confermano la proliferazione dei cardiomiociti indotta dal PAB nel ventricolo sinistro, che potrebbe essere un'evidenza che la procedura, tramite interazione meccanica ventricolo-ventricolare, stimola la rigenerazione miocardica del ventricolo sinistro, che può contribuire al recupero funzionale miocardico dopo la procedura.

Conclusioni: il PAB, associato ad una terapia medica antiscompenso aggressiva, è risultato essere una procedura con alta efficacia nel trattamento dello scompenso cardiaco terminale in lattanti e bambini selezionati, con funzione ventricolare destra conservata, funzionando da bridge al recupero funzionale o al trapianto.

I modelli sperimentali di cardiomiopatia dilatativa nel ratto sono difficili da ottenere, ma l'effetto positivo del PAB può essere confermato, almeno ecocardiograficamente, nel modello indotto da DOX. Il PAB, inoltre, sembra stimolare effettivamente la proliferazione dei cardiomiociti a livello del ventricolo sinistro. Ulteriori studi e nuove ricerche orientate sui meccanismi di trasduzione molecolare di segnale potranno confermare questi risultati preliminari incoraggianti.

2. ABSTRACT

Background: Pediatric Heart Failure (HF) represents an important cause of morbidity and mortality in childhood, caused first by congenital heart diseases (CHD) and secondly by cardiomyopathies, especially Dilated Cardiomyopathy (DCM). Its management is challenging; patients usually undergo mechanical circulatory support (MCS), with long hospitalization time and high risk of thrombosis and infection, waiting for the ultimate therapy represented by heart transplant (HTx). However, limited long-term survival, low donor availability, costs and life-long immunosuppressive treatment (with the risk of infections and malignancy) makes HTx a sub-optimal option for an infant or patient in early childhood. Based on the hypothesis that ventricular-ventricular interaction can benefit dilated left ventricle failure, following the Giessen protocol conceived by Schranz et al., since 2015 the Pulmonary Artery Banding (PAB) strategy has been used at our Institution to treat end-stage heart failure (ESHF) in patients with preserved right ventricle function, as a bridge to transplant or functional recovery.

Aim of the study: this study combines both clinical (1) and experimental (2) research.

1. The former aims to assess the current results of this innovative strategy across Europe (Padua, IT; Warsaw, PO; Ghent and Leuven, BE) and Asia (Manila, PH).
2. The latter aims to investigate the PAB's effect on a rodent model (Sprague-Dawley) of DCM through functional echocardiographic examination and histological and molecular analysis on heart tissue samples, in order to understand the molecular mechanisms behind the ventricular rehabilitation and to prove the potential PAB-induced myocardial regeneration due to cardiomyocyte proliferation.

Materials and methods: The clinical part (1) consists of a multicentric retrospective international study enrolling infants and children admitted for ESHF

and consequent DCM, not responding to conventional medical management. Exclusion criteria were the presence of biventricular failure, the age over 5 years, the lack of parental consent, concomitant structural (congenital) heart disease (except for repaired ALCAPA), moderate-severe tricuspid regurgitation, pulmonary hypertension out of proportion with left-ventricular end-stage cardiomyopathy. Clinical, echocardiographic and CMRI data were collected together on a common database (REDcap). Early and late complications were assessed during clinical Follow-up. The PAB-Efficacy-Index was defined as the overall freedom from death/VAD/HTx.

As for the experimental procedure (2), for the first time in literature, we evaluated, on 46 Sprague-Dawely "young" and "adult" rats, the PAB's effect in two models of DCM: one induced by doxorubicin (DOX, cardiotoxic chemotherapy drug) intraperitoneal administration, the other induced by temporary surgical LAD-ligation's ischemic effect, through echocardiographic, histological and genetic examinations. In parallel, in collaboration with the ICGEB of Trieste, we assessed the PAB-induced cardiomyocyte proliferation on 6 healthy juvenile Sprague-Dawley rats, through the injection of Adeno-Associated-Virus (AAV) vector fluorescent-based reporter systems and a nucleoside analogue, later incorporated into newly synthesized DNA (EdU, 5-ethynyl-29-deoxyuridine).

Results: Part 1. 31 patients (18 males) underwent PAB at a median age of 210d (IQR: 131-357), with pre-operative ejection fraction (EF) <30% in 21 pts (68%), ≥30 in 5 (16%) and not specified in 5 (16%). 3 patients underwent surgical associated surgeries: PDA closure, ALCAPA repair and ECMO+atrial septostomy. After the procedure, complications occurred in 14 pts (47%), including low cardiac output syndrome in 5, infections in 4 e AKI in 2. As for the early reinterventions, 1 pts underwent ECMO and 3 pts VAD application; delayed chest closure occurred in 7 pts and surgical PAB tightening in 4. Early deaths were 4 (13%): 3 for congestive heart failure (CHF), 1 for sepsis and MOF after VAD. At a median follow-up of 2,9 years (IQR: 1,20-4,85) one late death and 3 effective HTxs (two preceded by VAD, one by rescue ECMO+VAD) occurred. PAB dilation was performed in 14 (60%) out of 23 patients who survived with

their native heart. Medical management prior and after PAB consisted of ACE-inhibitors or ARBs, beta-blockers and diuretics, as well as Levosimendan infusion cycles. At the last follow-up echo (median time 2,7 years, IQR: 1,25-4,6), EF improved unitl the median value of 60% (IQR: 53.75-63.25) from 25% (IQR: 17.5-27.75) on admission ($p_{holm\text{-}adj.}= 2.69e^{-09}$). Left ventricular end-diastolic diameter's Z-score reduced from a median value of 9.72 (IQR: 6.55-12.63) on admission to 2.52 (IQR: 0.07-3.07) ($p_{holm\text{-}adj.}= 1.91e^{-05}$).

The PAB-Efficacy-Index was 74.2%, while the overall survival (HTx included) was 84%. Females had greater overall survival from death/VAD/HTx than males (p=0.045). Patients under 12 months of age faced less risk of undergoing VAD application than patients over 12 months of age (p=0.012).

Part 2. The preliminary results of our animal research show that the DOX-induced rat DCM model is only partially adequate. The impaired contractile function was confirmed through two-dimensional echocardiography, whereas no clear evidence was detected histologically or by molecular tests. However, contractile function of rats undergoing PAB after DOX administration improved. Instead, temporary LAD-ligation was not sufficient to estabilish a model of rat DCM. The preliminary results of the study involving fluorescent reporter systems confirm the PAB-induced cardiomyocyte proliferation in the left ventricle, which might be a proof that the procedure stimulates myocardial regeneration of the left ventricle thanks to mechanical ventricular-ventricular interaction, contributing to functional myocardial recovery.

Conclusions: PAB, together with aggressive anti-congestive medical management, turned out to be an effective procedure in the treatment of ESHF in infants and children with preserved RV function, serving as a bridge to functional recovery or transplant.

Establishing experimental rat DCM models is challenging. However, the positive effect of PAB can be confirmed, at least through echocardiographic assessment, in the DOX-induced model. PAB-induced cardiomyocyte proliferation in the left ventricle seems corroborated. These preliminary, but promising results may

subsequently be confirmed by more research and investigations regarding the involved molecular transduction mechanisms.

3. INTRODUCTION

3.1. PEDIATRIC HEART FAILURE

Pediatric Heart Failure (HF) is a progressive clinical and pathophysiological syndrome caused by cardiovascular and non-cardiovascular abnormalities that results in characteristic signs and symptoms including oedema, respiratory distress, failure to thrive, exercise intolerance, associated with circulatory, neurohormonal, and molecular derangements (1).

Although most cases of children with HF are associated with congenital heart diseases (CHD), the second most common cause of pediatric HF is cardiomyopathy, especially dilated cardiomyopathy (DCM), usually with a preserved right ventricle (RV) function.

In contrast to HF secondary to CHD, the outcome of children with cardiomyopathy remains poor, with a 5-year risk for death or cardiac transplantation of around 50% for patients with DCM. (2)

Large amount of research has been published on the management of HF in adults, whereas there are only few small, retrospective studies about infants and children. Apart from this limited literature, pediatric HF therapy has largely been based on clinical experience and extrapolation of adult HF data, which is not ideal given the differences in etiology.

Considering the small number of patients affected, pediatric HF may seem a less crucial public health concern as compared to adult HF. However, its economic and social consequences should not be neglected, such as the high costs of surgical or catheter-based interventions for children, the great demands of medical care and the loss of potentially productive years per death of a child. (1)

As a result of the recent flourishing research on pediatric HF, nowadays improved outcomes of medical and surgical therapies are allowing more and more children with HF to reach adulthood.

3.2. PEDIATRIC DILATED CARDIOMYOPATHY

Dilated cardiomyopathy (DCM) is a progressive and almost always irreversible disease of the heart muscle, characterized by left ventricle dilation and reduced systolic function, leading to HF and, later, to multiple organ failure, with a 20% mortality rate at 1 year and a 56% mortality rate at 4 years. Thus DCM is the main indication for heart transplant in children and adults. (3)

3.3. EPIDEMIOLOGY

The overall annual incidence of pediatric cardiomyopathies is about 1.13-1.24 per 100.000 children according to the data collected by two population-based registries, where DCM accounted for more than half of the primary causes. (4)

In the United States, the annual incidence of DCM in children is 0.57 cases per 100.000 population per year. Similar statistics have been reported in Finland (0.65 per 100.000 aged under 20 years) and in Australia (1.09 per 100.000 aged under 10 years).

In children, DCM occurs most commonly within the first year of life. In fact, infants are afflicted at a rate 13 times higher than older children.

Boys have a greater incidence of DCM as a result of both X-linked causes and neuromuscular disorders.

Moreover, African American children have a higher incidence of DCM as well. (5).

3.4. ETIOLOGY

While adult DCM is preferentially linked to an ischemic myocardial disease, the etiology in children is multifactorial. Despite the association with genetic mutations, myocarditis, neuromuscular disorders, inborn errors of metabolism, endocrine and hematologic disorders, and drug exposures (e.g., chemotherapy), in most cases there is no evident cause, which is why idiopathic DCM accounts for more than 70% of pediatric DCM (*Table 1*).

Table 1. Etiology of dilatd cardiomyopathy during childhood. (3)

Etiological groups	**Subgroups**	
Infections	Viral	Group A and B coxsackievirus, Echovirus, Adenovirus, Mumps, Rubella
	Bacterial	Streptococcus and Staphylococcus, Salmonella, Neisseria, Mycobacterial, mycoplasma and Chlamydia
	Fungal	Candida, Aspergillosis
	Protozoan	Trypanosoma cruzi, Toxoplasmosis
	Spirochete	Lyme disease
Metabolic	Endocrine	Hypo-/hyperthyroidism, Diabetes mellitus and born to a diabetic mother, Pheochromocytoma, Neuroblastoma, Congenital adrenal hyperplasia
	Storage	Glycogen storage, Mucopolysaccharidosis, Sphingolipidosis, Hemochromatosis

	Nutritional deficiency	Kwashiorkor
	Hypernatriuria	Carnitine
Systemic	Connective tissue system	Lupus erythematosus, Juvenile rheumatoid arthritis, Polyarteritis nodosa, Kawasaki disease, Pseudoxanthoma
	Infiltrations and granulomas	Leukemia, Sarcoidosis, Amyloidosis
	Others	Hemolytic uremic syndrome, Reye syndrome, Mitochondrial disease
Genetic	Muscular dystrophies and myopathies	Duchenne muscular dystrophy, Steinert muscular dystrophy and Barth syndrome, X-linked cardiomyopathy, Progressive juvenile spinal muscular atrophy, Myotubular myopathy
	Neuromuscular disorders	Friedreich's ataxia
	Gene mutations of cardiac structural proteins	Sarcomere, cytoskeleton, desmosome, sarcoplasmic reticulum, nucleus, mitochondrion, extracellular matrix, and ion channels
Toxic	Drugs	Sulfa drugs, Penicillins, Anthracyclines
Tachyarrhythmias	Supraventricular tachycardia, Atrial fibrillation, Ventricular tachycardia	

3.5. PATHOPHYSIOLOGY

Dilation of the left ventricle (LV) and its systolic dysfunction, viariably associated with right ventricle (RV) dysfunction, are the main characteristics of DCM.

In the early stages, the ventricular dilation, driven by the reduced sarcomere contractility and left ventricular dysfunction, still manages to maintain the cardiac output, despite increased left ventricular end-diastolic pressure and volume.

In fact, according to the Frank-Starling law, the stretching of the myocardial fibers increases ventricular stroke volumes in order to preserve cardiac output.

However, Frank and Starling (6) demonstrated that, although increased ventricular preload augments contractility, excessive pressure and volume induce an exceeding fibre stretching and a plateau, with a consequent reduction in myocardial contraction, leading to ventricular dysfunction.

Hence, this early attempt to compensate ultimately failes, contributing to producing the thin-walled dilated LV.

Due to the ventricular dilation and reduced systolic function, the pressure-volume curve shows a right-ward displacement, with an increase of both left ventricular end-diastolic volumes and pressures. (7)

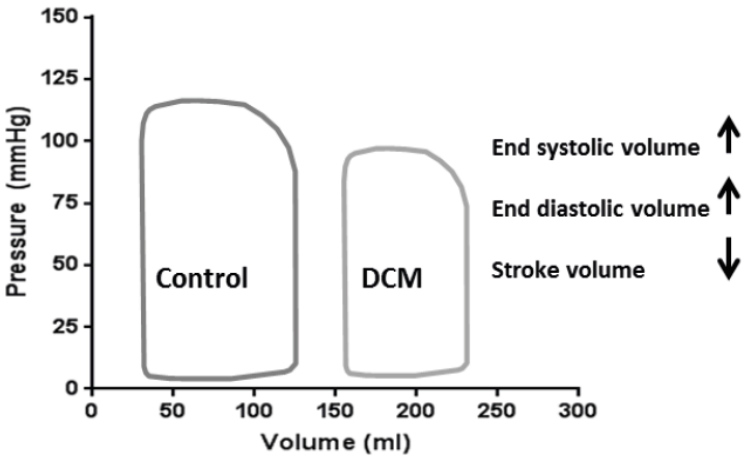


Figure 3. Pressure-Volume relationship in DCM

Despite increased preload, stroke volume may be reduced, as well as the index of contractility (end-systolic pressure to volume ratio).

In addition to this, there is an upward shift of the curve owing to diastolic dysfunction due to inadequate ventricular relaxation and increased stiffness due to interstitial fibrosis.

When the preload reserve (i.e., the maximal increase in left-ventricular end diastolic volume that is possible during an attempt to increase cardiac filling) is exhausted, the stroke volume becomes sensitive to alterations in the afterload, which depends on blood viscosity, vascular resistance, vascular distensibility and mainly myocardial wall tension.

The grade of hypertrophy associated with LV dilation is not enough to maintain in the range the wall tension.

According to the Laplace law:

$$T = \frac{P\ x\ R}{2\ x\ h}$$

Where T is wall tension, P is pressure, R is the radius and h is wall thickness.

This formula shows that the wall tension is directly proportional to the radius of the cavity and inversely proportional to the wall thickness. In the case of DCM, a dilated ventricle (↑R) with its thin wall (↓h) is clearly characterized by elevated afterload. Thus, to create the same pressure (P) during blood ejection, a much higher wall tension (T) has to be developed by the cardiac muscle.

This means that dilated heart requires more energy to pump the same amount of blood as compared to normal-sized heart. The increased oxygen consumption is accompanied by further ventricular deterioration and reduction in cardiac output.

Due to LV dilation, papillary muscles become dislocated, therefore making the mitral vale insufficient. The increased end-diastolic ventricular pressure and the mitral regurgitation together induce an increase of pressure in the upstream

cavities (left atrium, pulmonary veins and capillaries), with patients manifesting signs and symptoms of left heart failure. Progressively, pulmonary arterial hypertension (PAH) appears, contributing to right ventricular deterioration and tricuspidal regurgitation.

The tricuspidal regurgitation, together with the increased right ventricular end-diastolic pressure, increases the pressure in right atrium and peripheral veins, with consequent signs and symptoms of right heart failure.

Considering other compensatory mechanism to DCM, the neurohormonal activation mediated by the sympathetic nervous system increases heart rate and contractility in the attempt to maintain the cardiac output.

All these compensatory mechanisms, that ultimately lead to LV remodeling, are also the reasons why patients could be asymptomatic during the early stages of ventricular dysfunction, with the symptoms becoming evident only when the cardiomyocyte degeneration progresses, and the volume overload increases.

3.5.1 Cardiac Remodelling

Cardiac remodelling is the pathognomonic aspect of DCM, consisting of an abnormal ventricular architecture and chamber configuration (increased volume), driven histologically by:

- pathologic myocyte hypertrophy
- myocyte apoptosis
- myofibroblast proliferation
- interstitial fibrosis.

Factors contributing to LV remodelling include:

- renin-angiotensin-aldosterone (RAA) axis
- adrenergic nervous system
- increased oxidative stress

- pro-inflammatory cytokines and endothelin.

Their role is proven by the fact that RAA system inhibition and beta-adrenergic blockade seem to ameliorate or reverse LV remodelling in patients with DCM. (7)

3.6. CLINICAL PRESENTATION

At the time of diagnosis, 71% of children present with clinical signs of heart failure and marked LV dysfunction (8), with non-specific symptoms simulating a respiratory disease. The presence of HF must be ruled out when typical symptoms (i.e, tachycardia, oedema, respiratory distress, growth failure, exercise intolerance) occur.

A population-based cohort study in Australia (9) showed that symptoms were present in most subjects at presentation and were usually severe. Congestive HF was the initial symptom in almost 90% of patients, half of whom were admitted to an intensive care unit. Sudden death was the first manifestation of dilated cardiomyopathy in nearly 5%, and a further 13% died during their initial hospitalization.

Regarding the definition of the severity of heart failure, the New York Heart Association (NYHA) Heart Failure Classification is not applicable to most of the pediatric population, so the Ross Heart Failure Classification was developed to provide a global assessment of HF severity in infants and has later been modified in order to apply to all pediatric ages. The modified Ross classification incorporates feeding difficulties, growth problems and symptoms of exercise intolerance into a numeric score comparable with the NYHA classification for adults (*Table 2*). (1)

Table 2. Modified Ross Heart Failure Classification for children

Class I	Asymptomatic
Class II	Mild tachypnea or diaphoresis with feeding in infants, dispnea on exertion in older children
Class III	Marked tachypnea or diaphoresis with feeding in infants, marked dyspnea on exertion, prolonged feeding times with growth failure.
Class IV	Symptoms such as tachynea, retractions, grunting or diaphoresis at rest

3.7. DIAGNOSIS

A detailed diagnostic workup for DCM includes:

- Case history
- Clinical examination
- Electrocardiogram: sinus tachycardia, increased left ventricular voltages and ischaemic changes are seen on ECG at presentation. The QRS complex may be broad due to conduction disturbance and evidence of right atrial hypertrophy and left atrial hypertrophy is sometimes evident. (10)
- X-ray: increased cardiothoracic ratio, with evidence of lateral bronchial displacement due to left atrial enlargement and pulmonary plethora in association with hepatomegaly. (10)
- Echocardiogram: measures of chamber dimensions, volumes, and wall dimensions, as well as functional assessment of myocardial performance, including Doppler traces of ventricular contractility (dP/dt), systolic-to-diastolic ratio, myocardial performance index, tissue Doppler imaging, and measurements of myocardial deformation (strain and strain rate).

Key measurements include the LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), LV end-diastolic volume (LVEDV), and LV posterior wall and septal thicknesses, all expressed as z scores to adjust for patient size. (11)

Usually LV-DCM, is characterized by LV-ejection fraction of less than 40% and LV-end-diastolic dimension (LVEDD) with z-values above +2; LVEDD with a z-value above +5 has a low incidence of spontaneous recovering. (12)

- MRI: Abnormal chamber dimensions, wall thicknesses, and ventricular mass can be determined by cardiac magnetic resonance imaging (cMRI), helping to define a specific morphological phenotype. In addition, cMRI can provide functional assessment of myocardial performance, including flow rates, shunts, and regional wall motion abnormalities. Tissue characterization of fibrotic scar, interstitial fibrosis, edema, and hyperemia can help determine the cause of cardiomyopathy (e.g., IDCM versus myocarditis). (11)
- Coronary-angiography: especially to exclude anomalous origin of the left coronary artery form the pulmonary artery.
- Myocardial biopsy: this is not always possible, but it is highly useful to determine the underlying cause of dilated cardiomyopathy.

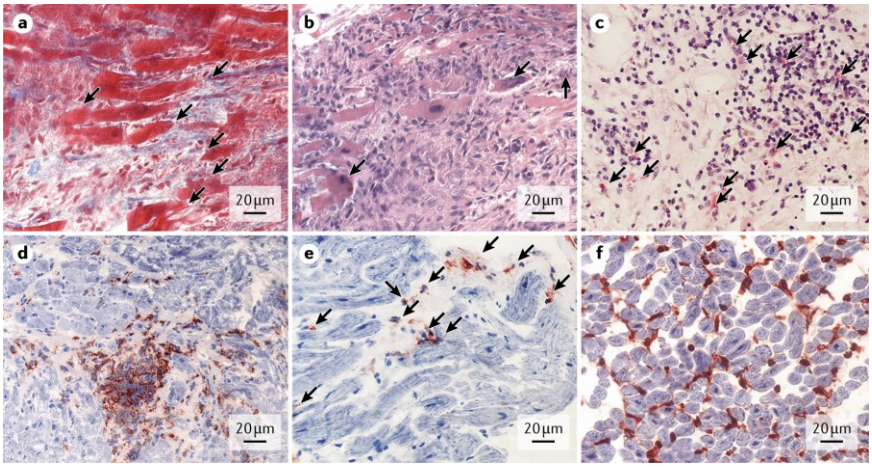


Figure 2: possible differential diagnosis of the underlying causes of dilated cardiomyopathy. A) Active myocarditis with immune cell

infiltration and myocytolysis (arrows), histological azan staining. B) Giant cell myocarditis with massive immune cell infiltration around multinuclear giant cells (arrows), histological haematoxylin and eosin (H&E) staining. C) Eosinophilic myocarditis with immune cell infiltration and eosinophils (arrows), histological H&E staining. D) Immunohistochemical staining depicting CD3+ T cells (red-brown staining) in a focal pattern in borderline myocarditis. E) Immunohistochemical staining of increased perforin-positive cytotoxic cells (arrows) in inflammatory cardiomyopathy. F) Immunohistochemical staining of increased cell-adhesion molecule HLA1 (red-brown staining) in inflammatory cardiomyopathy. (13)

- Laboratory data (BNP or NT-proBNP, CRP, hemoglobin, sodium, potassium, creatinine, albumin, and if possible, plasma levels of aldosterone, norepinephrine, angiotensin, renin respectively).
- Genetic testing: mutations in genes encoding components of the sarcomere or costamere and related binding proteins, Z-band, nuclear membrane, desmosome, mitochondrial, and calcium-handling, as well as neuromuscular disorders, inborn errors of metabolism and genetic syndromes (14).

3.8. CLINICAL MANAGEMENT

3.8.1 Medical therapy

While medical treatment recommendations for chronic HF in adults have been based on controlled randomized studies, there can be significant obstacles to the implementation of clinical trials methodology in children, as illustrated by the fact that the first “large” multicenter randomized trial of a therapeutic agent in children with HF was not published until 2007.

Shaddy et al. (1) enrolled 161 children and adolescents in a trial where they compared 2 dosages of carvedilol to placebo. However, the fact that enrolling 161

children required 26 centers and nearly 5 years illustrates the difficulties with pediatric heart failure research.

Rossano and Shaddy (15) emphasized the missing data in children and stated that extrapolating evidence from adult patients to children with HF may have limited utility.

In general, the goals for pediatric chronic HF (cHF) treatment are the optimization of (16):

- Heart rate
- Preload, by avoiding volume depression
- Afterload, by lowering systemic resistance
- Myocardial oxygenation and contractility
- Sinchrony
- Ventricular-ventricular interaction
- Exogenous and endogenous repair mechanisms.

Conventional pediatric heart failure therapy typically consists of a combination of ACE-inhibitors, beta-blockers and diuretic and is heavily reliant on adult studies as evidence when available. The International Society of Heart and Lung Transplantation (ISHLT) updated consensus pediatric heart failure guidelines in 2014 (17). As for the heart failure with reduced EF, recommendations include (18):

Table 3: ISHLT recommendations for pediatric HFrEF

Drug/Class	**Recommendation**
Diuretics	Should be used to treat fluid retention associated with ventricular dysfunction to achieve euvolemia (2014: Class I, LOE C; 2004: Class I, LOE C)
ACE-I	Recommended (unless specific contraindication) for the treatment of symptomatic LV dysfunction. Start at low doses, and up-titrate to a

	maximum tolerated safe dose (2014: Class I, LOE B; 2004: Class I, LOE B) Recommended (unless specific contraindication) for the treatment of asymptomatic LV dysfunction (2014: Class IIa, LOE B; Class I, LOE B) Consider for individuals with DMD unless specific contraindication, although the optimal age of institution of therapy is unclear (2014: Class IIa, LOE B) Should not be routinely instituted for all patients with single ventricle morphology, but could be considered in specific cases, e.g. valve regurgitation or ventricular dysfunction (2014: Class IIb, LOE B)
Beta-blockers	Consider in symptomatic children with systemic LV systolic dysfunction, particularly if the systemic ventricle has a left ventricular morphology. Start as low dose and slowly up-titrate (2014: Class IIa, LOE B) Consider in asymptomatic children with systemic LV systolic dysfunction. Start as low dose and slowly up-titrate (2014: Class IIa, LOE B)
Aldosterone Receptor Antagonists	Consider in systemic ventricular systolic dysfunction (2014: Class I, LOE C)
ARBs	Generally reserved for systemic ventricular systolic dysfunction that would benefit from RAAS blockade but are intolerant of ACE-I. (2014: Class IIa, LOE A; 2004: Class IIa, LOE C)
Digoxin	Not recommended with asymptomatic LV dysfunction because no survival benefit seen in adults (2014: Class I, LOE C; 2004: Class IIb, LOE C) Used for symptom relief. Consider doses targeting lower serum digoxin concentrations (e.g., 0.5–0.9 ng/mL). Dose reductions in patients on carvedilol and amiodarone or those who have or are at risk for renal dysfunction (2014: Class IIa, LOE C; 2004: Class I, LOE B)
Hydralazine and Isosorbide	Not recommended (2014: Class III, LOE C)

dinitrate	
Antiarrythmics	May be needed in select cases where dysrhythmias persist after normalization of electrolyte disturbances or metabolic issues (i.e., hyperthyroidism) and the dysrhythmias are poorly tolerated (2014: Class IIb, LOE C) Should not be used routinely (2014: Class III, LOE C)
Statins	Not indicated (2014: Class III, LOE C)
Renin inhibitors	Not recommended (2014: Class III, LOE C)
Anticoagulants	Patients with intracardiac thrombus should receive anticoagulation with heparin or warfarin (2014: Class I, LOE B) Consider anticoagulation with heparin or warfarin if history of prior thrombus or thromboembolic event with EF <25 % (FS <15 %) (2014: Class IIa, LOE C) Recommend anticoagulation with unfractionated heparin, LMW heparin or warfarin if persistent or uncontrolled paroxysmal atrial fibrillation or flutter (2014: Class IIa, LOE C) Not recommended if no history of thrombus or thromboembolic event, as insufficient evidence (2014: Class III, LOE C) Not recommended for routine use but may be considered in select situations to lower CVP when other interventions have been unsuccessful (2014: Class IIb, LOE C)
Nesiritide	Consider for symptomatic relief in palliative setting (2014: Class IIa, LOE C)
Pulse or chronic inotropic support	Not recommended for use other than as a bridge to transplant (2014: Class III, LOE C)
Vasopressin receptor antagonists	Not recommended for routine use (2014: Class III, LOE C)

ACE-I angiotensin-converting enzyme inhibitors, ARBs angiotensin II receptor blockers, CVP central venous pressure, DMD Duchenne muscular dystrophy, EF ejection fraction, FS fractional shortening, HFrEF chronic heart failure with reduced ejection fraction, LMW low molecular weight, LOE level of evidence, LV left ventricle, RAAS renin–angiotensin–aldosterone system.

According to the strategy used by Schranz et al., at the Giessen Institution, pediatric HF therapy should consists of specific long-acting β1-adrenoreceptor blocker (Bisoprolol), long-acting tissue angiotensin-converting enzyme inhibitor (Lisinopril) and mineralcorticoid-receptor blocker (Spironolactone), aiming to reduce the continous endogenous neuro-humoral activation which leads to chamber dilation and progressive dysfunction, driven by myocardiocyte apoptosis, necrosis, and cardiac fibrosis.

In particular, the hypothesized goals for the treatment of LV-DCM with this triple therapy are (16):

- Heart rate control in order to improve the ratio of myocardial oxygen consumption to demand and prolong the time for diastolic ventricular filling (β1-specific β- blocker)
- Diminishing apoptosis and myocytes necrosis (β1-specific-β blocker)
- Diminishing interstitial fibrosis by blocking sympathetic- and Renin-Angiotensin-Aldosterone- systems (three first line drugs)
- Reduction of cardiac afterload together with preservation of the coronary perfusion pressure by adequate (preload) intravascular, in particular, arterial vascular filling (avoidance of diuretics, effectively dosed ACE-I + β1- specific blocker, preserving the β2 receptor function)
- re-estabilishment of ventricular synchrony and ventricular-ventricular interaction as prerequisite for cardiac regeneration (all three first-line drugs)
- Low-risk benefit ratio and high parental compliance by daily single-dose therapy together with easy doing of 0.1-0.2 (0.3) mg/Kg x day for both Bisoprolol and Lisinopril and once application of Spironolactone 1-2 mg/Kg, respectively.

This combination of drugs might also help transiting unstable patients to a stable hemodynamic status.

In addition:

- Digoxin could be used as a fourth-line HF drug with a targeted plasma level of 0.5-0.8 ng/mL
- Chronic treatments with Furosemide (or other loop-diuretics) should be avoided and used only if necessary, at a dosage of 0.5 mg/kg
- Hydroclorothiazide in low dosages of 0.5-1 mg/Kg could be applied once or twice per day, if really needed
- In the presence of inappropriate ADH secretion with severe hyponatremia, tolvaptan (V2-receptor antagonist) could be used once per day in a dosage of 0.1- (0.3) mg/Kg, targeting a sodium serum level of 140-145 mmol/L.
- Inotropic agents and vasoconstrictor should be used only if necessary, as short as possible or as a bridge towards heart transplantation. Positive inotrope vasodilators (milrinone, levosimendane) have to be preferentially used if myocardial perfusion pressures are not compromised. During cathecolamine infusion therapy, a combination of epinephrine or norepinephrine infusion with β1-receptor blockers (metoprolol, bisoprolol) is recommended.

Miyamoto et al. (19,20) recommended the use of ß1-selective BAR blocker in children. They showed that ß1-ARs are downregulated in both adults and children with chronic HF but that the ß2-AR is downregulated only in pediatric DCM, stating that further inhibition of the already downregulated ß2-ARs may override the benefit of ß1-AR inhibitor therapy, because preservation of ß2-AR function is beneficial. As a long-acting, highly cardio-selective BAR-blocker, Bisoprolol is recommended. Additionally, by blocking renal ß1-adrenergic receptors, ß1-specific BAR-blockers also reduce the concomitant renin release caused by HF, enhancing the effect of ACE inhibitor therapy.

Sinergistically with BAR blockers, long-acting tissue angiotensin-converting enzyme inhibitors (like Lisinopril) block stimulated RAAS, lowering systemic

vascular resistance and, together with the mineralocorticoid-receptor blocker Spironolactone, avoiding myocardial fibrosis.

The Giessen Institution is also recently using the triple drug combination in all LV-DCM patients undergoing reversible Pulmonary Artery Banding.

3.8.2 Mechanical Circulatory Support

In case of no response to medical therapy, Ventricular Assist Devices (VADs) can be used as a bridge to heart transplantation, to recovery or as a destination therapy for patients with ESHF in DCM. (21)

Multiple studies have demonstrated the benefit of VAD support on mortality and overall condition at transplant. (22,23)

In this context, a useful classification is represented by The Pediatric Interagency Registry for Mechanically Assisted Circulatory Support (PEDIMACS) profiles, which helps identify ambulatory patients with advanced HF who may benefit from current mechanical support devices under existing conditions.

The Fifth Annual PEDIMACS Report (24) showed that 58% of pediatric patients who underwent MCS were affected by cardiomyopathy, with DCM representing almost the totality (94%).

Most patients (55%) underwent VAD implantation at PEDIMACS level 2 (progressive decline), with 31% being at Pedimacs Level 1 (Critical Cardiogenic Shock). The most common devices implanted were Implantable Continuous (IC, 41%), followed by Paracorporeal Pulsatile (PP, 27%), Paracorporeal Continuous (PC, 26%) and Percutaneous (5%).

Overall, at 6 months after VAD implantation, 83% had a positive outcome (transplant, explant, or alive on device).

Survival differs by device type with patients on Implantable Continuous (IC) VADs having the best overall survival and those on PC having the lowest overall survival.

When it comes to overall survival, infants (those under the age of one year at the time of VAD implantation) have the lowest overall survival when compared to older children. (24)

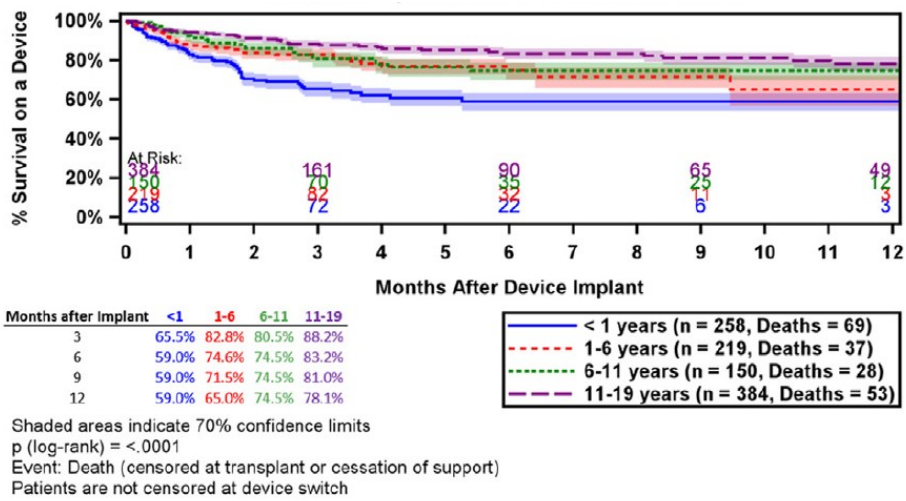


Figure 3: Kaplan-Meier Survival on a Device Stratified by Age Group (n=1011); Pedimacs: Semptember 19, 2012 – December 31, 2020 (24)

These findings underline the difficulties that this population faces and the urgent need for new treatments. In our facility, patients under 12 months of age represent the best candidates for Pulmonary Artery Banding application, as a bridge to transplant or functional recovery.

Table 4: PEDIMACS profiles (25)

Profiles	Description	Details	MODIFIERS (see *table 5* below)
PEDIMACS 1	Critical cardiogenic shock (Crash and burn)	Life-threatening hypotension despite rapidly escalating inotropic support, with critical organ hyopoperfusion confirmed by worsening acidosis and lactate levels	A or TCS
PEDIMACS 2	Progressive decline (Sliding fast on inotropes)	Decline function despite intravenous inotropic support	A or TCS
PEDIMACS 3	Stable but inotrope dependent (dependent stability)	Stable on continuous intravenous inotropic support	A; TCS (if the patient is in the hospital with circulatory support); FF (if at home in case of frequent accesses to hospital)
PEDIMACS 4	Resting symptoms on oral therapy at home	Patient experiences daily symptoms of congestion at rest or during activities of daily living	A and/or FF
PEDIMACS 5	Exertion intolerant	Patient is comfortable at rest and with activities of daily living but anable to engage in any other activity	A and/or FF

PEDIMACS 6	Exertion limited	Patient has fatigue after the first few minutes of any meaningful activity	A and/or FF
PEDIMACS 7	Advanced NYHA Class III	Patients living comfortably with meaningful activity limited to mild physical exertion	A

Table 5: Modifiers of the Pedimacs Patient Profiles(25)

A - ARRYTHMIA	This modifier can modify any profile. Recurrent ventricular tachyarrythmias that have recently contributed substantially to the overall clinical course. This includes frequent shock from ICD or requirement for external defibrillator, usually more than twice weekly.
TCS – TEMPORARY CIRCULATORY SUPPORT	This modifier can modify only patients who are confined to the hospital, Patient Profiles 1 or 2 and 3, but a patient who is listed as Patient Profile 3 stable on inotropes who has been at home until elective admission for implantable VAD cannot have a TCS modifier; Support includes, but is not limited to, IABP, ECMO, Rota flow, Tandem Heart, Levitronix, BVS 5000 or AB5000, Impella, Sorin Revolution, Biomedicus.
FF – FREQUENT FLYER	This modifier is intended for Patient Profiles 4, 5, and 6. If the patient is frequently at home, this modifier can be used to change Patient Profile 3 Frequent Flyer is a term used to describe a patient who requires frequent emergency room visits or hospitalizations for intravenous diuretics, ultrafiltration, or short-term inotropic treatment. At least two emergency visits/admissions in the previous three months, or three times in the previous six months, would be considered frequent. If admissions are caused by tachyarrythmia or ICD shocks, the modifier to use is A rather than FF.

As for the medical therapy, the development of pediatric assist devices took way more time for children than for adults.

Extracorporeal membrane oxygenation (ECMO) was the primary supporting device for children with ESHF until 2010.

Then, in 2011, the Berlin Heart EXCOR® was approved by the US Food and Drug Administration (FDA) after a study comparing its outcomes versus those of ECMO. This pulsatile pneumatically driven paracorporeal VAD became the most commonly used pediatric VAD throughout the world and only long-term FDA approved VAD available for neonates and infants in the United States as a bridge to transplant (22), allowing to reduce the waitlist mortality. The variety of pump sizes (10, 15, 25, 30, 50, 60, and 80 mL stroke volume) made it possible to support children ranging from neonates to adulthood.

However the incidence of major lethal or disabling complications is not negligible and the management of Berlin Heart often requires long-term hospitalization with increased hospital costs and patient discomfort (26).

Other two devices that may be used for ESHF are HeartMate 3 and The Jarvik Infant Heart.

The first, approved in late 2018, allows centrifugal continuous flows between 2.5 and 10 L/min and creates an artificial pulse. Despite its low pump thrombosis incidence, it has a size limitation since it can be implanted only in older children as small as 19 Kg (BSA 0.78 m^2). (27)

The latter is an axial continuous-flow VAD specifically designed for small children, but it is still being evaluated in the so-called Pump for Kids, Infants, and Neonates (PumpKIN) trial. Although it is very small (15mm), about the size of a battery, and provides incremental blood flow, by increasing the pump speed as the child grows, first implantations showed a high risk of hemolysis, which required several structural modifications of the device until the 2015 version of the Jarvik infant Heart. (28)

Lastly, biventricular assist device (BiVAD) or a total artificial heart (TAH) may be the best option in case of both right and left-sided heart failure.

Currently, SynCardia TAH (SynCardia Systems Inc., Tucson, AZ, USA) is the only approved available TAH. It is an implantable biventricular device that anatomically replace both ventricles, but due to its large size, it cannot be used for small children. (21)

Unfortunately, it is important to underline that LVADs carry some important risks of complications such as bleeding, stroke, infection, pump thrombosis, pump malfunction and RHF (29).

The freedom from stroke at 3 months was highest in IC VADs (93%), compared with PP VADs (84%) and with PC VADs (75%).

According to the 5th PEDIMACS annual report, for patients who died on support, the most common mode of death was multiorgan system failure (26%), followed by neurologic (22%). However, for patients supported on PC VADs, the most common mode of death was gastrointestinal (28%) and for those supported on IC VADs, the most common modes of death were circulatory (23%), multiorgan system failure (23%) and neurological (23%).

In addition, although ventricular assist devices have been increasingly utilized in developed countries, the availability of such therapies is limited in underserved regions.

3.8.3 Heart transplantation

The ultimate therapy for ESHF is heart transplant (HTx). However, survival rate after HTx is suboptimal since it drastically decreases with time, with an overall 25-year survival as low as 37%. Together with the low donor availability, the costs, and the life-long immunosuppressive treatment with its negative long-term consequences, including risk of infections and malignancy, this makes HTx a not so appealing option for an infant or child.

According to ISHLT registry, DCM is the overall main indication for pediatric heart transplantation (HT).

Even though CHD has remained the commonest underlying diagnosis in infants younger than 1 year of age, the proportion of infant recipients with cardiomyopathy has doubled to almost 40% in the most recent era. In older patients, cardiomyopathy remains the predominant diagnosis.

The most recent analysis from the ISHLT registry shows a median survival of 22.3 years for those <1 year of age at transplantation, 18.4 years for those 1 to 5 years, 14.4 years for those 6 to 10 years and 13.1 years for those >11 years, numbers that continue to improve almost annually. Remarkably, survival to 10 years in the most recent era conditional on survival to 1-year post-transplant is now 83% for all age groups. Outcomes at 1 year and 5 years have improved when comparing 1982-1991 to 2010-2017. Based on data from 2002 to 2009 the current 10-year survival is 68% with 15-year survival at 58.9%.

Considering the impact of the diagnosis on HTX outcomes, the overall survival of cardiomyopathy patients by 3 years post-transplant was 88% compared to 79% for patients with CHD, but this difference was absent by 10 years post-transplant with 70% and 68% survival respectively. (30)

Outcomes are also greatly affected by the need and type of mechanical support while waiting for an organ. The 5-year survival for patients who needed no mechanical support or those who needed only a VAD or total artificial heart (TAH) had improved outcomes (85%) than those who needed ECMO (77%). (31)

Some other factors that have been shown to affect 1-year mortality include being on a ventilator or dialysis at the time of transplant, other markers of renal insufficiency (creatinine and glomerular filtration rate), recipient body mass index, recipient total bilirubin, ischemic time, and transplant center volume (30,32).

Graft failure, rejection, infection, and cardiac allograft vasculopathy (CAV) continue to be the major causes of death within the first 5 years post-transplant. (33)

Due to these complications, patients may die or require multiple transplants. Also, in the long-term follow-up, the immunosuppressive regimen increases the risk of infection and cancer.

In conclusion, HTx is not curative, but it actually turns the primary cardiac pathology into a chronic systemic disease.

3.8.4 Novel strategies

Randas Batista proposed a bold surgical strategy in Brazil where HTx was not available. First described in 1996, The "Batista procedure" was applied in adult patients with DCM as an experimental procedure in the hope of reversing the effects of remodelling in cases of end-stage dilated cardiomyopathy refractory to conventional medical therapy. It consisted of a partial left ventriculotomy (PLV), along with mitral valve repair, designed to reduce LV wall tension by decreasing the short-axis radius. Due to its initial bad outcomes, the procedure was then modified with the apex preservation, associated with better long-term survival, and the papillary muscle approximation (PMA), associated with further improvement in LV geometry. Kawaguchi et al. reported in a cohort of adult patients undergoing PLV that 83% showed improvement in ventricular dimensions and ejection fraction. (34)

In 2009, Westaby et al. presented a case of a 5- month-old who developed severe ventricular dysfunction due to anomalous left coronary artery from the pulmonary artery (ALCAPA). This child underwent a PLV, made an excellent recovery, and was free of heart failure symptoms at the ten years follow-up. Unfortunately, apart from this case report, most PLV literature only concerned adults. However, the procedure was then abandoned, so there is very few information on pediatric patients with DCM.

Another possible option is represented by the electric cardiac resynchronization therapy (CRT), which may improve LV morphology and function in selected adult and pediatric DCM patients.

Indeed, up to 65% (35) of pediatric DCM patients show a wide QRS morphology and an electrical dyssynchrony, such as left bundle branch block, resulting in altered myocardial mechanics and LV stroke volume. Biventricular pacing may provide a more coordinated cardiac contraction.

In a study enrolling 19 pediatric DCM patients, 68% of them showed clinical improvement with CRT, although not all showed improved LV remodeling. (36)

Another treatment approach for DCM may consist of regulating the mesenchymal stem cell activity of a diseased heart by the delivery of progenitor cells, especially bone marrow-derived progenitor cells (BM-PCs), with the goal to stimulate the intrinsic repair ability of the human heart. (37)

This possibility was taken into account after Mollova et al. described cardiomyocyte mitosis and cytokinesis until the age of 20 years, with the highest percentage in infants (38). A 3.4-fold increase of cardiomyocytes in the left ventricle between the age of 1 year until 20 years suggests that myocardium can regenerate and in the diseased heart, proliferation might be able to be stimulated. It was also demonstrated that human neonatal myocardium is enriched with cell populations representing human cardiac progenitor cells (hCPCs). Not only the number was increased but also marker genes of proliferation and differentiation.

Intracoronary administration of BM-PCs into one of three coronary arteries in adults with DCM leaded to improvements in cardiac contractile (increased LV-EF) and microvascular function, as well as decreased NT-proBNP suggesting a beneficial effect on remodeling. (37)

Regarding pediatric patients, Rupp et al. showed that cardiac function improved as well with autologous BM-PC intracoronary infusion in a case-report of a 2-year-old patient with DCM. (39)

While in adults Cellular Based Therapy (CBT) addresses mostly ischemic cardiomyopathy, the published literature in pediatric left ventricular failure includes DCM, either idiopathic, anthracycline-induced, and post-myocarditis (39–45).

It is still unclear how exactly cellular cardiomyoplasty improves ventricular function in the ischemic or other types of heart disease. Angiogenesis and neovascularization play an important role and there is good evidence that release of paracrine factors and cell-cell contact or cell-to-cell fusion counteract apoptosis for myocardial protection and influences remodeling.

The most frequent delivery of precursor cells in children is intracoronary during cardiac catheterization by using the stop-flow-technique to allow adhesion and engraftment. The group in Riga/Latvia decided for percutaneous intramyocardial application into the interventricular septum or LV wall (40,41), while in HLHS, Burkhart et al. (46) decided for a subepicardial injection of umbilical cord derived cells to the free wall of the RV with six single injections arranged in a radial pattern at the time of stage II of the palliative track of single ventricle repair. It is also planned to administer the cells also at the time of bidirectional cavopulmonary anastomosis via the cardioplegia needle after completion of surgery.

The quantity of cells that are injected varies greatly across the published series, and only a small percentage of them will be engrafted in the recipient's heart. This does not indicate ineffectiveness, underscoring once more the critical role played by paracrine systems.

Measures to demonstrate the effect of CBT in pediatric HF have mostly been improvement of the clinical status, increase of LV-EF and decline of BNP plasma levels.

It is unclear from the data included in the case reports whether the younger DCM patients responded better in terms of functional improvement.

Other stem cell lineages currently under investigation include adipose originating stem cells (AD-SCs), umbilical cord/ Wharton's jelly, cardiospheres (stem cells isolated from myocardial biopsies), and novel induced pluripotent cells from mouse fibroblasts. (5)

Although traditional medical management of ESHF because of DCM is common, as said before, other innovative strategies have been tried. Among them, a crucial role may be played by a reversible pulmonary artery banding.

3.9. PULMONARY ARTERY BANDING

Pulmonary artery banding (PAB) is an old surgical procedure tipically used for the palliation of certain congenital cardiac defects, such as functionally univentricular hearts, multiple ventricular septal defects, and complete atrioventricular septal defects, but now performed as a novel therapeutic strategy for the treatment of DCM, since it potentially induces cardiac remodeling and regeneration.

3.9.1 THE OLD FASHION

Pulmonary artery banding is usually performed with two goals (47):

1. Reducing pulmonary blood flow in patients with significant left-to-right shunting and pulmonary overcirculation, as palliation before definitive surgical repair.
2. Increasing afterload to a right-sided morphological left ventricle, which functions as a low-pressure pulmonary ventricle, so as to prepare or "train" it to become a systemic ventricle prior to an ASO (Arterial Switch Operation) in case of transposition of the great arteries (TGA).

3.9.1.1 Limitation of pulmonary blood flow

In 1951, the first PAB procedure was performed by Muller and Dammann in a 5-month-old infant with a large ventricular septal defect (VSD) and a large left-to-right shunt with pulmonary over circulation. (48)

It was later carried out in patiens with congestive HF due to tricuspid atresia, large VSDs, and atrioventricular canal defects (AVC). (49–57)

By restraining the pressure load to the pulmonary bed, PAB protects the pulmonary vasculature from the excessive blood flow which could induce

pathologic changes of the pulmonary vascular walls, leading to stiffness and consequent pulmonary hypertension. Patients with a large ventricular septal defect and low pulmonary vascular resistance will firstly develop pulmonary over-circulation, clinically confirmed by signs of congestive heart failure, including tachypnea, poor feeding, and pulmonary edema.

In the short term, the management of this condition may consist of diuretics, systemic afterload reduction, and potentially nasogastric feeding supplementation. But, in the long term, pulmonary over-circulation may lead to progressive vascular medial hypertrophy and pulmonary hypertension from increased vascular resistance. This happens in conditions of high pressure and high-volume flow as in case of large VSD, as opposed to low-pressure but high-volume flow, for example due to large ASD.

A PAB creates a physical restriction in the mid-segment of MPA which reduces pulmonary blood flow and, by increasing subpulmonic ventricular afterload, reduces left to right shunting. In this way, the pulmonary vasculature is protected from being exposed to high pressures and from the consequent deleterious remodeling. The reduction in shunt volume will consequently increase systemic cardiac output and blood pressure.

In other cardiac defects where mixing of pulmonary and systemic blood is needed to maintain reasonable systemic oxygen saturation, for example in TGA, PAB might not be tolerated, especially in the presence of a restricted atrial septal defect. Therefore, surgical septectomy or balloon septostomy might need to be performed before attempting PAB. (47,58,59)

Clinical scenarios in which pulmonary blood flow requires limitation via PAB include (47):

1. Muscular "Swiss cheese" VSD which would require technically challenging procedures.

2. VSD with surgical comorbidities, such as low birth weight, sepsis, pneumonia, intracranial hemorrhage, MOF etc.
3. Unbalanced AVC, with borderline left ventricular hypoplasia as palliation in preparation to potential biventricular repair.
4. High-risk infants with hypoplastic left heart syndrome which requires bilateral PAB and PDA stenting for short term surgical palliation.

3.9.1.2 Ventricular training

The potentiality of PAB procedure to relieve systemic right ventricle failure was first used in three groups of patients: simple and complex TGA who have undergone atrial swtich procedures (Mustard and Senning) and patients with congenitally corrected TGA (cc-TGA). Nowadays, after the introduction of the Arterial Switch Operation, it mainly concernes the latter group.

Some cc-TGA patients with a good systemic mRV, managing the systemic workload, have a good quality of life, bust most develop progressive systemic ventricular dysfunction and/or sever tricuspid (which is systemic) atrioventricular valve regurgitation early in life and require surgical intervention. Patients with a systemic mRV and intact ventricular septum, unlike those with ventricular septal defect (VSD), has a “deconditionated” mLV, which has been working against a low-resistance pulmonary circulation. It has a reduced ventricular volume and wall thickness and may also be distorted by the volume-loaded mRV. Also, the malposition of the interventricular septum may contribute to systemic AV valve regurgitation owing to distorted subvalvar tension apparatus.

Surgical options for symptomatic patients with a systemic mRV and systemic ventricular dysfunction and/or AV valve regurgitation include:

- AV valve replacement
- Correction of discordant AV and ventriculoarterial connections (Double Switch Operation, which combines both the atrial and arterial switch)

It is unlikely though that the mLV could effectively support the systemic circulation before undergoing a period of prior training, achieved by PAB in order to increase mLV afterload.

Early improvement in tricuspid regurgitation (TR) and in the clinical status of the patient is associated with the procedure.

In particular, examples of PAB applications are (47):

1. Training of left ventricle, which has become “deconditioned” due to its subpulmonary position, in patients with D-TGA presenting late (>1 month) before staged arterial switch operation.
2. Preparation of left ventricle in patients with L-TGA for a subsequent staged double switch (atrial switch-arterial switch) procedure.
3. Reduction of tricuspid regurgitation in patients with L-TGA without VSD, thanks to the increased subpulmonary LV pressure which produces a shift of interventricular septum that improve coaptation of tricuspid valve leaflets in the systemic RV.
4. In patients with single ventricle anatomy at the time of bidirectional Glenn shunt placement to maintain some antegrade flow but a low superior vena cava pressure.

3.9.2 PAB TECHNIQUE

From an anatomical point of view, it is important to consider two aspects (47):

1. The length of the main pulmonary artery (MPA): the band must be inserted in the mid-portion of the MPA, carefully avoiding impinging the pulmonary valve in the proximal segment and branch pulmonary arteries (PA) distally. Right PA is at higher risk of impingement and distortion because of its proximal origin from the MPA with an acute angle.
2. The thin wall of the MPA which is also larger than aorta due to the higher pulmonary to systemic blood flow ratio. This exposes to a higher risk of wall rupture during the surgery.

Echocardiography, cardiac magnetic resonance imaging and/or cardiac catheterization are needed before surgery for the detailed anatomic definition.

Systemic afterload reduction and vigorous diuresis should be used to reduce the left to right shunt prior to PAB, especially in patients with congestive HF

symptoms. Furthermore, patients with severe pulmonary over circulation and pulmonary edema may require respiratory assistance, including appropriate oxygenation and ventilation via mechanical ventilator support.

Prior to surgery, two aspects of the band must be taken in consideration:

1) The material: PTFE, Dacron or umbilical tape, the latter preferred by few surgeons due to the low probability to erode through the vessel wall and to the fact that it can be adjusted by passing it through a silastic snare.
2) The circumference: the optimal value can be calculated through the "Trusler formula" (60). This formula has specified the following dimensions as a good initial guide:
    - non-cyanotic mixing lesions = 20 mm + 1 mm/kg;
    - mixing lesions = 24 mm + 1 mm/kg;
    - single ventricular physiology with a plan for future palliative Fontan surgery = 22 mm + 1 mm/kg.

    Then, the definitive band circumference is fixed on the basis of the pressure gradient through the band, distal pulmonary artery pressures, and potentially by the degree of desaturation if the patient has single ventricle physiology.

Concerning the surgical approaches to PAB, they include:

1. Anterior left thoracotomy through the $2^{nd}/3^{rd}$ intercostal space
2. Left lateral thoracotomy through $3^{rd}/4^{th}$ intercostal space
3. Median sternotomy

In case of anterior/lateral thoracotomy, retraction of thymus is needed, followed by dissection of the pericardium anterior to the left phrenic nerve. Median sternotomy is preferred in case of TGA or single ventricle anatomy and if PAB is carried out together with other procedures (such as atrial septectomy) through a median sternotomy, cardiopulmonary bypass is recommended.

After exposure of the aorta and MPA, the adventitia between them is dissected and the band is first passed across the transverse sinus encircling the pulmonary trunk then carefully passed between aorta and MPA through the previous dissection site, avoiding the need to pass a clamp across MPA and potentially injuring the vessels. The marked sites of the band, located on the anterior wall of MPA, are snared by passing it through polyethylene tubing. Hemoclips may help fix the snare, increasing or decreasing the circumference of the band, and a pericardial pledget may avoid injury to the vessel.

Cardiovascular hemodynamics to be achieved post-PAB include (47):

1) PA pressured to 50% of systemic pressures

2) Oxygen saturation of approximately 90% at 50% FiO2

3) Increase in systemic blood pressure by 5 to 10 mmHg.

In patients with single ventricle physiology, lower arterial oxygenation targets are acceptable.

When PAB is taken down, the scar tissue around the PAB is dissected and the band removed, with possible evidence of stenosis of MPA. This is corrected either by resection of the stenotic segment followed by end-to-end anastomosis or vertical incision of the stenotic segment with patch augmentation of the segment, although this PA plasty is often not necessary.

Controindications to this procedure include (47):

- Patients with pressure gradient across the systemic outflow tract/subaortic region >15-20 mmHg, potentially creating a "double banding" scenario, with pressure overload on both ventricles.
- Severe AV valve regurgitation of the pulmonary ventricle or of the systemic AV valve in single-ventricle patients, which would get worse for the increased afterload caused by the band
- Truncus arteriosus: in the type 1 the short MPA may be difficult to band, and the band may impinge into the right PA. Types 2 and 3 would require

bilateral PAB. In addition, PAB placement in scenarios of both systolic and diastolic pulmonary flow, such as truncus arteriosus, may be less effective in limiting pulmonary flow due to the continuous nature of flow (as opposed to purely systolic flow in patients with a VSD).

Success of PAB can be highlightened by significant improvement in cardiac hemodynamics, in signs and symptoms of congestive HF, in pulmonary over circulation, and by the reduced ventricular end-diastolic volume.

The mortality rate among patients undergoing PAB is more dependent on the underlying congenital heart defects rather than the procedure itself. While early studies reported approximately 25% mortality with PAB (61), now it is reduced approximately to 5%, thanks to improvement in surgical technique and perioperative care. (62)

Potential complications of pulmonary artery banding include (47):

- Stenosis or distortion of one or both branch PAs
- Alterations in the function of the pulmonary valve
- Impingement of circumflex artery
- Erosion of band into the pulmonary artery
- Pseudoaneurysm of pulmonary artery
- Hemolysis
- Thrombosis
- Ineffective band placement leading to irreversible changes to pulmonary vasculature cause pulmonary hypertension
- Local infection

3.9.3 THE GIESSEN PROTOCOL

Learning from the retraining of the subpulmonary ventricle in congenitally corrected transposition of great arteries, in 2007 Schranz et al. (Giessen Institution, Germany) (63) described for the first time the case of a 2-month-old infant with progressive idiopathic dilated cardiomyopathy (iDCM) who was listed for heart transplantation and recovered dramatically from end-stage heart failure

after pulmonary artery banding (PAB). Until that time, the use of PAB to treat left-sided iDCM had not been described in the literature.

The patient presented a massive dilation and reduced systolic function of the left ventricle (LV). At 35 weeks of gestation, delivery became necessary because of progressive deterioration of heart function. A post-natal echocardiogram showed an extremely dilated LV with an LV-EF of 13%. Following initial treatment with ventilation, inotropics and prostaglandin E1, the patient was listed for heart transplantation, but 2 months after Schranz et al. decided to perform a central PAB, knowing that PAB is an established approach for patients with a morphologic RV in systemic position to retrain the LV and to improve RV failure and ameliorate tricuspid regurgitation.

Rehabilitation of the LV shape and improvement of function accompanied by a diminution of the mitral regurgitation were observed by repositioning of the ventricular septum as an immediate LV-RV crosstalk.

Six weeks after banding, echocardiographic assessment revealed a decrease of the LV end-diastolic diameter to 30 mm (from 41 mm on admission) with a significant improvement of systolic function. At the age of 6 months, CMRI showed a concentric hypertrophy of the LV with a normal systolic function, instead of the expected RV hypertrophy with a remaining LV dilation. LV-end-diastolic diameter was 24 mm and the EF 62%.

11 years after this first experience, in 2018, Schranz et al. (64) reported a worldwide experience of PAB treatment of end stage LV-DCM with preserved right ventricular function.

Fifteen centers of 11 countries from America, Asia and Europe introduced PAB in the effort to diminish the need for LV assist device support and HTx.

The following figure (Figure 3) shows the flowchart of 70 patients who received PAB between March 2006 and 2017.

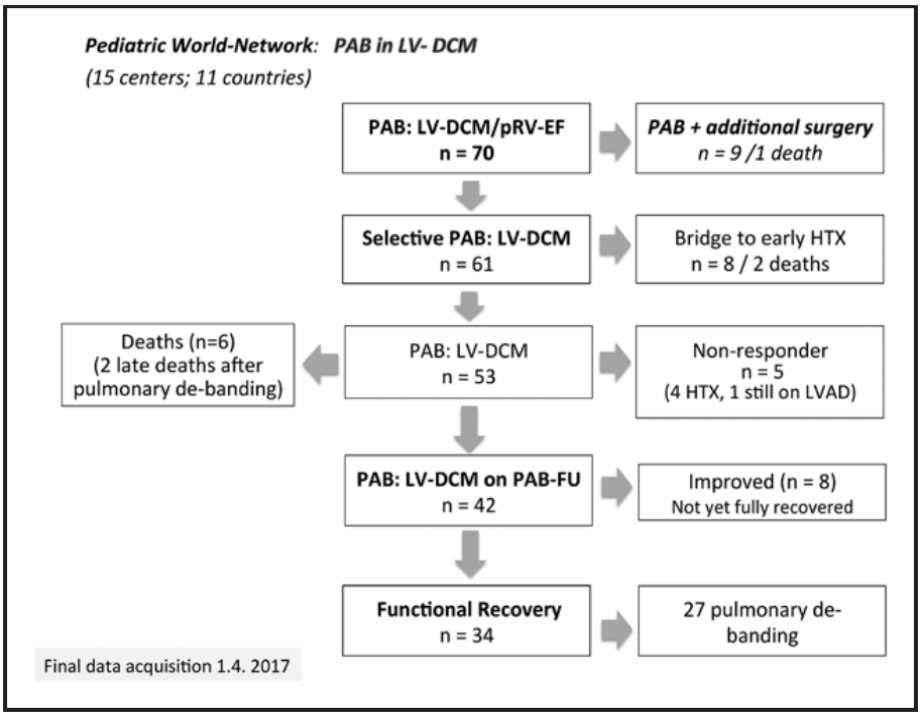


Figure 4. Seventy patients with left ventricular dilated cardiomyopathy and preserved right ventricular ejection fraction: treatment by PAB. Nine patients received PAB after additional open-heart surgery. In 61 patients, PAB was performed by a selective open-chest approach; detailed FU analysis was performed in 42 PAB responders

Nine patients received PAB after additional open-heart surgery. So, focusing on the 61 patients who received PAB as the only operation, outcomes are promising. Overall medium-term mortality is confirmed to be modest (8/61, 13%) and the rate of complete LV recovery is brilliant (34/61, 56%). In addition, 8 patients (13%) experienced a partial improvement in LV function, although not a fully recovery, while 13 patients (21%) required an HTX, as planned strategy or because of PAB non-responders.

About the improvement of these patients, Schranz speculates that (64):

- Geometric rearrangements of LV dimension is achieved by re-estabilishing the interventricular septal position with gradual restoration of LV ejection fraction;
- Cardiac improvement with potential for regeneration reciprocal to the patient's age is promoted by PAB-induced right ventricular hypertrophy.

3.9.3.1 THE PAB EFFECT

The left and right ventricle do not work indipendentely; instead, they are connected by a shared septum, myofibers and pericardial space.

Reminding that the determinants of cardiac output are heart rate, contractility, preload, afterload, parameters of ventriculo-ventricular interaction (VVI), interatrial and atrial-ventricular as well as ventriculo-arterial coupling, the potential stimulus of VVI driven by reversible PAB is the rationale of its application in LV-DCM patients, especially those who manifest left heart failure with reduced ejection fraction (HFrEF) but preserved RV-function.

Indeed, rPAB shifts the interventricular septum back to the left, reshaping the left ventricle from a pathological "apple-like" morphology to its normal, ellipsoid, "pear-like" one.

Mechanisms of reversible PAB in LV-DCM include (12):

- Increase of RV contractility (Anrep Effect), wall stress and isovolemic contraction
- Stimulation of RV hypertrophy and matrix remodelling
- Improvement of RV diastolic inflow characteristics
- Leftward shift of the IVS and restoration of electromechanical RV-LV synchrony
- Reduction of LVED volume with reshaping from spherical to ellipsoid morphology
- Reduction of LV-filling dynamics (preload) and subsequent LVED pressure
- Improvement of LA-LV interaction by reduction of left atrial volume overload

- Restoration and improvement of MV regurgitation and LV ejection fraction
- LV reverse remodelling (co-hypertrophy) by favouring endogenous repair potentials

The diagnosis of DCM must be certain, before rPAB can be used. Clinical and imaging examination must exclude other causes of dilated LV (for example pressure overload, as in aortic coartaction o critical aortic stenosis), as well as possible coronary anomalies, obstruction or fistulae.

Inclusion and exclusion criteria were developed for young children with LV-DCM and preserved right ventricular function and are shown in the *table 6*.

Table 6: PAB inclusion and exclusion criteria (12)

Inclusion criteria	Exclusion criteria
Age 0-5 years	Age greater than 5 years
Left-sided ventricular dilated cardiomyopathy	No parental consent
Left ventricular end-diastolic diameter (LVEDD) Z-score > +4.5	Biventricular end-stage DCM
Ejection fraction (EF) <30% despite inotropic & anti-congestive therapy	Acute/per-acute myocarditis
Acceptable candidate for mechanical circulatory support and/or heart transplantation (HTx)	Concomitant structural (congenital) heart disease, except for repaired ALCAPA
Clinical functional status III-IV (hospitalized)	Moderate-severe tricuspid valve regurgitation
Parental consent	Pulmonary hypertension out of proportion with left-ventricular end-stage cardiomyopathy
Preserved RV function (EF>45%)	Hereditary disease associated with bi-

ventricular dysfunction

3.9.3.2 PAB PROCEDURE

Prior to PAB, the patient is prepared to be listed for HTx and submitted to perioperative medical therapy, consisting of (12):

- Already established triple therapy with Bisoprolol, Lisinopril and Spironolactone;
- Continuous intravenous infusion of Milrinone, usually established together with B-L-S days before PAB in a dosage adapted to 1 µg/kg × min.
- Levosimendan additionally infused in a dosage of 0.1 µg/kg × min without loading dose, twelve hours before surgical PAB; the infusion should be continued for further 12 hours during anesthesia for PAB surgery and in PICU.

Heart rate (HR), systemic atrial blood pressure (SAP), right atrial pressure (RAP), pulse oximetric oxygen saturation, and cerebral near infrared spectroscopy (cNIRS) calibrated to oxygen saturation acquired from the upper caval vein are all monitored continuously during open chest surgery.

Midazolam, fentanyl, and vecuronium are commonly used as anesthetics in continuous infusions at small single doses, if needed.

Additionally, continuous infusion of clonidine (1–2 mg/kg/hour) is established or continued; infusion of epinephrine (0.01–0.05 µg/kg/min) and norepinephrine (0.01–0.2 µg/kg/min) are prepared to start several minutes prior to PAB-application, if not already used for treatment of cardiac failure before.

The approach for PAB is based on a sternotomy and partial pericardial incision. Through a transmural puncture, a polyethylene 21G (arterial) cannula is inserted into the right ventricle, secured with a purse-string suture, allowing the RV pressure to be measured before and after PAB.

To avoid impairing systemic and, in particular, coronary blood flow, the pulmonary arterial trunk must be carefully banded.

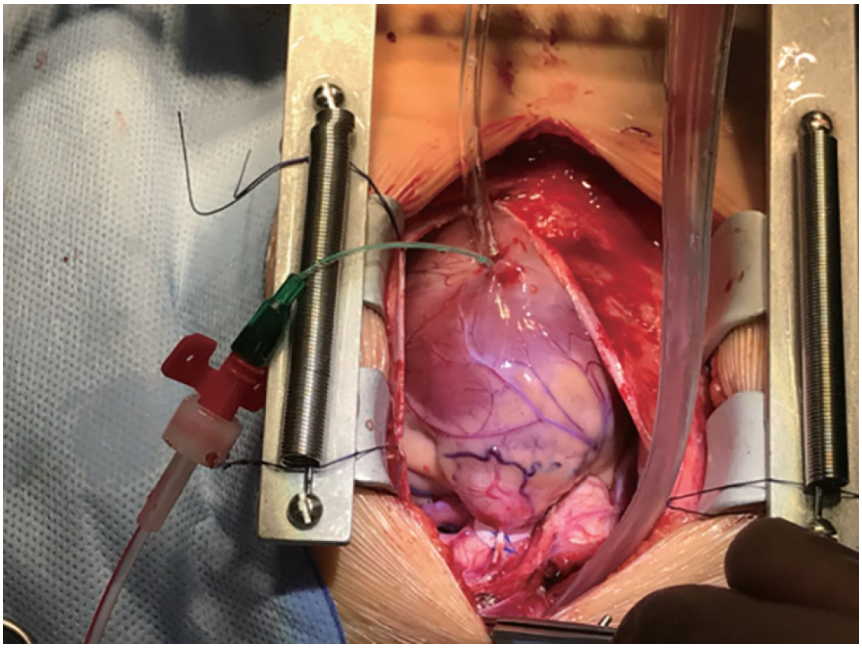


Figure 5: Open chest and pericardial approach; PAB is already placed, the 21 gauge (arterial) cannula is still in place measuring the RV pressure. (12)

Before the PAB is tightened, hemodynamic and oxygen transport parameters have to be stable (12):

- Heart rate (HR, influenced by long-acting bisoprolol, and clonidine infusion) < 145/min, preferred <125/min, despite catecholamine support;
- Right atrial pressure (RAP) > 5 mmHg (sufficient intravascular volume and hemoglobin >12 g%), but < 12 mmHg;
- Systolic arterial pressure (SAP), age and anesthesia-dependent, > 70 - [80] mmHg, if the RVP is < 35 mmHg;
- Arterial-venous oxygen saturation difference (Sa- vDO2) should be <40%, preferred <30%;
- TAPSE (tricuspid annular plane systolic excursion), degree of the mitral valve regurgitation (MR) as well as the interventricular septal position

(IVS) are monitored by TEE before, during, after PAB. LAP-RAP gradient should also be monitored by TEE, in case of persistent foramen ovale or percutaneous created restrictive ASD.

The band should be tightened under continuous hemodynamic monitoring and TEE control until the leftward shift of IVS appears. Without affecting SAP or raising RAP above 10 mmHg, the PA-circumference can usually be lowered to roughly 50-60% of the diameter of the pulmonary valve annulus. Given the PAB-induced decrease in TAPSE, a systolic RVP/SAP ratio of 0.6 may be reached.

The pressure gradient across the PAB is normally “only” 20–25 mmHg following band placement, but the true pressure gradient can be recorded when the RV-function is re-adapted, soon before before the patient is discharged.

Two sutures with 6.0 (or 5.0) prolene are utilized to facilitate subsequent partial or total de-banding by transcatheter balloon dilation. Another suture is put 3-5 mm above the first double suture-line to allow not only patient growth in the PAB, but also to establish a residual band effect, which may be important in the event of LV-DCM accompanied with a non-compaction morphology.

Myocardial function is boosted with catecholamines and inodilators during open chest approach; if the postoperative hemodynamics are instable due to hypotension caused by patient’s agitation, the patient needs to be muscle relaxed until stability is established.

Usually, the patient is extubated early after surgery and the supportive medical therapy (*Table 5*) is started. Even during continuous catecholamine support, cardiac protective drugs (B-L-S) are quickly re-established. The highly specific β-1-adrenoreceptor blocker bisoprolol, as well as the intravenous variant metoprolol or esmolol, should already protect against the endogenous and exogenous β1-adrenergic activation which has harmful cardiac effects (myocyte apoptosis, necrosis). Because of their heart-protecting, hypertrophy-supporting, and stem cell mobilizing actions, β2-receptors should be retained (65). If they’re additionally stimulated by continuous β2-agonists, their side effects such as the chronotrope

action can sufficiently be blocked by β1-receptor blockers combined with the anti-tachycardia effects of clonidine.

Digoxin or even ivabradin (0.1 mg/kg) may be utilized in some cases. Catecholamines have to be weaned off gradually over a short period of day, whereas milrinone is commonly weaned over 1–2 (or 4) weeks, by reducing the dosage of 0.1 µg/kg × min per day, depending on clinical condition (Ross-status, and BNP-values).

The separation from mechanical ventilation happens within the first few days or weeks post-rPAB. Enteric feeding is promptly restarted, and all recommended oral drugs are reintroduced in a sufficient dosage.

The infant is subsequently discharged on oral medications (B-L-S), avoiding furosemide, but using low-dosage of hydrochlorothiazide (0.5–1 mg/kg) in 1 or 2 oral applications per day in some patients; supplemental drugs are applicated long-term.

Every child is kept under meticolous follow-up. Parents should carefully keep a close eye on their child's breathing rate at sleep and administer oral medications cautiously, especially B-L-S once per day. Intermittent clinical examinations are necessary to keep track of height, weight, electrocardiogram, echocardiogram, cardiac MRI, plasma BNP levels, as well as parents' surveillance of the child; oftentimes, an invasive examination, such as heart catheterization for re-myocardial biopsies or PAB de-banding by PAB-ballooning, is ordered (12)**.**

Table 7: PAB supportive medical therapy (65)

Medication	Dose	Comments
Bisoprolol	0.05-0.1/(0.2) mg/kg 1x day	Dose adaption to SAP/HR
Lisinopril	0.05-0.1/(0.2) mg/kg 1x day	Dose adaption to SAP
Spironolactone	2-3 mg/kg 1x day	For remodeling
(Methyl-) Digoxin	Loading: 0.01 mg/kg(8h,	If necessary, in addition to beta-

	followed by 0.008 mg/kg 1x day (serum level 0.5-0.8 nmol/liter)	blocker to control HR
Furosemide Hydrochlorotiazide	0.5-1 mg/kg SD 1/ (2) mg/kg SD	Treatment of lung edema, goal: no chronic diuretic therapy to achieve sufficient dosing of B + L
Supplement-drug therapy		
Coenzyme Q	10-15 mg/kg/day	All DCM patients
Riboflavin	3-20 mg/day	Mithocondrial disease
Carnitine	25-100 mg/kg/day	If deficient
Nicotinamide	50 mg/kg/day	Mithocondrial disease
Erythropoietin	100-150 U/kg 3 x week	Goal: 12-14g % hemoglobin

3.9.3.3 DEBANDING

Between 3 and 9 months after PAB, significant clinical and morphological improvements due to reverse remodeling of the LV are frequently recognized in PAB-responders, including the normalization of:

- LV-size;
- LV-ejection fraction percentage;
- Mitral valve regurgitation.

Most patients could be removed from the transplant waiting list and, complete or partial de-banding of the PA could be accomplished, using transcatheter balloon dilation with high-pressure balloons, based on their improved clinical and hemodynamic status.

When symptoms of exercise intolerance appear despite a fully functional LV, PAB debanding is recommended. In this setting, moderate tricuspid valve

regurgitation accompanies RV-distress with ventricular dilation, high-pressure gradient across PAB and elevated plasma BNP levels. Band enlargement may be accomplished in stages, with a minor residual right ventricle-main pulmonary artery pressure gradient of 15–30 mmHg as the end goal.

The technique of PAB-ballooning is determined by the annulus of the pulmonary valve. Balloons are usually smaller than the PA-valve annulus. The procedure begins with a high-pressure balloon with a diameter that is approximately 50% larger than the smallest measured PAB-diameter (for example 4–5 mm PAB vs. 8 mm balloon diameter); after that a bigger balloon is adopted, so as to achieve a partial or complete PA- debanding**.** Schranz et al. demonstrated therapeutic benefit only in infants and young children whose myocardium may have high potential for regeneration. However, until now, it has not been clearly defined who may benefit from this novel V-V interactive procedure (12)**.**

3.9.4 PADUA EARLY EXPERIENCE

Since 2015 (66), our Institution applied the Giessen methodology to treat ESHF in selected infants and children through PAB, which was found to be a valid strategy to avoid or at least postpone HTx in infants with DCM and preserved right ventricular function.

At our Institution, we enrolled seven selected patients, 4 males and three females, with a median age of 248 days (range 57 – 1288 days), with three of them manifesting fever, malaise, and upper respiratory tract infection before admission.

On admission their EF was 16.7 ± 6%, with mostly moderate mitral valve regurgitation, increased TAPSE and trivial or mild tricuspid regurgitation.

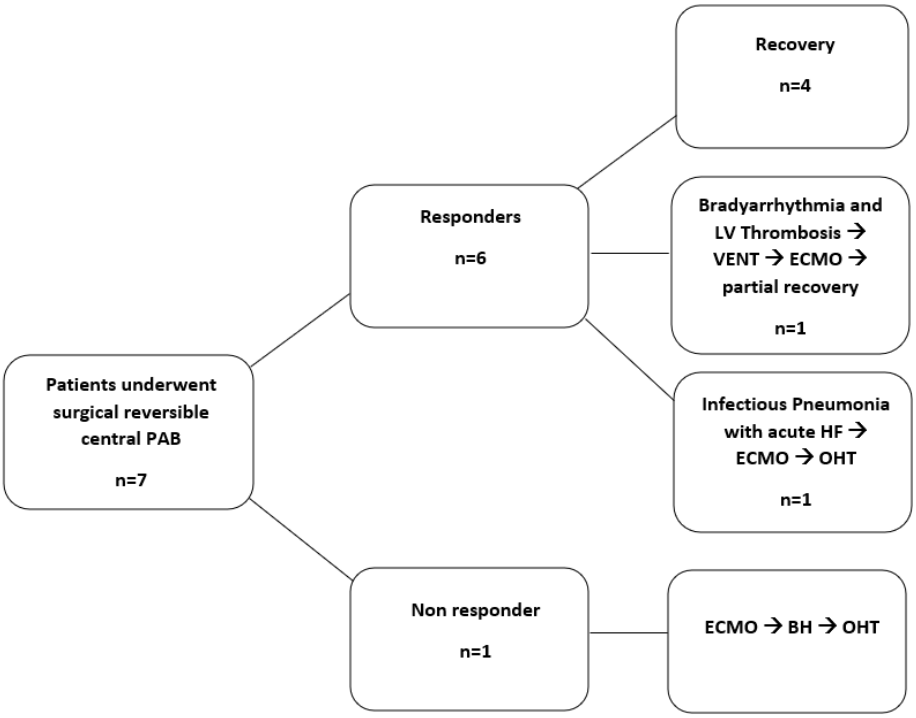


Figure 6: Synopsis. PAB, Pulmonary Artery Banding; FU, Follow-Up; ECMO, Extracorporeal Membrane Oxygenation; BH, Berlin Heart; OHT, Orthotopic Heart Transplant

After PAB, as expected form the Giessen experience, TEE showed decreased mitral valve regurgitation and leftward shift of interventricular septum in all.

One patient did not respond to PAB, whereas the other six did, but with different outcomes.

The "non-responder" (patient #7) was a 3.5-year-old male who was admitted emergently with left atrial massive dilation and underwent PAB associated with a decompressive atrial septostomy on pump. Although TEE showed some changes in intracardiac mechanics after the procedure, the clinical condition and cardiac function did not ameliorate, requiring ECMO support for 7 days. Despite its weaning off, the hemodynamic condition remained unstable with consequent need

of massive inotropic support and mechanical ventilation. Eventually, he underwent elective left Berlin Heart EXCOR® implantation 33 days after PAB and heart transplantation.

As for the other six patients, all of them initially responded well to PAB, which showed to have an immediate effect in supporting left ventricle function, by the acute increase in right ventricle pressure. Mechanical remodeling, such as ventricular septum shift to the left, with a change in the shape of the left ventricle and a decrease in its preload and mitral valve regurgitation, was observed immediately after PAB. However, they had different clinical post-operative courses.

Two patients experienced acute heart failure during pneumonia:

- Patient #6 (DCM in non-compaction cardiomyopathy), because of the distance from our center, was urgently admitted to another hospital due to severe low cardiac output syndrome, where he required emergent venous-arterial (VA) ECMO support, underwent Berlin Heart EXCOR® implantation and, after 7 days of VAD support, was successfully heart transplanted.
- Patient #2 (DCM in chronic myocarditis) was successfully treated in our hospital with intravenous inotropic infusion.

Similar clinical problem occurred in other two patients, without pneumonia:

- #1 with acute myocarditis, who presented a late acute deterioration of cardiac function, and required hospitalization with infusion of inotropic agents.
- #5, who suffered from bradyarrythmia and left ventricle thrombosis (surgically removed) and underwent VENT and ECMO implantation. Weaning from ECMO was later possible thanks to the improved haemodynamic conditions, with a partial recovery afterwards.

It is clear that, in order to avoid acute left ventricular deterioration driven by common pediatric problems as pneuomonia, a very strict follow- up with weekly evaluation and TTE assessment for the first months after PAB is strongly

suggested.

Lastly, two patients (#3 and #4) had an uneventful post-operative course and could be easily discharged.

Therefore, excluding the two patients who required HTx (#6 and #7) after PAB, the remaining five patients recovered with their native heart, manifesting a progressive improvement of symptoms, and left ventricular function, with three of them (#1, #2 and #4) having a complete cardiac function recovery, although #1 and #2 had early relapsing signs of CHF, concomitantly to lung infections, which required rehospitalization and inotropic therapy with levosimendan 3 and 2.5 months after PAB, respectively.

Patient #3 improved his cardiac function significantly, though without complete recovery at the time of follow-up, despite an uneventful clinical course. Similarly patient #5's cardiac status moderately ameliorated after PAB, despite the temporary early need of ECMO support following surgery.

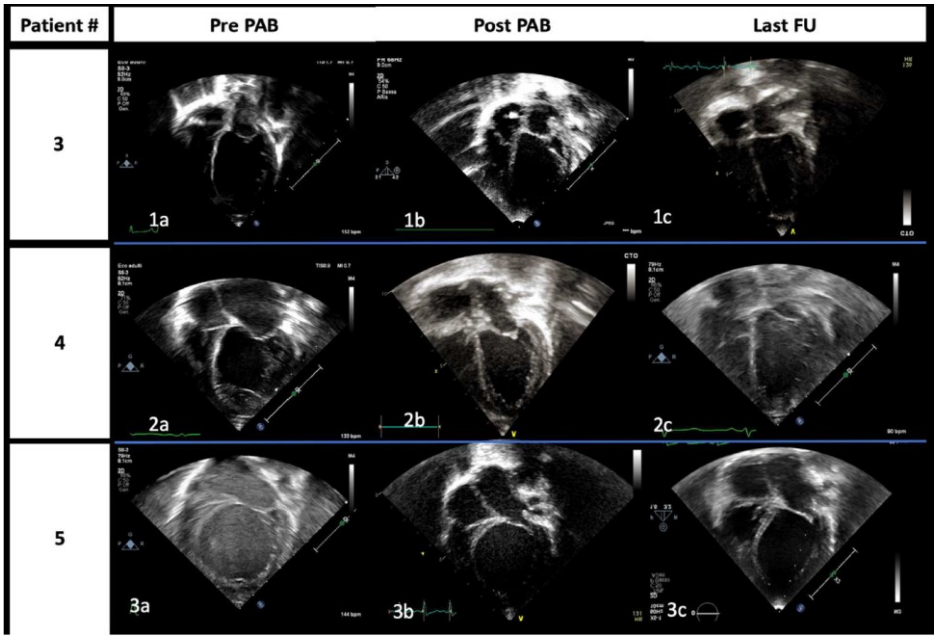
Three out of five patients who recovered with their native heart underwent an elective percutaneous PAB balloon dilation 18.5, 4.8, and 10.7 months (#4 and #5 missing) after PAB, respectively. The indication for PAB dilation is based on the presence of progressive right ventricle hypertension and hypertrophy, with increased tricuspid regurgitation.

At the last follow-up, these five patients were in Ross class I and thriving well, with echocardiogram showing improvement of EF, decrease of LVEDD, no or trivial mitral regurgitation, trivial or mild tricuspid regurgitation with preserved TAPSE values.

Moreover, plasma levels of B-type natriuretic peptide (BNP)/pro-BNP turned out to be decreased in all patients.

These findings, together with an average somatic growth and neuropsychological development out of the hospital, allowed to remove these patients from HT list.

Figure 7. End diastolic images from apical four-chamber view of patients #1 (1), #2 (2), and #3 (3) acquired at admission (a), immediately after PAB procedure (b), and at the last follow-up (c), respectively. The gradual improvement of left atrium and ventricle dilation is highlightened; it is of note that it is already evident immediately after PAB.



Considering the non responder patient, he was the only one older than 1 year of life (3.5 years) and had already a compromised hemodynamic state (cardiogenic shock) on admission in our center, where he arrived sedated and intubated. He presented a very dilated left atrium compressing the main left stem bronchus and causing air entrapment and lung damage. For this reason, he underwent PAB associated with atrial septectomy and ECMO support, but despite weaning off from ECMO, there was no improvement in cardiac function, and he finally underwent VAD support and HTx.

Because the presence of a dilated left atrium may imply a long-lasting myocardial dysfunction before the onset of symptoms, this may have been the cause that prevented a positive PAB effect.

On the other hand, this failure might be due to his age, because the regenerative myocardial capacity is inversely proportional to patients' age (37).

Learning from this experience, the advantages of the PAB procedure include:

1. The simplicity of the surgery, which does not require cardiopulmonary bypass, and can be tolerated hemodynamically by the patients as long as continuous echocardiographic monitoring is guaranteed.
2. The possibility to modulate PAB tightness both in the immediate post-operative period thanks to the delayed chest closure and by graded percutaneous balloon dilation of the band in the follow-up, when PAB is too narrow with high gradient trans-PAB, right ventricle dilation, and tricuspid regurgitation
3. Less risks as compared to VAD support. PAB is easier and less invasive, since it does not require left ventricle apicectomy, as for the VAD inflow cannula. Infections and thromboembolism, which may occur during MCS, are minimized as well.
4. Early management in the ward, patient's mobilization, and discharge home with weekly hospital evaluations.

PAB certainly avoided that four among five patients would undergo an invasive approach in the acute phase, such as MCS or emergent HT, but is uncertain if it was only a modality of transient support of the ventricular function that would have spountaneously recovered in any case.

Undoubtedely, it provided these children a possibility of complete remission with a simple procedure and an option to have a healthy life expectancy after recovery, without the negative consequences of life-lasting immunosuppressive therapy that come along with HTx.

4. AIM OF THE STUDY

PAB could represent a true “turning point” in the management of children affected by end-stage HF. However, many surgical institutions are still hesitant to apply this procedure due to the lack of comprehensive information about the ultimate clinical outcomes and the precise biological pathways activated by PAB. Further understanding of the efficacy of this technique and its underlying cellular and molecular working mechanisms is required to provide an evidence-based explanation in support of this approach and to promote the use of PAB for the treatment of pediatric HF.

For this reason, this study combines both clinical and experimental research.

The clinical investigation aims to report the current results of this innovative strategy across 4 centers in Europe (Padua, IT; Warsaw, PO; Ghent and Leuven, BE) and 1 in Asia (Manila, PH), through a multi-centric international retrospective study enrolling 31 patients.

The aim of the experimental study is to investigate the PAB’s effect on a rodent model (Sprague-Dawley) of DCM through functional echocardiographic examination and histological and molecular analysis on heart tissue samples, in order to understand the molecular mechanisms behind the ventricular rehabilitation and to prove the potential PAB-induced myocardial regeneration due to cardiomyocyte proliferation.

5. MATERIALS AND METHODS

5.1. CLINICAL STUDY

5.1.1 Sample

A total of 31 patients were enrolled in this multicentric international retrospective study, which involved five centers:

1. Padua, Italy (7 patients)
2. Ghent, Belgium (8 patients)
3. Manila, Philippines (4 patients)
4. Warsaw, Poland (7 patients)
5. Leuven, Belgium (5 patients)

In each Institution, infants and children admitted for ESHF due to any kind of DCM not responding to conventional medical therapy were selected.

Exclusion criteria included:

- Age > 5 years
- No parental consent
- Biventricular failure
- Concomitant structural (congenital) heart disease, except for repaired ALCAPA
- Moderate-severe tricuspid regurgitation
- Pulmonary hypertension out of proportion with left ventricular end-stage cardiomyopathy

5.1.2 Evaluation and measures

These patients were monitored by routine non-invasive imaging (2 D echocardiography and CMRI) to assess values of heart function and volumes.

Clinical, echocardiographic and CMRI data from the five centers were collected together on a REDCap (Research Electronic Data Capture) database (*Supplemental Tables 29-32*).

Each patient was investigated in regard to demographic data on admission, to operative data prior to and after-PAB, and to information related to the follow-up.

Table 8: demographic, operative and last FU main data.

Demographic data	***Operative data***	***Last FU data***
o Age at PAB	o Procedures prior to PAB	o Length of follow-up
o Weight at PAB	o Associated surgical procedure	o Possible late death
o Associated congenital heart disease (CHD)	o PAB peak gradient at the end of the procedure	o Adverse event (AE), other than death during follow-up
o Etiology of ESHF	o Leftward interventricular septum displacement after PAB	o Therapy at last FU
o Endomyocardial biopsy if performed	o MR reduction	o PAB dilation during FU
o Associated genetic syndrome	o Delayed chest closure	o Presence of Sinus Rhythm
	o INTERMACS level	o NYHA/Ross Class
	o Preoperative intubation	o Levosimendan infusion therapy after PAB (timing and number of cycle)

	○ Preoperative infusion of Levosminedan ○ Cardiopulmonary bypass ○ Postoperative complications after PAB ○ Length of ICU stay ○ Length of hospital stay ○ Intubation time after PAB ○ Early death ○ Early reintervention (date and which reintervention) ○ Date of D/C home ○ Home therapy ○ NYHA/Ross class at discharge (D/C) ○ PAB peak-pressure gradient at discharge	

Echocardiography and, wherever possible, Cardiac Magnetic Resonance Imaging (CMRI) assessment were performed on admission, and at discharge, 3 months, 6 months, 12 months and at last follow-up.

Table 9: evaluated Echo and CMRI parameters

ECHO PARAMETERS	CMRI PARAMETERS
○ LVEF (%)	○ LVEDV (ml/m^2)
○ PAB-peak pressure gradient at CW (mmHg)	○ LVESV (ml/m^2)
○ Mitral regurgitation and grade	○ LVEF (%)
○ LV dilation	○ LVSV (ml/m^2)
○ LV dimensions (M-mode, mm)	○ LVCO (ml/m^2)
○ LVEDV (Simpson rule, ml/m2)	○ LVCI (l/min/m^2)
○ TAPSE (mm)	○ LVCM (g/m^2)
○ Tricuspid valve regurgitation	○ RVEF (%)
	○ RVEDV (ml/m^2)
	○ RVSV (ml/m^2)
	○ RVCO (l/min)
	○ RVCI (l/min/m^2)
	○ RVCM (g/m^2)
	○ Presence of myocardial oedema
	○ Presence, site, and type of myocardial fibrosis (LGE)

We also defined the parameter “PAB-Efficacy-Index” as the overall freedom from death/VAD/HTx.

5.1.3 Statistical analysis

Data are expressed as as a percentage, as median and interquartile range or as mean and standard deviation. Comparisons between categorical variables were performed by Fisher's test, while for continuous variables the non-parametric Kruskal-Wallis test was used with pairwise comparison by Dunn's test and p-value adjusted by Holm's method.

5.2 EXPERIMENTAL STUDY 1

5.2.1 Animals

46 Wild-type Sprague-Dawley (SD) rats were used for the experiments, 42 males and 4 females, 3 young rats (5-6 weeks, 153-176 gr.), 20 young-adults rats (6-12 weeks, 356-466 gr.) and 23 adult rats (>12 weeks, 244-630 gr.)

Rats were divided as follows:

Table 10: Experimental groups

Procedure	***n***
DOX-injection	16
LAD-ligation	8
SHAM	3
DOX-injection + PAB	13
LAD-ligation + PAB	6

These animals were housed in standard conditions, allowing them free access to water and food according to international rules (67) and without putting them in fasting conditions the night before surgery.

This experimental study was approved by the OPBA ("Organismo Preposto al Benessere degli Animali", i.e. the entity in charge of animals' welfare) of the University of Padua and by the Ministry of Health.

5.2.2 Surgical instruments and sutures

A micro-surgery set of instruments was used to perform surgeries, including:

- Fine scissors (Rudolf Medical, RU-2422-11), for skin and muscles opening
- Bipolar forceps (Jewler 5, 30665) and electrosurgical equipment (Surton 2009), to cut tissues and/or to cauterize blood vessel.
- Alm retractor (RU 4651-07, Rudolf Medical), to open the chest and provide access to the heart
- Straight Micro Jewelers Forceps, 11 cm, 0,3 mm tip (RU 4240-04, Rudolf Medical)
- Straight Spring type micro scissors, 14 cm (RU 2380-14, Rudolf Medical)
- Ring tip micro forceps (RU 4079-14, Rudolf Medical), for delicate manipulation
- Fine-tip curved Vannas micro scissors (Aesculap, OC497R)
- Stainless steel needle holder, Castrovejo (J 4065)
- Curved Forceps (Galiazzo, OC-22), to pass the band during PAB.

Both LAD-ligation and PAB surgeries were performed with the help of a stereomicroscope M400E (Leica Microsystems Italia srl, Milano, Italia), with 6x and 10x magnification.

The main sutures used throughout the surgeries were:

- Non-adsorbable 6-0 Prolene (ETHICON) for LAD-ligation

- Non adsorbable 2-0 silk suture (ETHICON), used as the band for PAB
- Adsorbable 3-0 vycril suture (ETHICON) for muscle closure.
- Non-adsorbable 4-0 suture (ETHICON) for skin closure.

5.2.3 Anesthesiological protocol for surgery

Fifteen minutes prior to surgery, rats were treated with subcutaneous injections of the analgesic Tramadol (CONTRAMAL ®, Forementi Srl, Milano, Italia).

For the induction stage of anesthesia, a filtered vaporizer Fluovac Sevoflurane/Halotane (Harvard Apparatus Ldt, Kent, England) was used, pumping 4% Sevoflurane (SEVORANE, Abbott SpA, Campoverde, Italia) into a Plexiglas box, together with an oxygen flow of 1 L/min.

Once asleep, animals were kindly pulled out of the box and underwent oro-tracheal intubation through a 16G cannula.

After intubation, animals were ventilated with 1 L/min of oxygen and 2.5% Sevorane, using the Rodent Ventilator 7025 (Ugo Basile, Italia). The parameters were set as follows:

- Respiratory frequence (RF) = 65-75 breathes per minute
- FiO2 = 100 %
- PEEP = 2 cmH2O
- PEAK = 12 cmH2O.

To avoid the risk of pneumothorax, a 14 G needle-cannula connected to a syringe was frequently left in the chest at a negative pressure until the last muscle suture was completed.

After chest, muscle and skin closure, the alogenated anesthetic administration was ultimately suspended and, with the resumption of spontaneous ventilation, the tracheal cannula was removed.

5.2.4 Procedures

5.2.4.1 Doxorubicin injections

Doxorubicin (DOX) is a chemotherapeutic agent, whose cardiotoxic effects could be used to establish a model of dilated cardiomyopathy in rats.

Many protocols are described in literature and among them we chose a short-term protocol, consisting of six intraperitoneal injection of DOX (2.5mg/0.5mL/Kg i.p.) every other day for two weeks for a total cumulative dose of 15 mg/Kg (68), allowing a short duration of experiment and reducing costs.

The DOX-injection was done under The Esco Airstream® Class II Microbiological Safety Cabinet (AC2-4S model), with the use of proper DPIs to protect eyes, face and skin in general.

Some of these rats, during the follow-up, underwent blood sampling (100 μL) through tail vein puncture.

5.2.4.2 LAD-ligation

Another option to induce a rat model of DCM consists of the evolution of an ischemic injury induced by the temporary ligation of the Left Anterior Descending artery (LAD).

The anesthesiological protocol was carried out as previously described.

After tricotomy and a standard skin preparation, a left-anterolateral thoracotomy was practiced in the 3rd intercostal space. Then, pericardium was opened by pulling it up to avoid heart's injury, the left lung boarder was pushed to the side using a sponge gauze and the left anterior descending artery (LAD) was localized 1-2 mm below the junction of of the pulmonary conus and the left atrial appendage. A 6-0 silk suture with a curve needle was used to ligate the LAD from the left border of the pulmonary conus to the right border of the left atrial appendage.

By doing so, the ventricular region below the artery usually changed its color, becoming pale.

After 30 minutes of ligation, the surgical knot was cut off allowing the reperfusion of the entire ventricle.

Chest, muscles and skin were ultimately closed through 3-0 and 4-0 sutures.

During the surgery, blood samples (100 µL) were collected through tail vein puncture.

5.2.4.3 Pulmonary Artery Banding

For the procedure, we looked back at a previous experimental experience of our centre (69), where PAB was carried out in order to induce mechanically right ventricular hypertrophy.

Anesthesia, intubation, and thoracic opening was identical to that described for the LAD-ligation surgery.

In case of rats undergoing both LAD-ligation and PAB, the two surgeries were performed together within the same operation, in accordance to European laws which forbid performing multiple surgeries on rats separated in time.

Once the main pulmonary artery and the aorta were recognized and isolated, a curved forceps was passed between them in order to encircle the MPA with the band, which consisted of a a 2-0 silk suture thread. Once the extremities of the thread were located on the anterior wall of the MPA, they were placed near and tied together with a 7-0 prolene suture in order to tight the band, whose length is 3.5 mm for young and 4.5 mm for adult rats.

Then chest, muscles and skin were closed as described for LAD-ligation procedure.

During the surgery, blood samples (100 µL) were collected through tail vein puncture.

5.2.4.4 Sham

The SHAM operation consisted of a procedure that included anesthesia, intubation, opening and closing of the chest, without performing the band placement. The rats of this group only experienced surgical stress.

5.2.5 Post-operative course

In the pre-operative period and during the post-operative course, animals were housed in cages, in a controlled lit and air-conditioned space, in accordance with law. Tramadol 5 mg/Kg s.c. was administered twice a day for the first 72 hours post-surgery, then if needed.

Animals were checked daily by animal care personnel and researchers, during the post-operative course.

An evaluation system called “score pain system” was used for the identification of clinical signs of possible animals’ suffering *(Table 11)*

Table 11. Pain scoring system

Clinical manifestation	Scoring
None	0
Ruffled fur	1
Ruffled fur, reduced physical activity	2
Ruffled fur, reduced physical activity and loss of weight between 10 and 15%	3
Ruffled fur, reduced physical activity, respiratory distress, and loss of weight between 15 and 20%	4
Ruffled fur, reduced physical activity, respiratory distress, loss of weight > 20%, kyphosis	5

In case of moderate illness, a supportive medical therapy was tried out only in some of the rats, consisting of 0.3 mg/Kg/day Bisoprolol dissolved in water.

5.2.6 Sacrifice

In case of evident illness, toxicity, physical deterioration, weight loss (>20% loss, compared to the weight measured the day of the surgery), animals were immediately euthanized through their exposure to CO2 for few minutes.

Sacrifice was also performed for each rat as they reached the endpoint of the study.

Simultaenously, the abdomen and chest of these animals were opened so as to take samples of blood (from the abdominal aorta) and organs including spleen, liver and heart, which were submitted to further molecular and histopatological studies.

5.2.7 Evaluation and measures

Echocardiography

During follow-up, cardiac function was assessed through high resolution EcocolorDoppler VEVO® 2100 (13-24MHz probe). Echocardiogram was performed at baseline, after Doxorubicin-injection and LAD-ligation (to assess the cardiac damage), after PAB and whenever possible at 15 and 30 days or just before the sacrifice.

Using M-Mode mode with short axis, we investigated the following measures:

- EF (Ejection Fraction, %)
- FS (Fractional Shortening, %)
- LVPWd (End-diastolic left ventricular posterior wall thickness, mm)
- LVIDd (End-diastolic left ventricular internal diameter, mm)
- IVSd (End-diastolic interventricular septal thickness, mm)

Histopathology

During 2021-2022, the Cardiovascular Pathology Unit of the University of Padua, received blood and cardiac samples from 38 rats, including:

- 3 SHAM
- 17 post-DOX administration
- 7 post-LAD ligation
- 5 post-PAB on DOX-treated rats
- 6 post-PAB on LAD-ligated rats

All cardiac samples were photographed, weighed and then prepared for histological, ultrastructural and molecular examinations.

As for the histological exam, samples were fixed in 10% buffered formalin and then routinely processed. 5 μm sections were stained with Hematoxylin and Eosin (H&E) for initial morphologic evaluation of:

- Cardiomyocyte diameter
- Perinuclear halos
- Cytoplasmic vacuolization

Sections were also stained with Azan-Mallory in order to detect fibrosis.

Sections were observed through Olympus BX51 optical microscope, connected to Image Pro-Plus program for the measurement of cardiomyocyte diameter and the quantification of possible fibrosis.

As for the ultrastructural examination, 2x2 mm samples were fixed in Karnovsky solution and routinely processed for electron microscopy. Ultra-thin section were observed through an Hitachi Transmission Electrone Microscope (TEM) to assess potential loss of cardiomyocytes' contractile components and mitochondrial alterations.

The molecular analysis was performed on 5 mm fragments of heart tissue which were frozen according to the "Snap Frozen" method and then preserved at -80°C.

Molecular study

Isolation of mi-RNAs from frozen whole blood

RNA was isolated from blood samples preserved in EDTA test tubes at -80°C. Total RNA, including small-RNA, was isolated using the MagMAX mirVana Total RNA Isolation kit (ThermoFisher Scientific, Waltham, MA, USA). First, enzymatic digestion of the samples through Proteinase K (PK) was performed to remove proteins present in the blood: 35 ul of PK Digestion Mix (PK + PK Digestion Buffer) were added to 50 ul of whole blood. The preparation was then homogenized through Termomixer compact (Eppendorf, Hamburg, Germany) for 5 minutes at 950 rpm and then incubated at 65 °C for 10 minutes.

Before proceeding with the subsequent extraction steps, 3.5 µl of "cell-39 working solution" were added to each sample, at a concentration of 1.6x108 copies / µl. It is a solution containing C. elegans miR-39 (Qiagen, Hilden, Germany): an exogenous control miRNA, defined as "spike-in control", derived from the nematode C. elegans and used for the normalization of the expression profile of miRNAs from blood or plasma. Magnetic RNA-binding beads were then added to each sample (20 µl of Binding Beads Mix, previously prepared) in order to isolate and retain the nucleic acid. The preparation was stirred for 5 minutes at 850 rpm. The samples were subjected to cell lysis by adding 65 µl of lysis mix (β-mercaptoethanol + lysis buffer), mixing for 5 minutes at 800 rpm. 135 µl of isopropanol was added and the preparation was further stirred for 10 minutes at 700 rpm.

The tubes were then inserted into the magnetic support for 5 minutes: in this way the magnetic beads are retained on the wall of the tubes and it is possible to aspirate the supernatant without taking the RNA bound to the beads. After removing the supernatant and extracting the tubes from the magnetic support, 150 µl of Wash Solution 1 were added to proceed with the first wash. The beads were resuspended in the washing solution until a homogeneous mixture was obtained and the supernatant was then removed again with the use of the magnet. This step was repeated with 150 µl of Wash Solution 2. The samples were shaken for 2 minutes at 1150 rpm with the cap of the tubes open to facilitate the evaporation of isopropanol residues and dry the beads.

The TURBO DNase Solution was at this point used to eliminate the DNA present in the samples. 50 µl of this solution (previously prepared) were added and the

beads resuspended until a homogeneous mixture was obtained. The preparation was stirred for 15 minutes at 1050 rpm. Avoiding mixing the two reagents together, the Rebinding Buffer (50 µl) and isopropanol (100 µl) were added, and the total content was stirred for 5 minutes at 950 rpm. The tubes were reinserted into the magnet for 5 minutes, the supernatant was removed, and two washes were performed with Wash Solution 2 (as described above).
Finally, the Elution buffer was used to elute the RNA: 50 µl of buffer previously heated to 65 ° C were added and the beads resuspended in it. The samples were mixed for 2 minutes at 1150 rpm, incubated at 65 ° C for 5 minutes and stirred again for 2 minutes at 1150 rpm. After inserting them into the magnet for another 3 minutes, the supernatant containing the RNA was collected.

Quantification of RNA
The extracted total RNA underwnt quantitative and qualitative analysis before being processed.

1) Spectrophotometric method

The determination of the sample concentration was performed with the NanodropOne spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) which provides a quantitative and qualitative analysis of DNA, RNA and proteins. The instrument measures the absorbance of light at 260 nm by exploiting the ability of nucleic acids to absorb UV radiation with a peak of maximum intensity at this wavelength (λ). By applying the Lambert-Beer law it is possible to derive the nucleic acid concentration starting from the measured absorbance. Before proceeding with the measurement of the sample, it is necessary to perform a measurement, called "white reading", with H2O DNase and RNase free in order to calibrate the instrument and eliminate any background noise. After loading 2 µl of the sample in the indicated point, the Nanodrop automatically returns the RNA concentration and absorbance ratios A260 / A280 and A260 / A230. The latter provide information on the purity of the extracted molecule. Low values of the A260 / A280 ratio indicate possible protein contamination: the peptide bond absorbs UV radiation at 280nm. Low values of the A260 / A230 ratio, on the other hand, indicate contamination of organic

solution. Good RNA preparations show values of both ratios around the value 2 (A260 / A280 between 1.8 and 2.0; A260 / A230 between 2.0 and 2.2).

2) Fluorimetric method

The quantification of the extracted nucleic acids was also carried out by fluorimetric method. It is a highly sensitive method based on fluorimetry that allows for more accurate quantification than the spectrophotometric method described above. It uses a fluorophore that binds to specific target molecules allowing their detection.

The protocol for the quantification of miRNAs consists of the preparation of a "Working solution" (WS) comprised of 199 µl of buffer (Qubit microRNA Buffer) and 1 µl of fluorophore (Qubit microRNA Reagent) for each sample to be quantified. Reagents are provided by the Qubit microRNA Assay kit. The prepared solution must be kept in the dark because the fluorophore is sensitive to light.

Before analyzing the samples, it is necessary to perform two standard measurements with Standard 1 and Standard 2 solutions to calibrate the instrument. Two test tubes are then prepared in which 190 µl of WS and 10 µl of standard solution are placed. It is homogenized with the vortex for a few seconds and left to incubate at room temperature in the dark for 2 minutes. The measurement is then carried out using the Qubit 3.0 fluorimeter (Invitrogen, Carlsbad, California, United States) which determines a calibration line necessary to obtain the concentration of the samples measured subsequently. The analysis of the samples takes place in the same way but 199 µl of WS are used, adding 1 µl of sample RNA. The final volume of each tube under test should be 200 µl.

NEXT GENERATION SEQUENCING (NGS)

The rapid advancement of next-generation sequencing technologies (NGS) has made it possible to efficiently analyze the expression profile of miRNAs. The NGS provides thousands of sequences with a single experiment since it allows to process multiple samples in a single sequencing session, producing a large amount of data. With this technique it is possible to analyze known miRNAs by making a relative quantification to study their expression but also to identify new miRNAs.

NGS was performed with the Lexogen Small RNA for Illumina kit (Illumina Compatible, Lexogen) on 6 whole blood samples, 2 controls (sham, #P1001, #P1002), 2 Doxorubicin-injected rats (#P0802, #P0704) and 2 Doxorubicin + PAB rats (#P0801, #P0810). In general, the method applied for the analysis of miRNAs involves the following phases:

A) Preparation of miRNA libraries:
- Ligation of 3 'and 5' adapters to miRNAs
- Retro-transcription (RT) of miRNAs
- PCR amplification of the cDNA obtained from RT
- Library quality control
- Library purification

B) Sequencing

C) Data analysis

A) Preparation of miRNA libraries

Ligation of 3 'and 5' adapters to miRNAs

The first step necessary for the construction of libraries involves the ligation of a 3 'adapter (3' 4N Adapter Ligation) to the total extracted RNA to allow the recognition of the 3'-OH end of the miRNAs present in the sample. An initial amount of total RNA of 150 ng is used for each sample, diluted to a final volume of 10.5 µl with Nuclease-free H2O. As indicated in the user manual, a reaction mix is prepared with the following reagents for each sample: 10.5 µl of RNA, 1 µl of 3 'adenylated adapter, 7 µl of ligation buffer and 1.5 µl of enzyme of ligation. The reaction is incubated at 25 ° C for 2 hours in the thermal cycler.

Subsequently, the excess adapter 3 'is removed by adding, for each sample, 25 µl of adapter depletion solution and 40 µl of beads (Cleanup Beads). Then 60 µl of isopropanol are added and the samples are placed on a magnet for 5 minutes, removing the supernatant at the end. Two washes are then carried out with 80% ethanol and the samples are removed from the magnetic support. The beads are completely resuspended in 22 µl of Resuspension Buffer. 20 µl of supernatant are transferred to a new well and the above steps are repeated again.

The excess adapter is then inactivated by preparing the following reaction mix: for each sample, 11.5 µl of sample derived from the previous phase, 2 µl of adapter inactivation buffer and 0.5 µl are mixed of enzyme inactivating the adapter. The reaction is incubated at 12 ° C for 15 minutes, at 50 ° C for 20 minutes and kept at 4 ° C. To isolate the miRNAs present in the samples, a 5 'adapter (5' 4N Adapter Ligation) is also used, which binds the 5'-P end of the miRNAs. The reaction mix includes, for each sample, the following reagents: 14 µl of sample derived from the previous phase, 1.5 µl of 5 'adapter, 7.5 µl of ligation buffer and 2 µl of ligation enzyme. The reaction is incubated at 20 ° C for one hour.

Reverse transcription of miRNAs

The miRNAs must be back-transcribed in order to obtain the cDNAs to be amplified. 25 µl of the sample obtained are mixed, i.e. the miRNAs ligated by adapters 3 'and 5', with 13 µl of RT buffer and 2 µl of retro-transcriptase. The reaction is incubated at 42 ° C for 30 minutes and at 90 ° C for 10 minutes.
Before amplifying the obtained cDNAs, Cleanup Beads are used to purify the back-transcribed miRNAs.

PCR amplification of the cDNA obtained from RT

To carry out the PCR, a universal primer (Universal Primer) is used which binds to the 5 'end of the cDNA and a specific primer (Barcoded Primer) which recognizes the 3' end. A different Barcoded Primer is used for each sample for which multiple sequencing analyzes are performed. The reaction mix includes, for each sample, the following reagents: 18 µl of back-transcribed sample, 1 µl of Universal Primer, 1 µl of Barcoded Primer and 5 µl of PCR Master Mix. The reaction cycles (12-25 cycles) consist of a denaturation phase at 95 ° C for 2 minutes, an annealing phase at 95 ° C for 20 seconds, 60 ° C for 30 seconds and 72 ° C for 15 seconds, an elongation phase at 72 ° C for 2 minutes.

Library quality control

The PCR products were analyzed using the Agilent 4200 TapeStation (Agilent, Santa Clara, CA, USA) to verify the quality of the prepared library and the

distribution of fragments. It is a microfluidic platform that allows both quantitative and qualitative analysis of DNA through automatic capillary electrophoresis. The kit used is the Agilent DNA 1000 ScreenTape Assay (Agilent, Santa Clara, CA, USA) which analyzes DNA fragments by separating them based on their size and is designed to analyze DNA in a range between 35 bp and 1000 bp. The protocol initially involves loading the microchannel gel into the instrument. To determine the concentration of the samples, a marker is used that delimits the length range of the fragments to be analyzed. First, the ladder is prepared by mixing 3 µl of D1000 Sample Buffer with 1 µl of D1000 ladder in position A1 of a strip. In the subsequent positions of the same strip, the samples to be analyzed are prepared by mixing 3 µl of D1000 Sample Buffer with 1 µl of sample. After mixing with vortex for 1 minute and centrifuged, the strip is loaded into the instrument and the analysis proceeds. The quality of the DNA is determined by the interpretation of the distribution curve of the size of the fragments that make up the library. The presence of a peak at about 150 bp and the absence of a peak at about 130 bp indicate that the library has been correctly prepared, and it is possible to proceed with its purification.

Library purification

The prepared library was purified using the Gel-Free Size Selection & Cleanup procedure. Initially 32.5 µl of Cleanup Beads are added to 25 µl of each sample, leaving the samples on the magnetic stand for 5 minutes. The supernatant, containing the amplified product, is transferred to a new well and the plate is removed from the magnetic support. Subsequently, the same steps described above are repeated, adding 30 µl of Cleanup Beads to each sample and carrying out 2 washes with 80% ethanol. Then 13.5 µl of Resuspension Buffer is added to resuspend the beads, placing the samples in the magnetic holder for 3 minutes. Finally, 12 µl of supernatant are transferred to a new well.

The quality of the final library is determined using the Agilent 4200 TapeStation (Agilent, Santa Clara, CA, USA), by calculating the average size of the fragments in the range from 100bp to 300bp. The concentration of the samples is measured with the Qubit dsDNA HS Assay (Invitrogen, Life Technologies, Carlsbad, CA,

USA) and finally the molarity of each sample is calculated. The different samples are then combined in equimolar quantities.

B) SEQUENCING

Sequencing takes place inside a small flow-cell V3 with a single-read run performing 50 cycles in a MiSeq platform (Illumina, San Diego, CA, USA). Before being loaded into MiSeq, the final library is diluted with the Resospension Buffer to a final concentration of 1.5 nM, 10 µl are taken from the latter and mixed with 10 µl of 0.1N NaOH to denature the library. 1 ml of sample at a final concentration of 15 pM is loaded into the MiSeq cartridge.

C) DATA ANALYSIS

Following the run, the program generates a file in FASTQ format for each sample. Each file shows the sequenced reads and the quality scores for each sequenced base.

FASTQ files are processed through a bioinformatics pipeline which includes software:

• FASTQ Toolkit (BaseSpace Labs, Illumina) which allows to eliminate the adapters (trimming) introduced during the preparation of the library;

• Small RNA (Illumina Inc) which allows to align the reads with 4 reference databases (abundant, mature miRNA, other RNA and genomic) and create lists of mature miRNAs, isomiRs and piRNAs. This software allows both the analysis of the differential expression of miRNA, piRNA or precursors, and the discovery of new precursors (Isis Analysis Software: 2.5.52.11; SAMtools: 0.1.19-isis-1.0.2; Bowtie Aligner: 0.12. 8; miRDeep *: 3.2; DESeq2: 1.0.17).

5.2.8 Statistical analysis

Data are expressed as a percentage or as mean and standard deviation.

5.3 EXPERIMENTAL STUDY 2

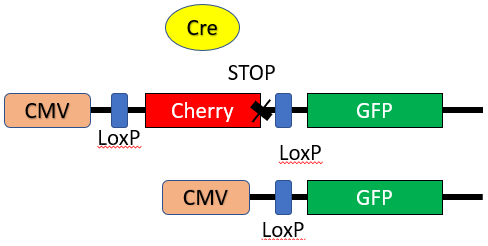
5.3.1 Procedure

As the main project proceeded, we also started a collaboration with the ICGEB (International Centre for Genetic Engineering and Biotechnology) of Trieste, with the aim to assess, through Adeno-Associated Virus (AAV) vectors, potential PAB-induced cardiomyocyte proliferation.

We selected 6 healthy young rats (5-6 weeks of age, weight 161-210 gr.), without any induced heart damage.

To track proliferating cardiomyocytes in rat hearts upon PAB, we established a pilot experiment in juvenile rats of 10 days. A cohort of animals underwent PAB (3) and simultaneously received an AAV fluorescent-based reporter system to monitor cell transduction and myocyte proliferation, while control animals received only the vectors without banding.

For each heart we injected twice 40 ul in the left ventricle for a viral titer of 3x10^11 vg/animal.



The AAV9 Cherry-GFP dual reporter system is allowing genetic labelling of proliferating myocytes.

Figure 8: AAV9 Cherry-GFP dual reporter system

The protein Cherry is under the control of the CMV ubiquitous promoter and was used as a marker of cell transduction, while the expression of lox-flaked GFP is normally inhibited.

Together with the reporter, animals were co-injected with an AAV9 expressing the Cre recombinase under the control of the Cyclin B promoter to label only GFP+ proliferating CMs or with an AAV9 expressing a CMV driven-Cre recombinase to monitor and quantify the recombination capacity of the system. Indeed, an ubiquitous expression of Cre recombinase will permanently allow the

GFP expression in all the transduced CMs. AAV vectors were produced by the AVU core facility of the ICGEB (https://www.icgeb.org/avu-core-facility/).

In parallel, all rats were also administered intraperitoneally with EdU (5-ethynyl-29-deoxyuridine), a uridine analogue that is incorporated into newly synthesized DNA. The injections were performed every other day up to sacrifice at a concentration of 30mg/kg. After 10 days, animals were sacrificed, the heart perfused with formalin and tissue were paraffin embedded.

5.3.2 Immunofluorescence

Cardiac proliferation was monitored by IF by staining for alpha actinin (CMs), DNA duplication (Edu) or by quantifying the number GFP+ labelled CMs. The CMV-Cre reporter was used as AAV transduction normalizer. In parallel, fiber hyperthrophy was evaluated by WGA lectin stain. Briefly, sections of rat hearts were deparaffinized, rehydrated and then underwent antigen retrieval by boiling in sodium citrate solution for 20 min. Slides were processed for immunofluorescence by permeabilizing with 0.5% Triton X-100 in phosphate buffered saline (PBS) solution for 15 min, followed by 1h min blocking in 2% BSA (Roche). Sections were then stained overnight at 4 uC with the following primary antibodies diluted in blocking solution: mouse monoclonal antibody against sarcomeric a-actinin (Abcam, 1:300), (1:300), Gfp (Roche, mouse), Cherry (Abcam, 1:1000, rabbit). Sections were washed with PBS and incubated for 2 h with the respective secondary antibodies conjugated to Alexa Fluor-488, -555 or -647 (Life Technologies). For wheat germ agglutinin (WGA)staining, heart sections were deparaffinized, rehydrated and then incubated for 1 h at room temperature with WGA conjugated to Alexa Fluor-594 (50 mgml21, Life Technologies) in PBS. Slides were then rinsed in PBS and mounted in Vectashield. When indicated, cells were further processed using the Click-IT EdU 594 Imaging kit to reveal EdU incorporation, according to the manufacturer's instructions, and stained with Hoechst 33342 (Life Technologies). Slides were then mounted in Vectashield with DAPI (Vector Labs).

5.3.3 Image acquisition and analysis

All acquisition was performed with a fluorescent microscope and eventually confocal microscopy was used to confirm GFP-EDU double positive cells. At least 9 different heart sections in 3 different level were used for quantification of CM proliferation, analysing separately left and right ventricle data (ongoing).

5.3.4 Statistical analysis

Data are expressed as a percentage or mean and standard error of the mean (SEM); Student's two-sided t-test was used for continuous variables' comparison.

6. RESULTS

6.1 CLINICAL STUDY

Table 11. Demographics of patients undergoing PAB surgery

VARIABLE	**Pts**	**%**
Total patients	31	100
Age at PAB (days, median, IQR)	210 (131-357)	
Weight at PAB surgery (kg, median, IQR)	6.4 (5.20-8.15)	
Male	18	58
Associated congenital heart disease (CHD)	3	9.7
ALCAPA	2	
PDA	1	
Etiology of ESHF		
Idiopathic dilative cardiomyopathy	**15**	**48.4**
-Mitochondrial cardiomyopathy	*1*	
-Covid IgG positivity	*1*	
Acute viral myocarditis	***2***	**6.5**
-Post-CMV	*1*	
Chronic viral myocarditis	**5**	**16.1**
Spongy myocardium	**1**	**3.2**
Genetic dilative cardiomypathy	**5**	**16.1**

-Heterozygotic carrier of new molecular variant in PRDM16 gene	*1*	
-Heterozygotic carrier of new molecular variant in MYBPC3 gene	*1*	
-Heterozygotic carrier of two molecular variants in MYL2 gene	*1*	
-Heterozygotic carrier of new molecular variant in MYH7 gene	*1*	
Post-ischemia	**2**	**6.5**
Other (not specified)	**1**	**3.2**
Endomyocardial biopsy	9	29
Normal	**1**	
Inconclusive, but excluding viral myocarditis	**1**	
Inflammatory DCM with endocardial fibroelastosis	**2**	
Chronic active myocarditis	**2**	
-Parvovirus-B19-induced	*1*	
Active lymphocytic myocarditis	**1**	
Interstitial disease	**1**	
Mitochondrial cardiomyopathy	**1**	
Associated genetic syndrome	2	6.5
Other (not specified)	*2*	

Legend: PAB, pulmonary artery banding; ALCAPA, anomalous left coronary artery from the pulmonarty artery; CMV, Cytomegalovirus, DCM, dilated cardiomyopathy.

Table 12. Operative data of patients undergoing PAB surgery.

VARIABLE	**Pts**	**%**
Total patients (n)	31	100
Echo assessment on admission	27	87.1
CMRI assessment on admission	10	3.2
Associated surgical procedure	3	9.7
Atriosettostomy + ECMO	*1*	
PDA transection 3.7 mm	*1*	
ALCAPA repair	*1*	
PAB peak-gradient at the end of procedure (mmHg, median, IQR)	29 (23-34) (n=30)	
Leftward interventricular septum displacement after PAB	27	87.1
MR reduction after PAB	13	41.9
Delayed chest closure	7	22.6
Within 48h	*3*	
Within 72h	*1*	
After 72h	*3*	
Procedure prior to PAB	6	19.3
ALCAPA repair	*2*	
Balloon atrial septostomy	*4*	

Listed for HTx	8	25.8
INTERMACS level		
Critical cardiogenic shock	*3*	*9.7*
Progressive decline	*6*	*19,4*
Stable but inotrope dependent	*19*	*61.3*
Resing symptoms	*1*	*3.2*
Exertion intolerance	*1*	*3.2*
Exertion limitation	*1*	*3.2*
Advanced NYHA Class III	*0*	*0*
Uunknown	*0*	*0*
Preoperative intubation	15	48.4
Preoperative infusion of levosimendan	22	71
Cardiopulmonary bypass during cardiac surgery + cardioplegic arrest	1 (n= 25)	4
Length of ICU stay (days, median, IQR)	13 (7-24) (n=30)	
Postoperative complications (n)	14 (n=30)	46.7
LCO syndrome	*5*	*35.7*
Bleeding requiring reoperation	*1*	*7.1*
AKI	*2*	*14.3*
Chilotorax	*1*	*7.1*
Pleural effusion requiring chest tubes stay > 3 days	*1*	*7.1*
Infection requiring antibiotic therapy for more than 7 days	*4*	*28.6*

Bradiarrythmia	*1*	*7.1*
Tachyarrythmia	*1*	*7.1*
Other:	*6*	*42.9*
Subglottic stenosis (Cotton grade III) > laser dilation	*1*	
Hypotension and LV thrombosis > VENT	*1*	
Pericardial effusion with surgical drainage	*1*	
Peripheral vein trhrombosis	*1*	
Progressive failure > VAD	*1*	
Intubation time after PAB		
< o = 24 hrs	*5*	*16.1*
24-72 hrs	*11*	*35.5*
>72 hrs	*15*	*48.4*
Early death (n)	4	12.9
Cardiac failure	*3*	
Sepsis > MOF	*1*	
Early reintervention (n)	13	42
Surgical PAB tightening	*5*	
Delayed chest closure	*7*	
ECMO	*1*	
VAD	*3*	
Other:	*2*	
Partial PAB tensioning	*1*	
Tamponade	*1*	

Home therapy	25	*8*
ACE-inhibitors or ARB	24	77
Beta-blokers	24	77
Diuretics	24	77
Warfarin	1	3.2
Aspirin or antiaggregation therapy	10	32
Other:	7	23
L-carnitine and Q coenzyme	1	
Clonidine	1	
Digoxin	2	
Clexane	2	
Immunosoppression after HTX	1	
NYHA/ROSS Class at D/C (n)	27	
I	*6*	
II	*17*	
III	*2*	
IV	*2*	
Pre-PAB BNP (pg/mL, median, IQR)	9927 (3346-14991) (n=23)	
PAB peak-pressure gradient at D/C (mmHg, median, IQR)	38 (28-44) (n= 23)	

Echocardiogram at discharge	22	74.1

Legend: CMRI, cardiac magnetic resonance imaging; ECMO, extracorporeal membrane oxygenation; PDA, patent ductus arteriosus; ALCAPA, anomalous left coronary artery from pulmonary artery; HTx, heart transplantation; INTERMACS, interagency registry for mechanically assisted circulatory support; ICU, intensive care unit; LCO, low cardiac output; AKI, acute kidney injury; MOF, multi-organ failure; VAD, ventricular assist device; ARB, angiotensin receptor blockers; D/C, discharge; BNP, brain natriuretic peptide;

Table 13. Follow up data of patients undergoing PAB surgery.

VARIABLE	**Pts**	**%**
Follow up (years, median, IQR)	2.9 (1.2-4.85)	
Total (n)	27	87.0
Late death (n)	1	3.7
CVA – stop VAD	*1*	
Adverse event (AE) other than death during FU (n)	8	29.6
Surgical AE	*7*	*87.5*
HTX	*2*	*28.6*
ECMO+VAD+HTX	*1*	*14.3*
VAD	*1*	*14.3*
Debanding PAB	*3*	*42.8*
Non-surgical AE	***1***	*12.5*
Progressive congestive heart failure	*1*	*100*

Therapy at last FU	26	
ACE inhibitors or ARB	*23*	*88.5*
Diuretics	*14*	*53.8*
Beta-blockers	*17*	*65.4*
Antiaggregation	*3*	*11.5*
Other	*7*	*26.9*
PAB dilation during FU	14 (n=23)	60.9
Once	*10*	*71.4*
More than once	*4*	*28.6*
Sinus Rhythm at last FU	27	100
NYHA class in survivors (n)	27	100
I	*22*	*81.4*
II	*4*	*14.8*
III	*0*	*0*
IV	*1*	*3.8*
Levosimendan infusion therapy after PAB	5	18.5
<u>Number of cycles</u>		
One cycle	*2*	*40*
Two cycles	*1*	*20*
More than two	*2*	*40*
<u>Timing</u>		

Within one month from discharge *Within 3 months from discharge*	*2* *3*	*40* *60*
CMRI at FU	5	18.5

Legend: CMRI, Cardiac magnetic resonance imaging; FU, follow-up; VAD, ventricular assist device;.HTX, heart transplant.

PAB was performed in a total of 31 patients (pts) from 5 international centers (Ghent, Leuven, BE; Manila, PH; Padua, IT; Warsaw, PO). Median age at PAB was 210 d (IQR: 131-357), median weight 6.4 Kg (IQR: 5.2-8.15). 8 patients were listed for HTx (25.8%) and most of them were on INTERMACS 3 (i.e., stable but inotrope-dependent, 61.3%).

As for the etiology of ESHF, we observed:

- Dilated Cardiomyopathy (idiopathic, genetic) in 20 pts (64%)
- Chronic viral myocarditis in 5 pts (16%)
- Other in 6 pts (20%)

Admission 2DEcho showed that LVEF was <30% in 21 ptz (68%), ≥30 in 5 (16%) and not specified in 5 (16%) and LV dilation > moderate in all (*Table 14, also showing the other assessed parameters*).

6 pts underwent procedures prior to PAB (19.4%):

  - ALCAPA repair in 2
  - Balloon atrial septostomy in 4

Associated surgical procedures were carried out in 3 pts (9.7%), consisting of:

- PDA closure
- ALCAPA repair
- ECMO + atrial septectomy

The PAB peak-gradient pressure at the end of the procedure was 29 mmHg (IQR=23-34). At the echo control, leftward interventricular septum displacement after PAB was observed in 27 (87.1%) and mitral regurgitation reduction in 12 (41.9%), as an immediate consequence of the surgery.

Pre-operative intubation was needed in 15 pts (48.4%), while preoperative infusion of levosimendan in 22 (71%).

Cardiopulmonary bypass was performed in only one patient who underwent associated ALCAPA repair.

Post-operative complications occurred in 14 pts (47%) and included:

- LCO syndrome in 5 (35.7%)
- Infections in 4
- AKI in 2

Intubation time after PAB was under or equal to 24 hours in 5 pts (5%), 24-72h (11%), >72 hours (15%).

Levosimendan infusion therapy after PAB was administered in 5 pts (18.5%).

Delayed sternal closure was performed only in one center (Padova), in 7 pts.

Median ICU stay was 13 days long (IQR 7-24).

Early death occurred in 4 pts (13%), caused by:

- CHF in 3 (age at PAB: 95, 124, 188 d)
- MOF in 1 (age at PAB 167 d)

As for the early reinterventions, they happened in 13 pts (42%), consisting of:

- Delayed chest closure in 7
- Surgical PAB tightening in 4
- Temporary ECMO in 1
- VAD in 3 (whose age at PAB were 1288, 481, 505 d)
- Other (partial PAB tensioning and tamponade) in 2

PAB peak-pressure gradient at D/C had a median value of 38 mmHg (IQR 28-44).

25 pts (81%) were discharged home on full anti-CHF therapy consisting mainly of ace-inhibitors, beta-blockers and diuretics.

Considering the remaining 27 pts (31 – 4 early deaths), they were all on Sinus Rhythm and most of them (81.4%) in class I NYHA.

During follow-up, there had been:

- 1 late death on VAD
- 3 late OHTx (two of them preceded by VAD, one of them preceded by rescue ECMO + VAD)
- 1 VAD application
- 3 PAB debanding

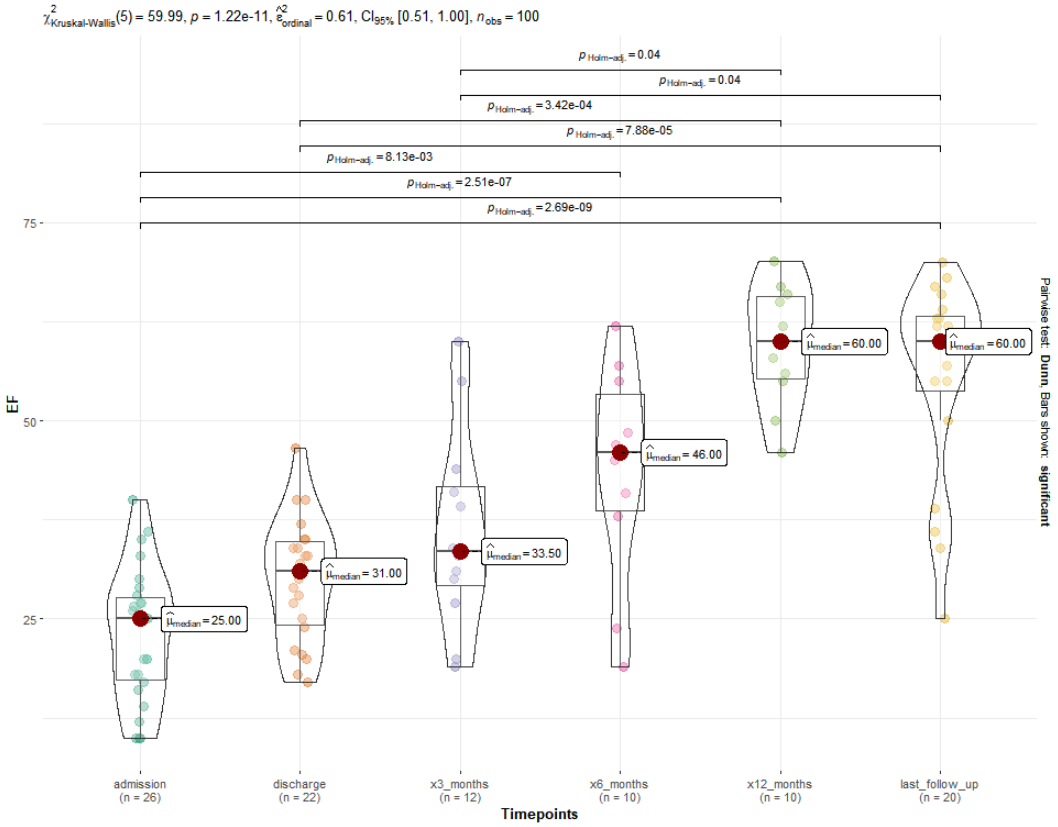
PAB dilation during follow-up was performed in 14 patients out of 23 (excluding from the total of 31 pts 5 deaths and 3 HTx).

26 pts were on therapy at the last follow-up.

The PAB-Efficacy-INDEX was 74.2% and the overall survival (HTx included) was 84%.

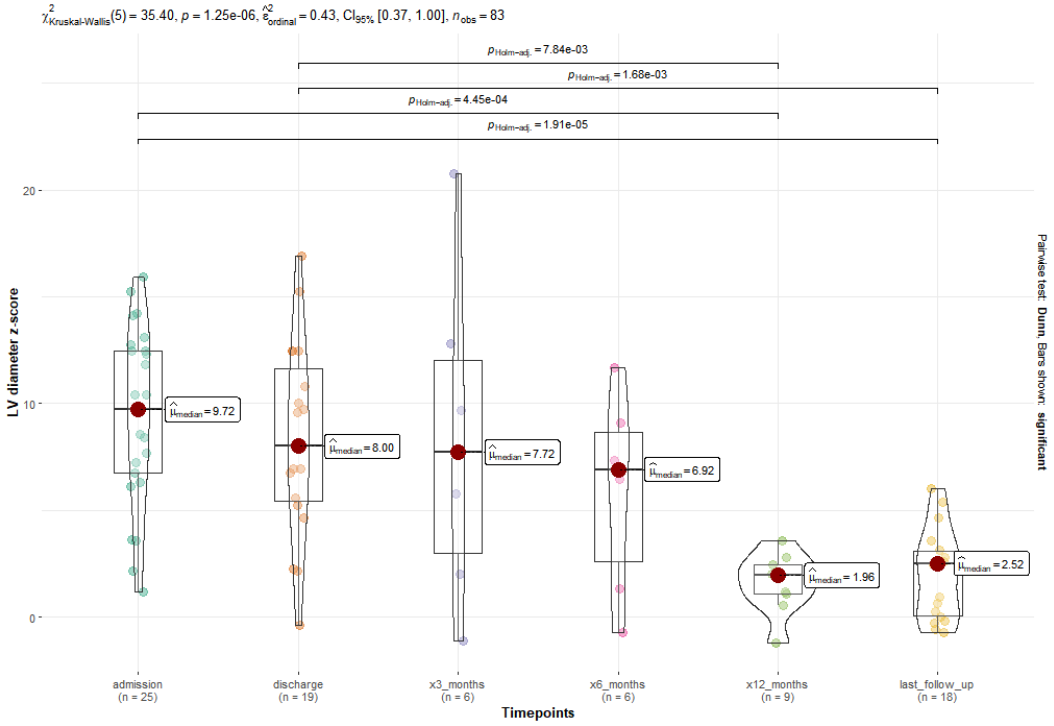
In all 23 survivors with their native heart, LVEF and LV dimensions gradually normalized from admission to discharge, 3 months, 6 months, 12 months and last follow-up control, as clearly shown in the following figure

The last echo follow-up control happened at a median time of 2.7 years (IQR: 1.25-4.6) from the PAB procedure.



Graph 1: Gradual normalization of LVEF (%) from admission to last follow-up- The non-parametric Kruskal-Wallis test was used together with Dunn's test for pairwise comparison and p-value adjusted by Holm's method.

As seen in the graph above, at the last follow-up, EF improved unitl the median value of 60% (IQR: 53.75-63.25) from 25% (IQR: 17.5-27,75) on admission, a variation which is statistically significant ($p_{holm-adj.}= 2.69e^{-09}$).



Graph 2: Left Ventricle diameter Z-scores from admission to last follow-up. The non-parametric Kruskal-Wallis test was used together with Dunn's test for pairwise comparison and p-value adjusted by Holm's method.

As seen in the graph above, left ventricle end diastolic diameter (LVEDD)'s Z-score reduced from a median value of 9.72 (IQR: 6.76-12.48) on admission to 2.52 (IQR: 0.07-3.07). This improvement was statistically significant too ($p_{holm-adj.} = 1.91e^{-05}$).

Table 14: Echocardiographic controls on admission, discharge and 3 months follow-up. Data are expressed as mean ± standard deviation.

	Admission			**Discharge**			**3 mo FU**		
VARIABLE	**n**	**Pts**	**%**	**n**	**Pts**	**%**	**n**	**Pts**	**%**
Total echo controls		**27**	87.1		**22**	70.9		**12**	38.7
LVEF (%, mean SD, range)	**26**	**23 ± 8.4** (10-40)		**22**	**29.8±7.9** (17-46.6)		**12**	**36.1 ±12.6** (19-60)	
PAB peak-gradient pressure at CW doppler (mmHg, mean, SD, range)	2	#12= 6 mmHg ; #13= 40 mmHg		20	34.75 ±14.5 (7-65)		7	52.6 ±26 (11-100)	
MR	26	23	88.5	21	20	95.2	12	12	100
None		*3*			*1*			*0*	
Mild		*3*			*6*			*5*	
Mild-moderate		*3*			*5*			*3*	
Moderate		*14*			*4*			*3*	
Severe		*3*			*5*			*1*	
LV Dilation	21	21	100	19	19	100	7	6	85.7
None		*0*			*0*			*1*	
Mild		*0*			*0*			*0*	
Mild-moderate		*0*			*0*			*0*	
Moderate		*2*			*5*			*3*	
Severe		*19*			*14*			*3*	

LVEDD (mm, mean, SD, range)	25	41.37 ±6.16 (23.8-52)		18	39.2 ±7.44 (21.7-51)		6	35.01 ±10.45 (21-46.7)	
LVEDV (ml/m^2, mean, SD, range)	3	153 ±59.9 (87-204)		3	131.7 ±66.5		-	-	
TAPSE (mm, mean, SD, range)	13	12.15 ±5.1 (8-25)		13	10.6 ±2.72 (7-16)		8	10.39 ±3.30 (7-16)	
TR	25	16	64	22	18	81.8	12	7	58.3
None		*9*			*4*			*4*	
Mild		*14*			*16*			*6*	
Mild-moderate		*2*			*2*			*1*	
Moderate		*0*			*0*			*0*	
Severe		*0*			*0*			*0*	
RV pressure calculation if TR (mmHg, mean, SD, range)	8	34 ±9.9		12	42.4±15.4 (24-75)		2	#1686-2= 52 mmHg; #1686-3=24 mmHg;	

Legend: LVEF, Left ventricle ejection fraction; CW doppler, continuous wave doppler; MR, mitral regurgitation; LV, left ventricle; LVEDV, left ventricle end-diastolic volume; TAPSE, tricuspidal annular plane excursion; TR, tricuspidal regurgitation; RV, right ventricle.

Table 15: Echocardiographic controls at 6 months, 12 months and last follow-up. Data are expressed as mean ± standard deviation

	Admission			**Discharge**			**3 mo FU**		
VARIABLE	**n**	**Pts**	**%**	**n**	**Pts**	**%**	**n**	**Pts**	**%**
Total echo controls		**9**	29		**10**	32.3		**20**	64.5
LVEF (%, mean SD, range)	**9**	**43.9 ±14.6** (19-62)		**10**	**59.5 ±7.9** (46-70.2)		**20**	**55.8 ±12.6** (25-70)	
PAB peak- gradient pressure at CW doppler (mmHg, mean, SD, range)	6	57.3 ±29.7 (24-100)		9	61.6 ±23.8 (20-85)		19	42.8 ±16.1 (15-73)	
MR	9	9	100	9	7	77.7	20	13	65
None		*0*			*2*			*7*	
Mild		*5*			*4*			*10*	
Mild-moderate		*1*			*1*			*2*	
Moderate		*2*			*1*			*1*	
Severe		*1*			*1*				
LV Dilation	6	4	66.6	19	19	100	7	6	85.7
None		*2*			*0*			*1*	
Mild		*0*			*0*			*0*	
Mild-moderate		*0*			*0*			*0*	
Moderate		*2*			*5*			*3*	
Severe		*2*			*14*			*3*	

LVEDD (mm, mean, SD, range)	6	36.68 ±10.1 (22-45.5)		9	31.17 ±4.23 (24-35.9)		17	35 ±5.15 (26.7-44)	
LVEDV (ml/m^2, mean, SD, range)	1	1 value only: 2 =165 ml/m^2		3	51.7± 13.9 (40-67.1)		4	36.3 ±14.9 (20.5-53)	
TAPSE (mm, mean, SD, range)	5	14 ±2.82 (10-16)			13.56 ±2.58 (9.8-16)		16	14.42 ±3.25 (4.8-19.5)	
TR	9	5	55.5	9	5	55.5	20	12	60
None		*4*			*4*			*8*	
Mild		*4*			*5*			*10*	
Mild-moderate		*0*			*0*			*2*	
Moderate		*1*			*0*			*0*	
Severe		*0*			*0*			*0*	
RV pressure calculation if TR (mmHg, mean, SD, range)	2	#2= 45; #1686-1=49;		3	54.3 ±28.7 (28-85)		8	42 ±22.4 (20-80)	

Legend: LVEF, Left ventricle ejection fraction; CW doppler, continuous wave doppler; MR, mitral regurgitation; LV, left ventricle; LVEDV, left ventricle end-diastolic volume; TAPSE, tricuspidal annular plane excursion; TR, tricuspidal regurgitation; RV, right ventricle.

Table 16: CRMI controls on admission and at last FU. Data are expressed as mean ± standard deviation.

	Admission			Last Follow-up		
VARIABLE	**n**	**Pts**	**%**	**n**	**Pts**	**%**
LVEDV (ml/m^2, mean, SD, range)	9	182.14 ±.70.49 (90-287)		5	78.65 ±.18.62 (50-98)	
LVESV (ml/m^2, mean, SD, range)	9	148.4 ± 60.3 (71-243)		5	35.2 ±.15.1 (17-58)	
LVEF (%, mean, SD, range)	9	18.6 ±4.8 (10-25)		5	56.7 ±.9.55 (41-66.4)	
LVSV (ml/m^2, mean, SD, range)	8	35.1 ±13.1 (19-58)		5	41.8 ±.5.90 (32-47.17)	
LVCO (l/min, mean, SD, range)	2	#2 = 1.3 l/min #4 = 2 l/min		4	2.17±.0.24 (2-2.5)	
LVCI (l/min/m^2, mean, SD, range)	2	#2= 3.42 l/min/m^2 #4= 5.1 l/min/m^2		5	3.99 ±.0.49 (3.47-4.8)	
LVCI (l/min/m^2, mean, SD, range)	2	#2= 3.42 l/min/m^2 #4= 5.1 l/min/m^2		5	3.99 ±.0.49 (3.47-4.8)	
LVCM (g/ m^2, mean, SD, range)	6	73.5 ±35.2 (16-118)		2	#3= 65 g/m^2; #1686-1= 51.72 g/ m^2;	

RVEF (%, mean, SD, range)	7	57.7 ±6.5 (45-64)		5	57.42 ±.10.74 (43.12-70)	
RVEDV (ml/m^2, mean, SD, range)	7	48.7 ±17.7 (33.6-83)		5	70.10 ±.3.06 (66-73.53)	
RVESV (ml/m^2, mean, SD, range)	7	17.9 ± 8.2 (12.6-36)		5	30.22 ±.8.28 (20.3-41.82)	
RVSV (ml/m^2, mean, SD, range)	9	29.8 ±10.4 (20-47)		5	34.82±.9.02 (23-46)	
RVCO (l/min, mean, SD, range)	2	#2 = 1.2 l/min #4 = 2 l/min		5	2.02 ±.0.3 (1.68-2.5)	
RVCI (l/min/m^2, mean, SD, range)	2	#2 = 3.15 l/min/m^2 #4 = 5.1 l/min/m^2		5	3.14 ±.1.42 (1.2-4.8)	
RVCM (g/ m^2, mean, SD, range)		-			-	
Myocardial Oedema		0	0		0	0
Myocardial Fibrosis <u>Site of fibrosis</u> *LV* <u>Type of fibrosis</u> *Subendocardial* *Intramyocardial*	9	3 *3* *2* *1*	33.3	5	4 *4* *2* *2*	80

Legend: LVEDV, left ventricle end-diastolic volume; LVESV, left ventricle end-sistolic volume; LVEF, left-ventricle ejection fraction; LVSV, left ventricle stroke volume; LVCO, left ventricle cardiac output, LVCI, left ventricle cardiac index, LVCM, left ventricle cardiac mass; RVEF, right ventricle ejection fraction; RVEDV, right ventricle end-diastolic volume, RVESV, right ventricle end-sistolic volume; RVSV, right ventricle stroke volume; RVCO, right ventricle cardiac outoput, RVCI, right ventricle cardiac index, RVCM, right ventricle cardiac mass.

CORRELATIONS

Considering as our outcome the overall freedom from death/VAD/heart transplant, we tried to correlate it with some variables to determine whether they had any statistically significant effects on the outcome of this treatment.

In particular, we examined the role of age (under and over 6 months and under and over 12 month), the etiology of ESHF (Idiopathic dilated cardiomyopathy vs all the others), gender (male vs female), PAB peak-pressure gradient at discharge (<=25 mmHg vs > 25 mmHg) and LVEF on admission (<=30 % vs > 30%).

Tables 17-22: correlations between the overall freedom from deaths/VAD/HTx (=PAB-Efficacy-INDEX) and age, gender, ESHF etiology, PAB peak-pressure gradient at discharge, LVEF on admission.

6 MONTHS

Characteristic	< 6 months, n=13			>6 months, n=18			p-value
	Pts	Tot	%	Pts	Tot	%	
Early death	3	13	23	1	18	5.6	0.3
Late death	0	10	0	1	15	6.7	>0.9
Late surgical adverse events							>0.9
Heart transplant	0	0	0	1	5	20	
VAD	0	0	0	1	5	20	
Other	0	0	0	3	5	60	
Early reintervention = VAD	0	13	0	3	18	17	0.2
Early reintervention = HTx	0	13	0	2	18	11	0.5
All adverse events							0.7
No Adverse Events	10	13	77	12	18	67	
One or more Adverse Events	3	13	23	6	18	33	

12 MONTHS

Characteristic	< 12 months, n=23			>12 months, n=8			p-value
	Pts	Tot	%	Pts	Tot	%	
Early death	4	23	17	0	8	0	0.5
Late death	0	18	0	1	7	14	0.3
Late surgical adverse events							>0.9
Heart transplant	0	2	0	1	3	33	
VAD	0	2	0	1	3	33	
Other	2	2	100	1	3	33	
Early reintervention = VAD	0	23	0	3	8	38	0.012
Early reintervention = HTx	1	23	4.3	1	8	12	0.5
All adverse events							0.2
No Adverse Events	18	23	78	4	8	50	
One or more Adverse Events	5	23	22	4	8	50	

ESHF ETIOLOGY

Characteristic	Idiopathic dilated cardiomyopathy, n=15			Other etiologies, n=16			p-value
	Pts	Tot	%	Pts	Tot	%	
Early death	3	15	20	1	16	6.2	0.3
Late death	1	12	8.3	0	13	0	0.
Late surgical adverse events							0.10
Heart transplant	1	2	50	0	3	0	
VAD	1	2	50	0	3	0	
Other	0	2	0	3	3	100	
Early reintervention = VAD	2	15	13	1	16	6.2	0.6
Early reintervention = HTx	0	15	0	2	16	12	0.5
All adverse events							0.3
No Adverse Events	9	15	60	13	16	81	
One or more Adverse Events	6	15	40	3	16	19	

GENDER

Characteristic	Male, n=18			Female, n=13			p-value
	Pts	Tot	%	Pts	Tot	%	
Early death	3	18	17	1	13	7.7	0.6
Late death	1	13	7.7	0	12	0	>0.9
Late surgical adverse events							>0.9
Heart transplant	1	4	25	0	1	0	
VAD	1	4	25	0	1	0	
Other	2	4	50	1	1	100	
Early reintervention = VAD	3	18	17	0	13	0	0.2
Early reintervention = HTx	2	18	11	0	13	0	0.5
All adverse events							0.045
No Adverse Events	10	18	56	12	13	92	
One or more Adverse Events	8	18	44	1	13	8	

PAB PEAK-PRESSURE GRADIENT AT DISCHARGE

Characteristic	<=25 mmHg, n=11			>, n=19			p-value
	Pts	Tot	%	Pts	Tot	%	
Early death	1	11	9.1	3	19	16	>0.9
Late death	1	9	11	0	15	0	0.4
Late surgical adverse events							>0.9
Heart transplant	0	2	0	1	3	33	
VAD	0	2	0	1	3	33	
Other	2	2	100	1	3	33	
Early reintervention = VAD	3	18	17	0	13	0	0.5
Early reintervention = HTx	2	18	11	0	13	0	>0.9
All adverse events							>0.9
No Adverse Events	8	11	73	13	19	68	
One or more Adverse Events	3	11	27	6	19	32	

LVEF ON ADMISSION

Characteristic	<=30, n=22			>30, n=4			p-value
	Pts	Tot	%	Pts	Tot	%	
Early death	3	22	14	0	4	0	>0.9
Late death	1	19	11	0	4	0	>0.9
Late surgical adverse events							>0.9
Heart transplant	0	0	0	1	3	33	
VAD	0	0	0	1	3	33	
Other	0	0	0	1	3	33	
Early reintervention = VAD	1	22	4.5	1	4	25	0.3
Early reintervention = HTx	0	22	0	0	4	0	0.2
All adverse events							0.2
No Adverse Events	18	22	82	2	4	50	
One or more Adverse Events	4	22	18	2	4	50	

As shown in the tables above, there are two statistically significant correlations:

1) Females have an overall freedom from death/VAD/HTx (7.7%) greater than males (44%) after PAB (p-value = 0.045)
2) The risk of an early reintervention after PAB consisting of VAD application is greater in patients over 12 months of age (38%) than patients under 12 months (0%) (p-value = 0.012).

Even if they are not statistically significant, certain associations are unquestionably biologically relevant. For example, it is significant that among the group under 12 months of age only 22% shad one or more adverse events compared to 50% of children above 12 months of age, evidence of the fact that infants (<12 months) have typically better outcomes.

Also, those with Idiopathic Dilated Cardiomyopathy suffer more adverse events (40%) than those with other etiologies (16%, DCM from acute viral myocarditis, chronic viral myocarditis, spongy myocardium etc.)

We believe that the lack of statistical significance is mainly due to the small numerosity of the sample, a clear limit we hope to overcome in the future by enrolling more and more patients in this study.

6.2 EXPERIMENTAL STUDY 1

EXPERIMENTAL MORTALITY

All rats treated with DOX exhibited decreased spontaneous motor activity, loss of body weight, ruffled dur and decreased survival in the days after the injection, whereas LAD-ligated rats always preserved better health status following the procedure.

Table 23: Mortality for each experimental procedure, excluding 3 rats who died due to intubation difficulties before surgeries.

Procedure	*Total rats*	*Deaths*	*%*
DOX	16	7	43.8
LAD	7	-	-
SHAM	3	-	-
DOX + PAB	11	5	45.5
LAD + PAB	6	-	-

As indicated in the *table 23* above, mortality was 43.8% for the DOX-injected rats, similar to that reported in literature (70), and 45.5% for the DOX-injected and banded rats.

No death occurred both in the LAD-ligation group and the LAD-ligation + PAB group, as well as the SHAM operation.

ECHOCARDIOGRAPHIC FINDINGS

Table 24: Echo parameters of rats undergoing DOX-injection, LAD-ligation, DOX-injection + PAB, LAD-ligation + PAB and SHAM. Data are expressed as mean± standard deviation (SD).

	PARAMETERS				
ECHO GROUP	**EF (%)**	**FS (%)**	**LVPWd (mm)**	**LVIDd (mm)**	**IVSd (mm)**
Baseline echo (n=20)	63.75±8.01	36.95±6.82	1.88±0.24	7.69±0.89	1.79±0.36
Echo after procedures					
Post-DOX (n=26)	46.58±13.72	24.27±8.41	1.83±0.35	7.38±0.95	1.60±0.29
Post-LAD (n=5)	62.8±9.41	33.6±7.44	1.62±0.35	7.43±0.55	1.6±0.52
SHAM (n=3)	61.3±8.14	33.67±6.11	2.12±0.19	7.99±0.40	2.36±0.04
Post LAD+PAB (n=5)	63.8±8.87	36±6.7	1.92±0.78	7.75±0.38	1.46±0.44
Post-DOX+PAB (n=3)	56±4.36	30±3.6	1.79±0.57	7.37±0.43	1.40±0.35
Echo at 15 days					
Post-DOX (n=7)	52.29±10.81	27.71±7.18	1.77±0.53	7.17±1.11	1.67±0.3
Post-LAD (n=6)	69.17±11.36	41.67±9.83	2.06±0.46	7.72±0.76	1.85±0.73
Post-DOX+PAB (n=1 → P0801)	64	35	2.15	6.51	2.08
Post-LAD+PAB (n=6)	61.33±7.55	34.5±6.41	1.74±0.57	7.79±0.69	1.46±0.51

SHAM (n=3)	59.33±5.03	35±2	1.68±0.6	8.61±1.17	1.71±0.04
Echo at 30 days					
Post-DOX (n=6)	56±10.86	30±7.04	1.84±0.53	6.66±0.77	1.67±0.39
Post-LAD (n=4)	59.5±8.35	33±6	2.42±0.24	8.13±1.55	1.8±0.48
SHAM (n=3)	72.67±0.58	43.33±1.15	2.03±0.53	8.61±0.16	1.89±0.3

Legend: EF, Ejection Fraction; FS, Fractional Shortening; LVPWd, Left Ventricle Posterior Wall end-diastole; LVIDd, Left Ventricle internal diameter end-diastole; IVSd, Interventricular septum at end-diastole.

As shown in the *table 21* above, the echocardiographic assessment revealed at baseline an EF of 63.75 ± 8.01% and a FS of 36.95 ± 6.82%, in the range of normality, as it has been reported in literature (71), since at baseline rats had not undergone any procedure yet.

As for the post-DOX assessment, EF and FS were 46.58±13.72% and 24.27±8.41 % respectively, whereas post-LAD ligation EF and FS were 62.8±9.41% and 33.6±7.44% each.

These data alone confirm that only the DOX-induced DCM model caused effective functional changes, whereas the LAD-ligated rats' EF and FS, as well as the other parameters, were not altered even at 15 and 30 days after the surgery. Also, PAB on LAD-ligated rats did not show any significant echocardiographic variation.

Thus, considering PAB on DOX-treated rats, out of the 6 banded rats which survived the procedure, only three echoes were done days after the procedure and only one echo at almost 15 days from surgery.

These instrumental examinations showed that the heart function was slightly ameliorated after the pulmonary artery banding as compared to post-DOX administration cardiac status.

Indeed, rat #P0801's EF and FS were respectively:

- 66% and 37% at baseline
- 51% and 28% post-DOX-injection
- 51% and 26% 4 days after PAB
- 64% and 35% 13 days after PAB

These parameters almost got back to baseline conditions, proving the beneficial effect of PAB.

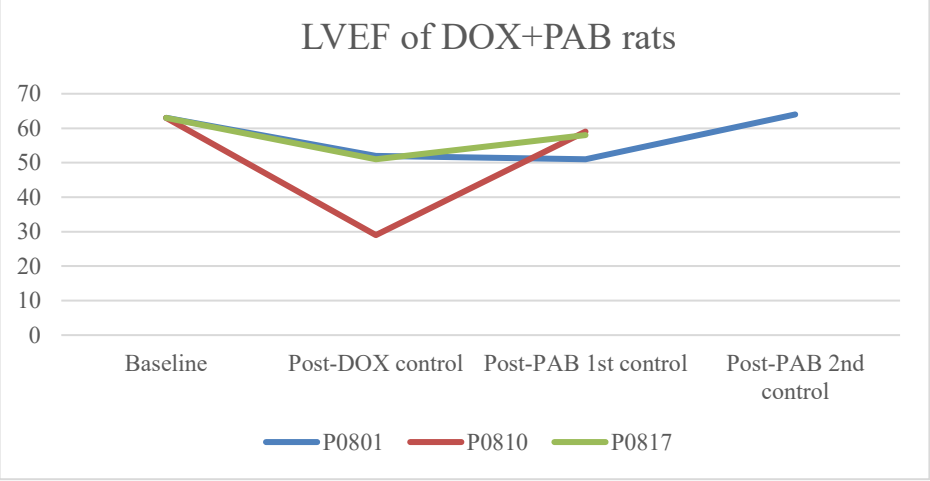
#P0810 suffered a significant cardiac deterioration after DOX-injection with EF and FS of 29% and 14% each; then, 8 days after PAB, echo showed a dramatic improvement, with an EF of 59% and a FS of 33%.

Lastly, #P0817 showed only slightly amelioration 6 days after PAB, with EF and FS of 58% and 31% respectively, from 51% and 27% after DOX administration.

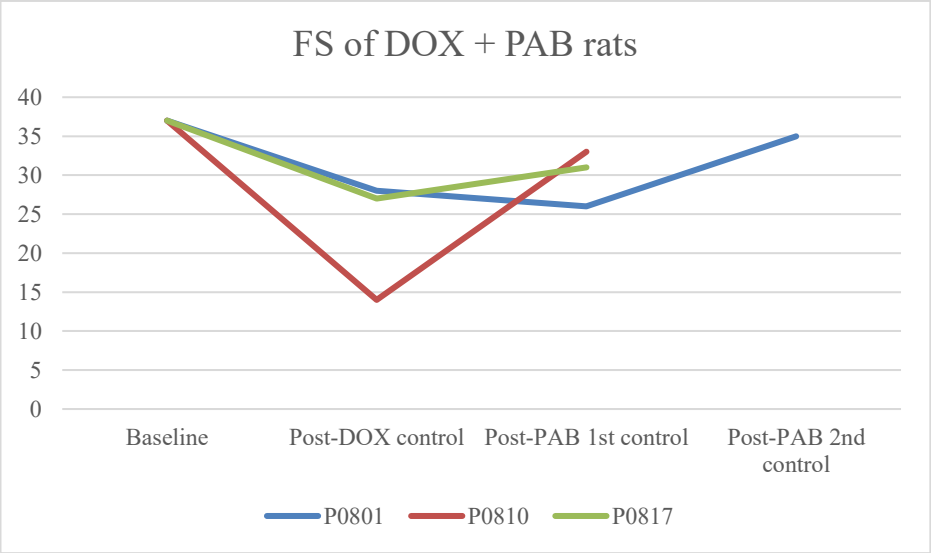
As for the other parameters, in all rats they showed a trend towards a reduction of LV internal diameter and increased posterior wall and interventricular septum thickness after PAB.

The following graphs (3-5) show in detail the trend of the 3 most significant echocardiographic parameters (LVEF, LVFS, LVIDd) assessed on these 3 bandend rats:

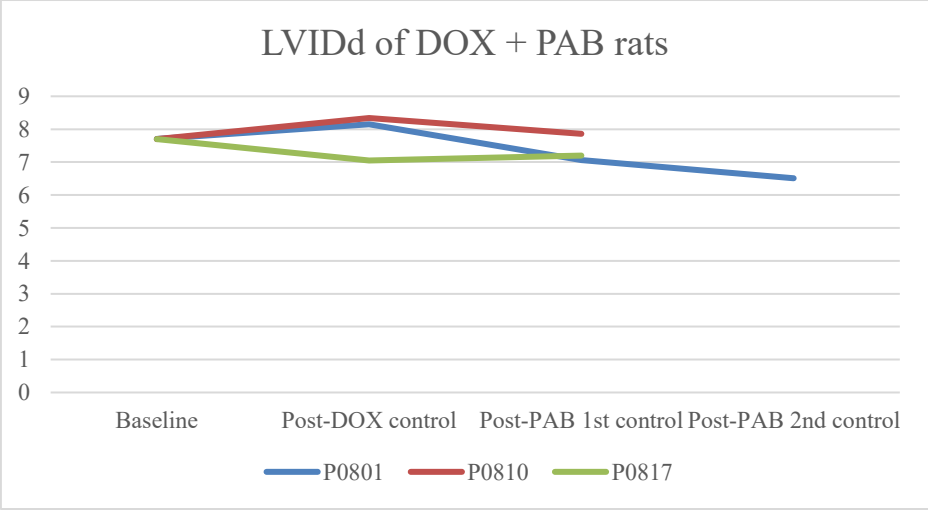
- At baseline
- On the control echo after DOX administration
- On the 1st control echo after PAB: 4 days after for P0801, 8 days for P0810, 6 days for P0817.
- On the 2nd control echo after PAB: 13 days for P0801



Graph 3: LVEF, Left Ventricular Ejection Fraction



Graph 4: FS, Fractional Shortening



Graph 5: LVIDd, Left Ventricular internal diameter end diastole.

Of course, a clear limit of this echocardiographic assessment is represented by the limited numerosity of ultrasound exams performed on banded rats.

For this reason, further experimental investigations are needed in order to reach the statistical significance we lack from these preliminary results.

Echo images

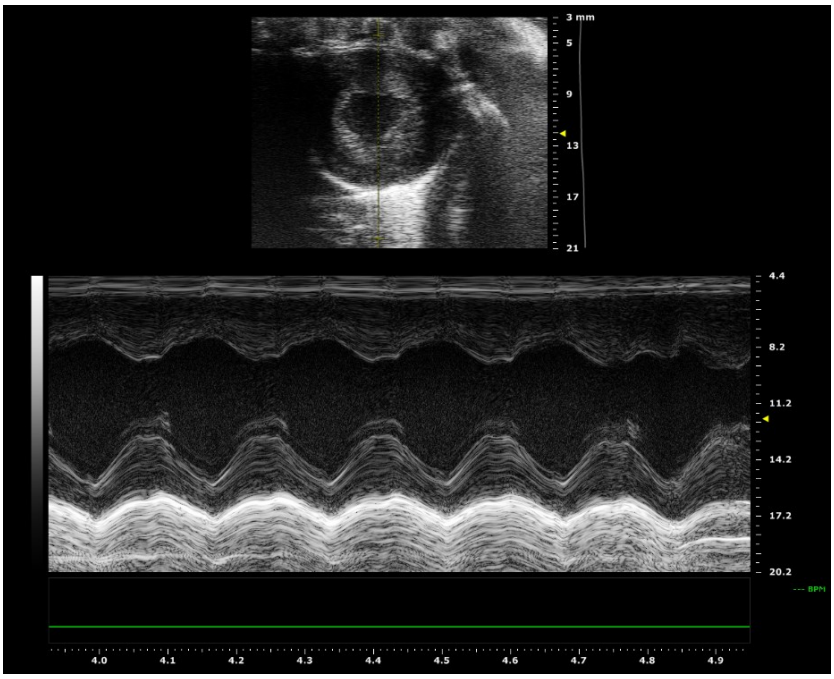


Figure 9: Basal echo (short-axis) of the rat #P0801 before DOX-injection and PAB, showing normal function.

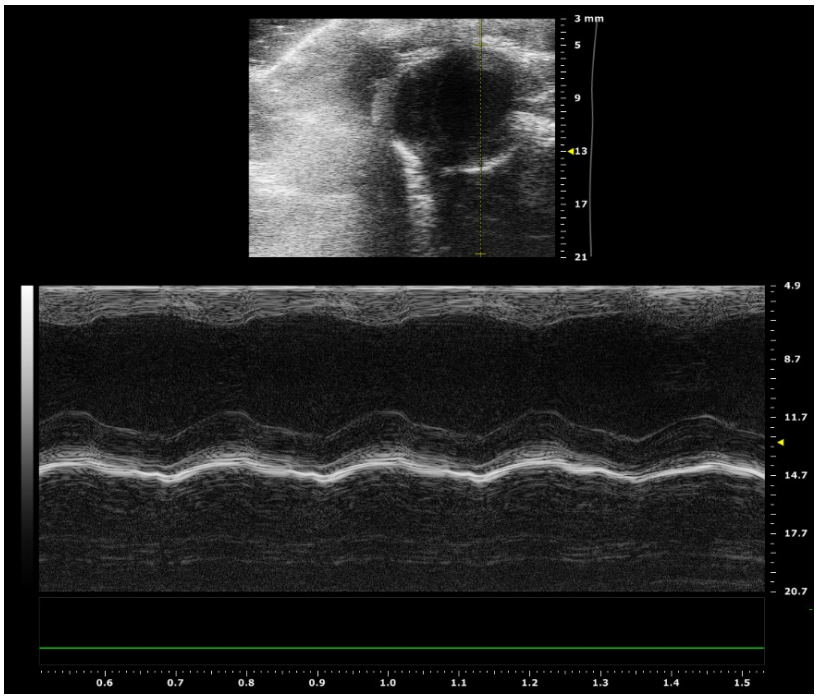


Figure 10: #P0801 echo after DOX-injection, showing systo-diastolic dysfunction

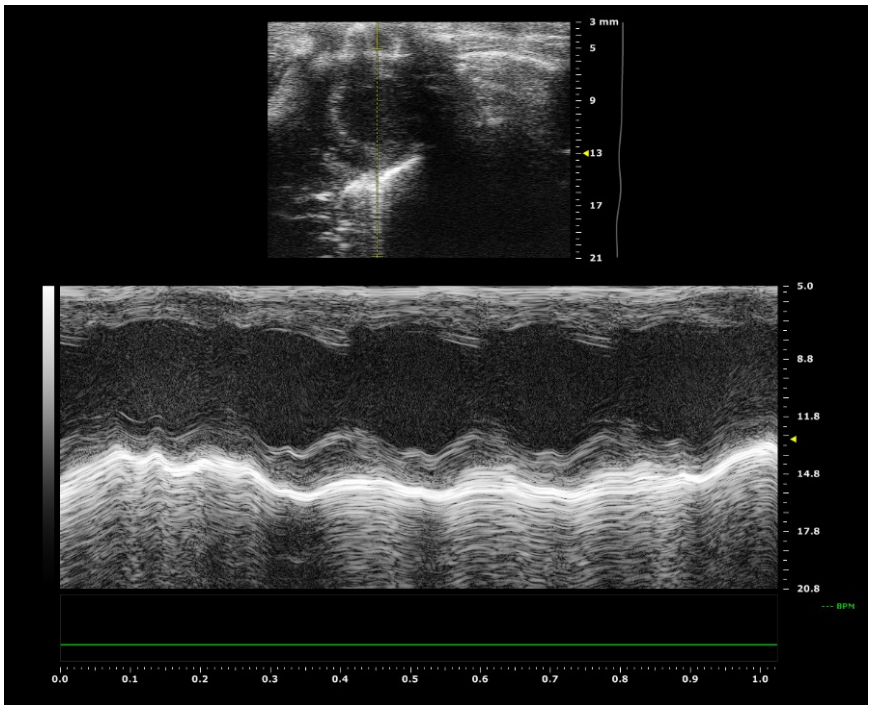


Figure 11: #P0801 4 days after PAB

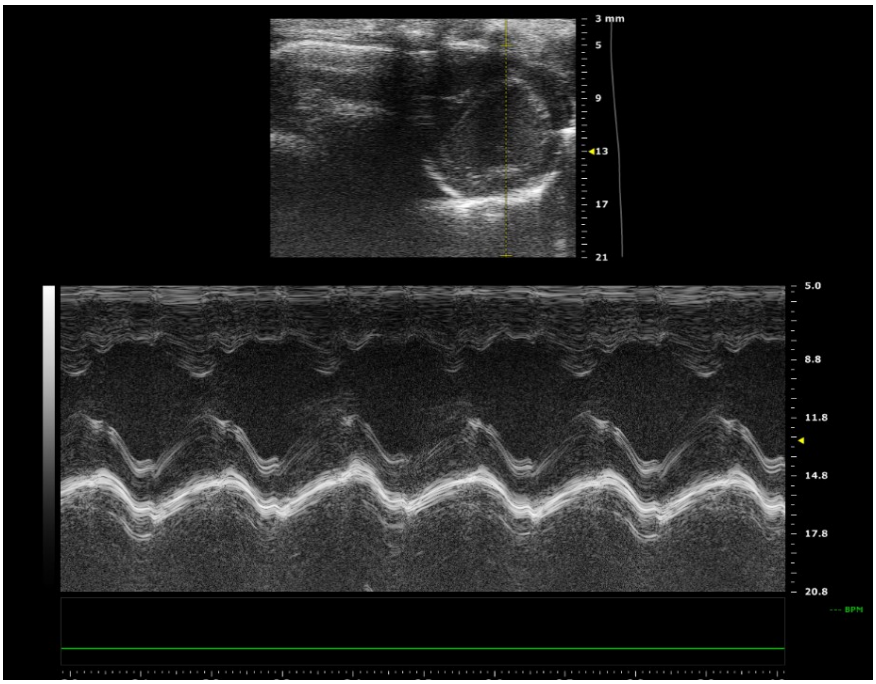


Figure 12: #P0801 13 days after PAB, showing improved systo-diastolic function

Histopathological findings

Heart weight did not differ between the groups, with a mean value of 1,38 ±0,37 g. Also, cardiomyocytes' diameter did not show differences among the groups, with a mean value of 13,38±0,87 micron.

LAD-ligated rats showed acute myocardial infarction located in the lateral wall of the left ventricle, with initial signs of repair as shown in the *figure 13* below.

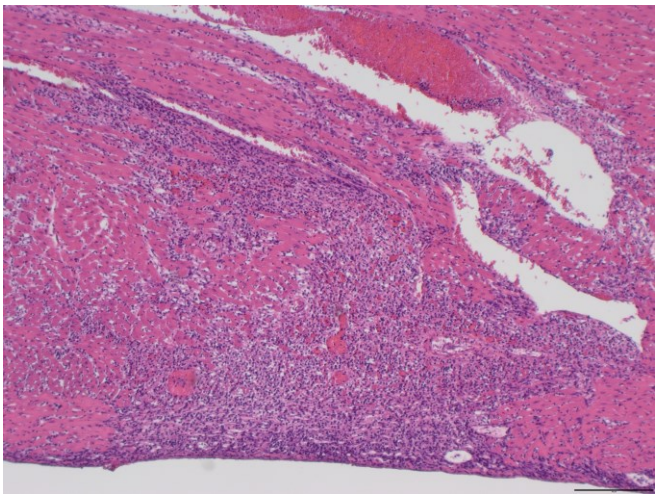
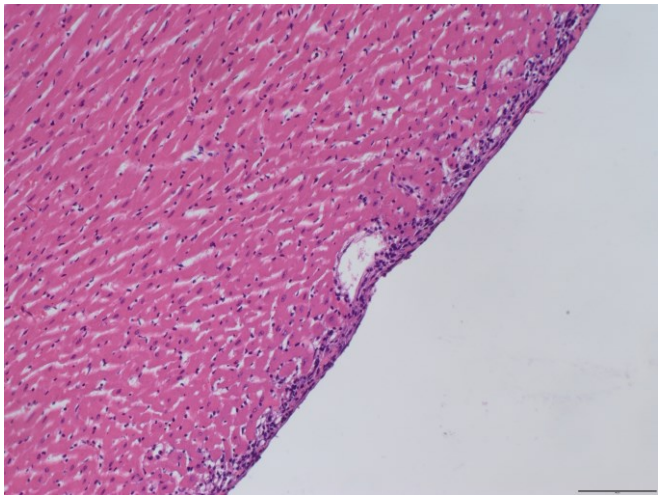


Figure 13: Acute Myocardial infarction of the LV lateral wall

Instead, DOX-treated rats did not display any morphological abnormalities that could explain the systo-diastolic dysfunction assessed through echocardiography. In particular, nuclear dysmetrias or dysmorphias, perinuclear halos and cytoplasmic vacuolization were not detected.



Animals which underwent PAB only showed signs of post-cardiotomy pericarditis, but no other significant alterations.

Figure 14: Post-cardiotomy pericarditis following PAB procedure

Even considering the ultrastructural point of view, all animals maintained normal contractile components and well-structured mitochondria.

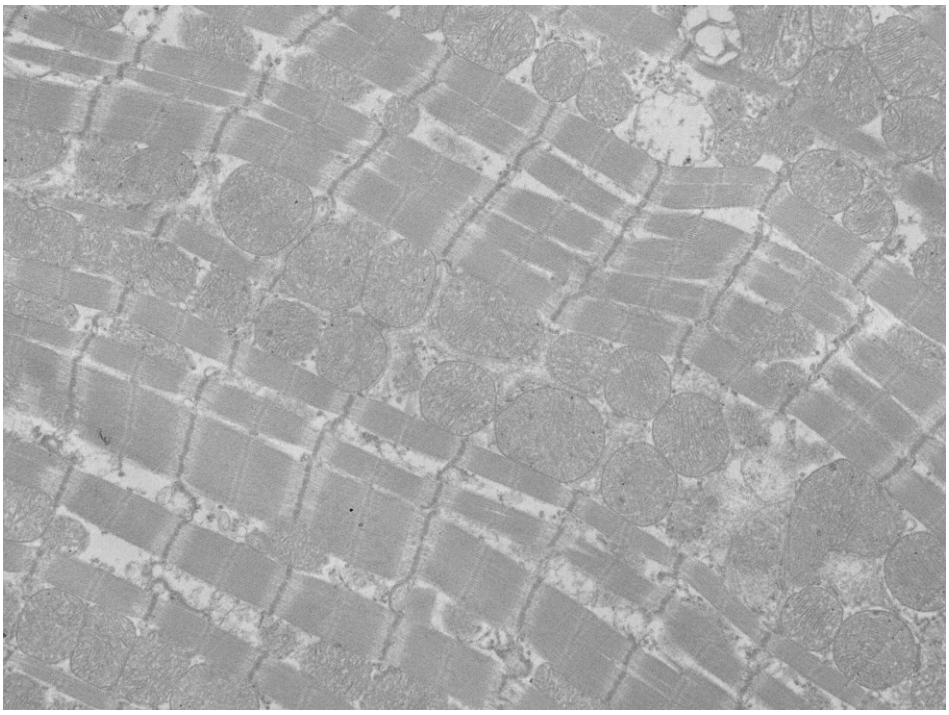


Figure 15: Transmission electron micrography of cardiomyocytes from banded rats post-DOX. No alterations of Z-lines, intracellular vacuoles, mithochondria or nuclei

Molecular results

Table 25: Number of PF (Pass-Filter) Reads for each rat

Sample ID	Sample group	Number of PF Reads
P0801	Doxo + PAB	2721823
P0810	Doxo + PAB	2776570
P0802	Doxo	3189829
P0704	Doxo	3589919
P1001	Sham	2481877
P1002	Sham	2929765

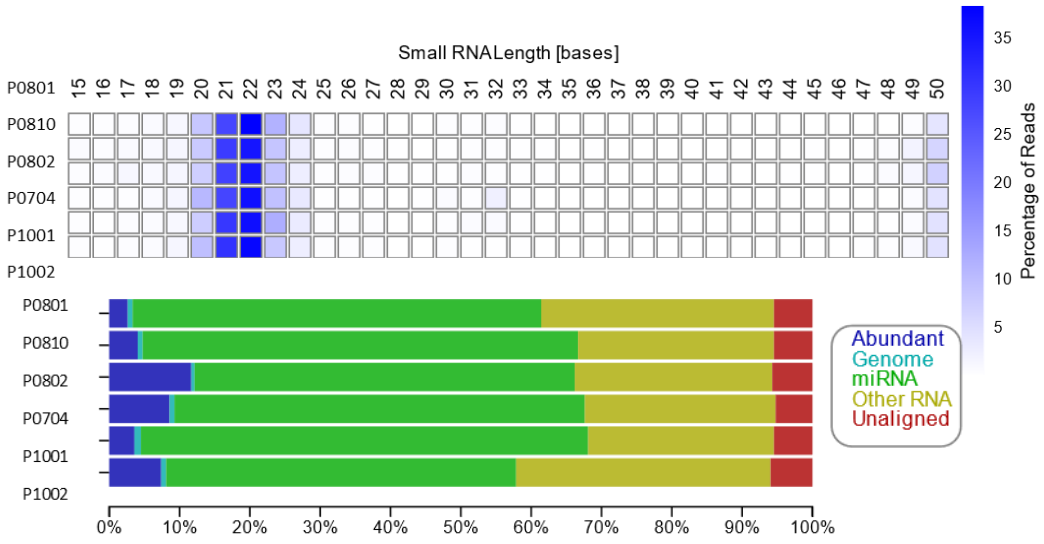


Figure 16: Reads distribution

Table 26-27: differentially expression analysis between DOX-treated rats and controls.

	Known	Novel	Total
Total miRNAs	5489	123	5612
Tested miRNAs	1076	20	1096
Differentially Expressed miRNAs	3	0	3

	Status	Mean Count	Log2FoldChange	SD	q-value
hsa-miR-485-3p	Low	8.31	1.29	0.416	NaN
hsa-miR-125b-5p	Low	1.89	1.25	0.465	NaN
38_TGTGTCCGGGAAGTGGAGGAGA_hsa-mir-4669	Low	1.23	-1.25	0.457	NaN

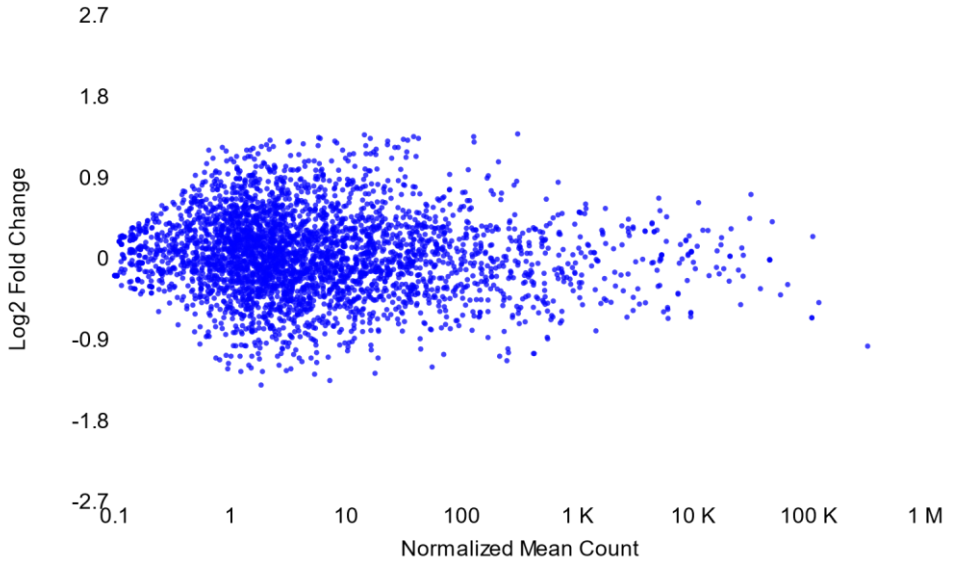


Figure 17: Differentially expressed miRNAs between DOX-treated rats and control rats. No over or under-expressione was detected. To consider an over/under-expression of miRNA the Log2FoldChange must be >1 or <-1

Table 28: Differentially expression analysis between DOX and DOX+PAB treated rats.

	Known	Novel	Total
Total miRNAs	5489	135	5624
Tested miRNAs	1180	16	1196
Differentially Expressed miRNAs	0	0	0

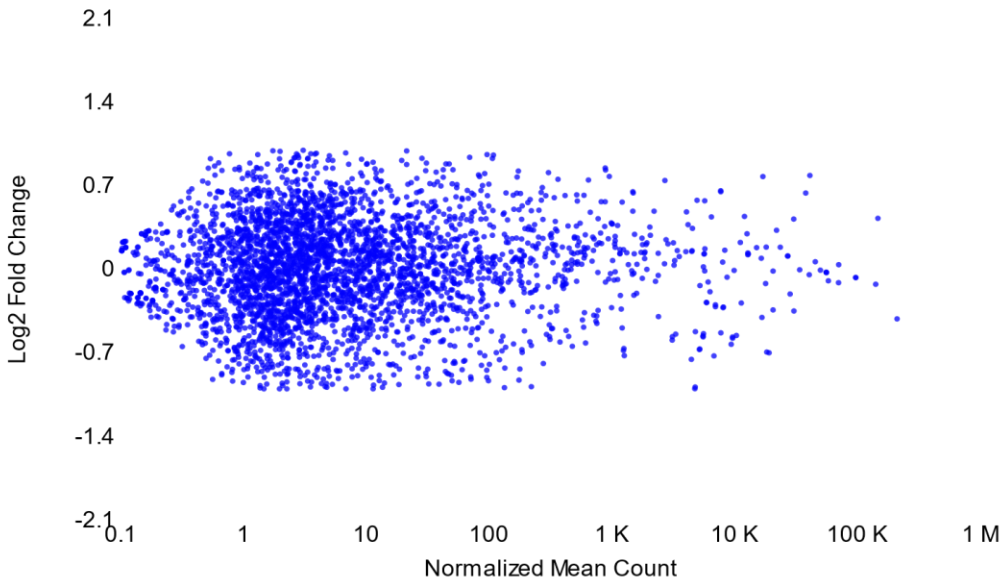


Figure 18: Differentially expressed miRNAs between DOX-treated rats and DOX+PAB treated rats. No over or under-expressione was detected. To consider an over/under-expression of miRNA the Log2FoldChange must be >1 or <-1

Unfortunately, the results from the molecular tests did not show any differentially expressed miRNA both between the controls and the DOX-treated rats and between the DOX and DOX+PAB treated rats.

This lack of results is mainly due to the small number of rats which underwent molecular testing.

We hope to overcome this limit with further experiments on more rats.

6.3 EXPERIMENTAL STUDY 2

After 10 days of experiment, the left ventricle walls of the hearts subjected to PAB appeared markedly thicker than the untreated samples. Analysis of EdU incorporation revealed a marked increase in the number of EdU+ cells in these hearts in comparison to control animals (*Figure 19*). Confocal microscopy analysis indicated that most of the proliferating cells were indeed alpha-actinin positive CMs.

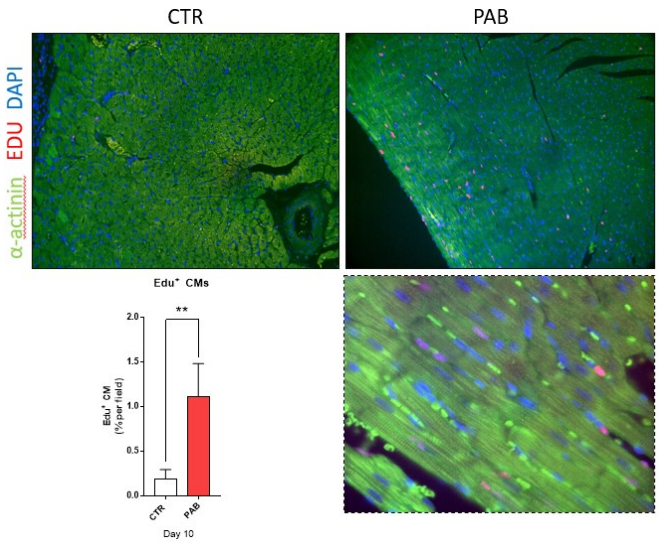


*Figure 19. Preliminary evidence that PAB induces cardiomyocyte proliferation. Representative images of EdU immunostaining in the left ventricles of rats 10 days after PAB, with relative quantifications. Cardiomyocytes are stained in green (a-actinin), proliferating nuclei in red (EdU incorporation in replicating cells during S-phase). The bottom panel show higher magnification image of proliferating cells. Data are mean ‡ SEM.: **P < 0.01; Student's two-sided t-test. Scale bar, 100 um.*

Analysis of the AAV fluorescent reporter system to monitor cell transduction and proliferation revealed that intracardiac injection is below 20% with the established viral titer and that the CMV driven recombination (counted as GFP+ cells vs Cherry+ cells) is approximately 60% (*Figure 20*, left panel). In the case of endogenous cardiac proliferation (monitored as GFP+ cells vs Cherry+ cells in the

presence of a CyclinB driven Cre recombinase), control animals showed less than 10% of proliferative events, while PAB animals showed a massive induction of GFP labelled cardiomyocytes (*Figure 20*, right panel), consistent with the Edu results. WGA staining was performed in order to monitor the fiber area in both left and right ventricles (ONGOING fiber quantification).

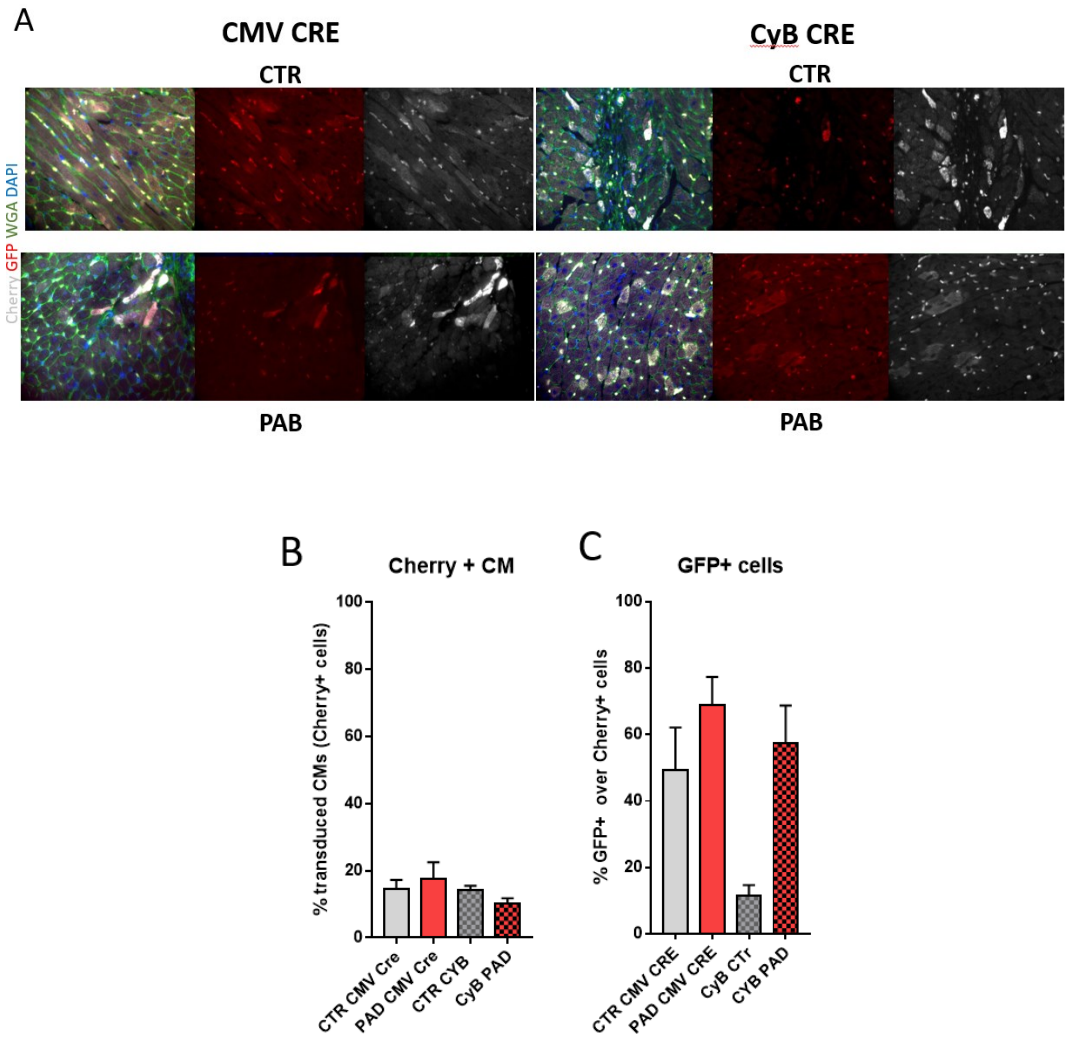


Figure 20. Preliminary evidence that PAB induces cardiomyocyte proliferation using an AAV fluorescent reporter. Representative images of immunostaining in the left ventricles of rats 10 days after PAB, with relative quantifications. Cardiomyocytes are stained in green (WGA), GFP-labelled CMs in red and transduced cells in white (Cherry protein). The left panel show high magnification image of animals transduced with the reporter and a CMV driven Cre recombinase, while the right panel showed the animals that received an AAV9 with a CyclinB-driven Cre recombinase (labelling specifically proliferating CMs with GFP).

7. DISCUSSION

7.1 CLINICAL STUDY

One of the challenges of the Pulmonary Artery Banding application is identifying, among eligible patients, potential responders and non-responders to this procedure.

The age of patients at PAB appears to have a critical impact on the outcome of the surgery.

Even if in our study there was no difference in the overall freedom from death, VAD and OHTx combined between patients < 12 months of age and patients > 12 months, we proved that the former underwent VAD application less frequently than the latter. Anyway, despite the lack of statistical significance due to the small sample numerosity, it was still clear that the younger the patients were, the better the outcomes. While Schranz et al. advocated a 6-year-old- age limit for PAB, our experience suggests that PAB should be considered as a surgical option only in children under 1 year of age, since a lower threshold seems to have better outcomes due to the link between age and cardiac proliferation that we assessed in the experimental part of this study.

Another factor we considered was the gender. In adult patients, it is known that women with DCM have better survival compared to men (72). In this study on pediatric patients, we demonstrated that after the PAB procedure females have greater overall freedom from death, VAD and HTx combined than males. The underlying reasons for this gender difference are not known, but might be related to genetic, hormonal or other causes.

The etiology of DCM may have a crucial impact on the outcome of PAB. In our study we observed that patients with Idiopathic Dilated Cardiomyopathy (iDCM)

suffer more adverse events (40%) than those with other etiologies (DCM from acute viral myocarditis, chronic viral myocarditis, spongy myocardium etc.). Among patients with iDCM, those with mithocondrial genome variations may benefit less from PAB's effects. Also, the presence of severe endocardial fibroelastosis may prevents them from reaching functional recovery after PAB.

Another important factor to consider is that each institution's approach to ESHF care undoubtedly has an impact on the procedure's outcomes.

In our center, for example, three patients were put on ECMO after experiencing abrupt cardiac deterioration due to the lack of response to PAB or complications following PAB, such as LV thrombosis and pneuomonia. The MCS application served as a bridge to OHTx in two cases and as a bridge to partial recovery in the other. In any case, it enabled them to overcome these acute difficulties and survive afterwards.

In contrast to that, other centers, for example Ghent, according to their management protocols for ESHF, do not provide mechanical support such as ECMO for children under two years of age, who unfortunately perish in the event of acute failure, as it happened for three of their patients enorolled in our study. Their decision comes from the observation that VAD application on infants showed the lowest overall survival when compared to older children (24), as reported previously in *figure 3*.

Still, we believe that a more aggressive management, which includes MCS devices, could play a critical role on the success of this therapy.

Indeed, as we observed in patients treated at our facility, the months following PAB are crucial, and children must be kept under strict follow-up. Even frequent pediatric insults, like pneumonia, can cause biventricular failure. In this case, a prompt instauration of MCS is the only chance to sustain the patient until a compatible donor is available.

From this point of view, PAB should be considered as a surgical option exclusively in patients who are eligible for long-term MCS. Thus, complete

patient assessment for MCS, family counseling, and notification to manufacturers must be parts of the preoperative planning of PAB.

Unfortunately, we are aware that at the current state of the art, this reccomendation could not be applied in countries, such as Ghent, where clinical conditions of these children do not meet criteria for MCS application.

Moreover, if we strictly followed this recommendation, we would lose the great potential of this procedure, especially in countries with limited resources (e.g, Manila), which lack MCS devices and/or transplant centers, where PAB could serve as the sole alternative for these children to recover from ESHF in DCM.

Thus, from this point of view, PAB may represent a turning point in the care of ESHF in many countries where MCS or HTx are not viable or feasible due to limited resources.

What we've discovered is that PAB is an effective surgery that in the best-case scenario serves as a bridge to full recovery. However, even if the recovery does not occur, PAB is still an excellent alternative to explore as a bridge to OHTx, since it drastically decreases some of the disadvantages of MCS, such as the need for long-term hospitalization and the high risk of thrombosis and infection.

The recovery associated with PAB procedure is certainly long, but shorter than potential spontaneous recovery.
One of the key questions surrounding the effectiveness of PAB is whether or not the left ventricle of the selected DCM patients would have healed anyway, even without undergoing PAB. In 10-20% of DCM patients, spontaneous healing is possible, particularly when a myocarditis-based cause is hypothesized (73,74). However, even if this is true, it takes years for natural functional recovery to happen, whereas PAB-induced LV reverse remodeling is evident already after a few months following surgery.

More studies are needed though to distinguish responders and non-responders to the procedure.

7.2 EXPERIMENTAL STUDIES

This is the first time that a pulmonary artery banding is performed in a rat model of dilated cardiomyopathy. We chose the rat as the experimental model because it is clearly easier to be housed and handled in our laboratory than other bigger animals. Despite their little size, dimensions of rat hearts and main vessels allowed us to perform the LAD-ligation and PAB procedures. Among the different breeds, we adopted the Sprague-Dawley strain, owing to its excellent reproductive performance, calmness, and ease of handling.

The goal of estabilishing an experimental rodent model of DCM was quite challenging and, unfortunately, not completely accomplished.

We chose essentially two models: pharmacological induction of DCM through Doxorubicin-injection and surgical induction through temporary LAD-ligation operation.

Other models are described in literature, as the experimental autoimmune myocarditis (EAM) induced-DCM model (75) or the DCM induced by pressure overload driven by ascending aorta constriction (76–80). Regarding this latter model, we could not apply this methodology since, as required by animal experimentation law, we could only operate on rats once.

Instead, this model requires two separated surgeries: the banding of the aorta, in order to induce LV compensated hyperthrophy which then progresses to decompensated heart failure, and the banding of the main pulmonary artery.

The initial idea of our study was to establish a young and adult model of both Doxorubicin and LAD-ligation induced-DCM to confront them and evaluate potential better outcomes of PAB on the young model.

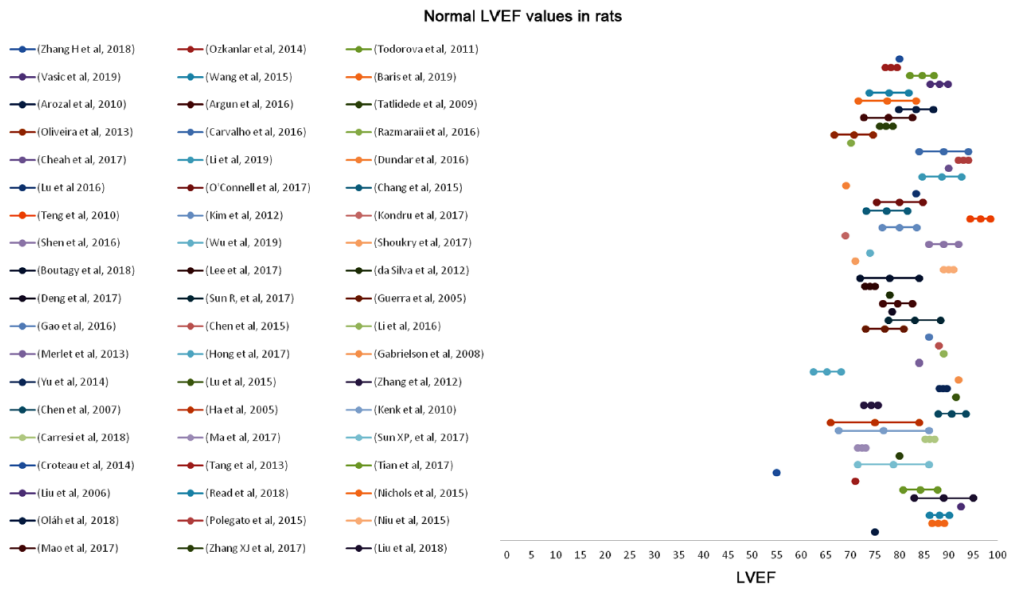
Thus, we were not able to reach this goal due to the lack of juvenile rats and the fact that young rats which underwent the two weeks protocol of DOX administration inevitably became older at the time of PAB.

DOXORUBICIN-INDUCED DMC

Sheep were used in previous attempts of experimental PAB on doxorubicin-induced HF by Yerebakan et al (82). In their study, the group treated with PAB had a better LVEF and FS, as well as reduced LV dimensions, three months following the procedure. However, significant differences in fibrosis or inflammatory infiltrates at terminal histopathology were not detected, probably because immunochemistry analysis was not performed, and the grade of hypertrophy and hyperplasia of cardiomyocytes was not assessed. But also, it is of note that the doxorubicin-based HF does not seem to reproduce exacty the pathology of DCM.

Similarly to these results, in our study the short-term DOX protocol (68) used to induce DCM in rats, although sufficient to detect alteration of cardiac function through 2D echocardiography, did not allow us to observe any histopatological damage of the heart tissue or any variation of miRNA expression.

In particular, comparing our echocardiographic data to those reported in literature (71), baseline values were similar, as well as LVEF and LVFS after Doxorubicin-injection, as shown in the following figures.



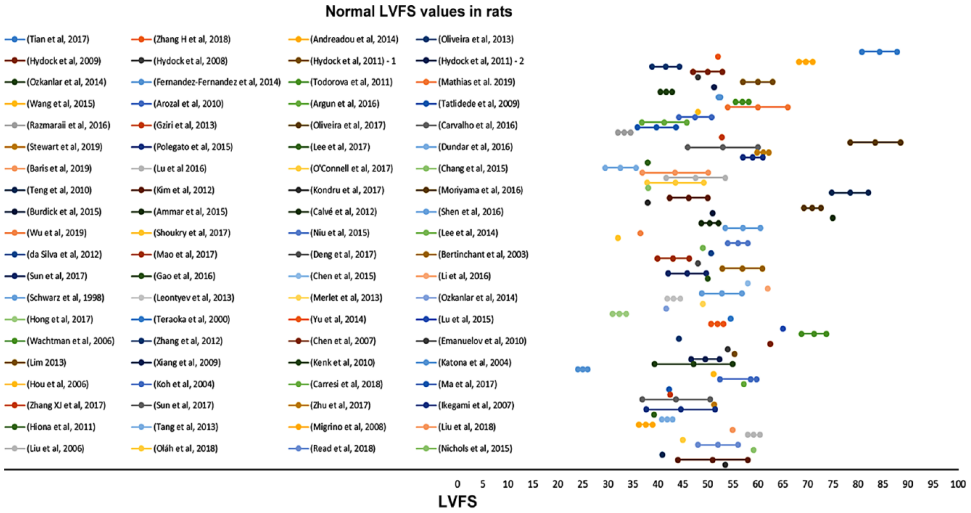


Figure 21 and 22: normal baseline LVEF and LVFS values in rats before anthracycline administration in 57 and 80 relevant studies (71)

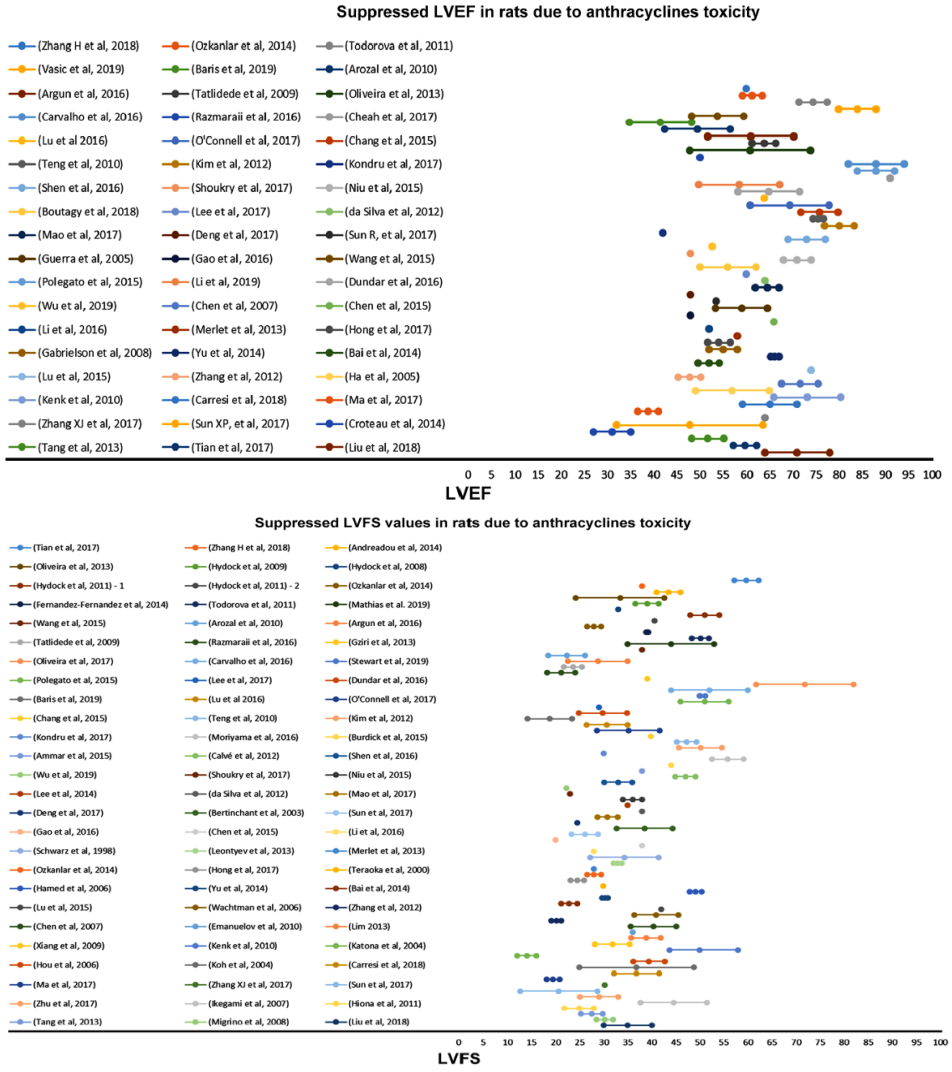


Figure 23 e 24: suppressed LVEF abd LVFS values in rats due to anthracycline toxicity as reported in 54 and 78 studies respectively (71).

The reason behind the lack of histopatologic changes, despite the cardiac functional deterioration confirmed through ultrasound examination, may be related to the injection protocol we used, which was appealing due to the reduced duration of the experiment and the lower associated costs, but it may not be sufficient to establish an adequate DCM model.

Digging into the current literature related to DOX-induced DCM rat models we found two major limitations (70):

- High mortality, approximately 50%
- Substantial individual variability in the magnitude of left ventricular remodeling and systolic dysfunction.

There are essentially two types of DOX administration protocols, the short-term (83,84) and the long-term (85,86) one and in both cases the total accumulated dose of DOX is the main determinant of the magnitude of left ventricular dysfunction (Maral et al., 1967) (87)

Apparently, both LT and ST DOX injection models should be effective in inducing typical myocardial histological changes of similar magnitude:

- Foci of swollen and vacuolated cardiomyocytes
- Disorganization of myofibrillis
- Pronounced intestitial fibrosis

However, short-term injection protocols usually show variable effects, inconsistently reproducing a heart failure. Indeed, in contrast to what has been reported in literature (70), in our study 2D echocardiography showed functional impairment, whilst no structural disturbance was detected through histopatological examinations.

In general, only the LT injections protocols seem to be associated with left ventricular remodeling and systolic dysfunction resembling those found in DCM.

For this reason, a long-term protocol, consisting of a cumulative dose of 18 mg/Kg (2 mg/Kg/dose once a week for 9 weeks) (70) may represent an adequate, cost-effective, and reproducible model for DOX-induced cardiomyopathy, the effects of which would replicate morphologic and functional cardiac changes observed in human patiens treated with this drug.

As the study proceeded, we also noticed that young rats were less subsceptible to doxorubicin's cardiotoxic effects. This feature is in accordance with literature, where an age-related difference in Doxorubicin pharmacokinetics is described (88). In fact, Doxorubicin and its metabolites are increased in old rats due to a reduced systemic clearance, which leads to a higher risk of anthracycline toxicity.

It was also observed that doxorubicin treatment resulted in sex differences, with females better protected from doxorubicin-induced HF, due to less mitochondrial damage associated with lower cardiotoxicity (89).

LAD-LIGATION-INDUCED DCM

As for the LAD-ligation, in our investigation, despite some signs of myocardial infarction of the LV lateral wall seen by histopathological analysis on heart tissue, the temporaray LAD-ligation actually did not show any negative variation in cardiac function and it did not help achieving a DCM model.

According to past research, ischemia for 30 min followed by 2 months of reperfusion results in (90):

- 15% infarcted left ventricle
- moderate impaired systolic function (reduced EF and FS)

Supposedely, the ligation should have caused moderate to extensive anterior myocardial infarction, leading to congestive heart failure in all rats, but in our

study, it was not sufficient to establish a model of rat DCM, despite the very low associated mortality of the procedure.

The lack of echocardiographic changes, as EF and FS alterations, is likely attributable to the fact that fibrosis caused by temporary LAD ligation occurs mostly in infarcted and border areas, rather than the remote myocardium.

As for PAB on temporary LAD-ligated animal, no changes were found as well through echocardiographic or histopathologic examination.

The great limitation in this case is represented by the fact that we could not perform more than one operation on rats, according to animal experimental law. In order to carry out one operation only, PAB had to be performed after the 30 min ischemia caused by LAD ligation. For this reason, we believe that the time was not sufficient to induce any damage, on which later evaluate the benefits of PAB.

In contrast to temporary LAD-ligation, a 2 months permanent LAD-ligation (91) protocol described in literature appears to result in significant cardiac function decrease. This procedure first induces large myocardial infarction, followed by progressive development of compensatory hypertrophy of non-infarcted areas in order to maintain LV systolic function. Only 2 months after ligation, rats actually provide a model of chronic ischemic dilated cardiomyopathy.

On the other hand, by performing both the permanent-LAD-ligation and PAB on the same occasion, PAB would exert its effects on a model still in development.

Further attempts are going to be tried out at our Institution, in order to reach an ideal experimental rodent model of DCM.

RAT HEART-FAILURE THERAPY

Some of the rats in this study, with poor health conditions after DOX-injections, LAD-ligation and/or PAB surgery, were treated with bisoprolol via drinking water

(0.3 mg/Kg/day), aiming to ameliorate symptoms related to these procedures and reduce mortality.

The rationale for this use was that rats on bisoprolol have a 38% lower rate of deaths(92). Infact, it improves survival by reducing heart rate and improving left ventricular function. According to the literature, other beta-blockers were not used in our study but seem to have a positive effect on survival, such as Carvedilol at low doses (93) or Metoprolol (94).

As for other drugs, Furosemide alone induces mortality in a rat model of chronic heart failure (95), but Furosemide plus Ramipril (as well as Captopril (96)) treated rats showed improvement in ventricular pump function and attenuation of ventricular dilation.

Torasemide, another diuretic, significantly reduces both LVDd and LVDs and increases FS, but it also attenuates LV fibrosis (hallmark of DCM), so it may prevent the correct estabilishment of the DCM model. (97)

Finally, Spironolactone improves cellular architecture of the left ventricle, reduces the activation of NFkB (cardiac inflammation) and reduces endoplasmic reticulum stress, but has lesser efficacy in antifibrotic effect. (98)

A combination of these drugs might improve survival and outcomes of the different procedures in rats. However, since the goal is to optimize the survival, and still to induce the disease in order to establish a DCM model, their use must be carefully pondered.

CARDIOMYOCYTE PROLIFERATION

In order to assess potential cardiomyocyte proliferation driven by PAB, the experimental procedure was carried out on healthy juvenile Sprague-Dawley rats.

The preliminary results are particularly encouraging, since they reveal that PAB-induced reverse remoedelling derive from cardiac repopulation driven by cardiomyocyte proliferation, through resident or circulating stem cells, rather than compensatory hypertrophy.

Furthermore, the great acknowledgment of this research is the fact that the cardiomyocyte proliferation occurs not only in the right ventricle, but also in the left ventricle.

Indeed, the increased afterload of the RV induced by PAB would lead one to believe that cardiac regeneration is limited to the right ventricle via directed mechanosensing and intracellular pathways causing cellular proliferation. Instead, as confirmed by this study, cardiomyocyte proliferation extends also to the left ventricle, corroborating the hyopthesis that this procedure stimulates mechanical ventricular-ventricular interaction (VVI), which is at the basis of the LV recovery after PAB.

Although preliminary results appear to confirm the PAB-induced cardiomyocyte proliferation, it would later be interesting to demonstrate this finding also after performing PAB on rats affected by induced DCM.

In humans, it is well-known the regenerative potential of the heart, which is a characteristic of newborns due to the presence of highly active cardiac progenitor. Though this potential decreases with age, with a significant reduction by one year of age and very low but still measurable levels after 20 years of life.

Actually, as demonstrated by a research in a 20-year-old lady with S/P Mustard in TGA, an adjustable PAB in selected patients may be able to produce a gradual but successful improvement of sub-pulmonary left ventricle function. (99)

In this regenerative point of view, the Hippo pathway, which is an evolutionarily and functionally conserved signaling pathway that controls organ size, by regulating cell proliferation, apoptosis and differentiation in the adult heart (100) (101), may be engaged. Recent studies demonstrate that cardiac-specific deletion of Hippo pathway kinase Mst (STE20-like protein kinases) co-activator WW45 (WW domain-containing adaptor 45), Mst1, or Lats2 (large tumor suppressor homologue 2) in mice result in over-grown hearts with elevated cardiomyocye proliferation. Furthermore, over-expression of YAP in the mouse embryonic heart

induces cardiomyocyte proliferation and increases heart size and promotes cardiac regeneration after myocardial infarction, whereas deletion of YAP in the mouse heart impedes cardiomyocyte proliferation, causing myocardial hypoplasia and embryonic or premature death.

We speculate that PAB, by modifying intracardiac pressure parameters, might stimulate the cardiac mechanosensing apparatus, which can regulate the Hippo signaling. Indeed, the YAP and TAZ, transcriptional coactivators, respond to cytoskeletal deformations related to cell shape and extracellular matrix architecture(102,103). These molecules are converging effectors of the Hippo signaling (104). As a result, mechanical stress stimulus may be translated into the activation of one of the most essential regulatory pathways of cardiomyocytes proliferation via the myocardial sensing machinery (101,105).
Thus, the cardiomyocyte proliferative potential would be reactivated by the augmented afterload driven by PAB. We believe that the identification of the microscopic and molecular causes of ventricular rehabilitation should be the focus of the future PAB research in DCM. For this reason, further investigations are going to be conducted at our institution.

8 CONCLUSIONS

The results from the clinical study basically show that PAB, together with aggressive anti-congestive medical management, may be an effective procedure to treat ESHF in selected infants and children, as an alternative strategy for bridge to transplant or functional recovery.

In fact, at mid-term follow-up, survivors presented with significant improved left ventricular function and reduced LV volumes and mitral regurgitation.

In this experience, cardiac remodeling seems to be better achieved in patients <12 months, probably due to a preserved myocardial regenerative potential.

Unfortunately, early postoperative period is delicate, and requires strict follow-up and long hospitalization.

Further research is required to differentiate between “responders and non” to such strategy, that may normalize the long-term prognosis of these children.

However, because the particular biological pathways recruited by PAB and the ultimate therapeutic results are uncertain, several surgical institutions are still hesitant to employ this technique.

To provide an evidence-based explanation in support of this strategy, we tried to understand the cellular and molecular working mechanisms of PAB.

We attempted to create a model of DCM on rats, which turned out to be a difficult challenge, since we only achieved a partial model of DOX-induced DCM, while LAD-ligation was not helpful. On this model we assessed, in a small number of rats, the deterioration of cardiac function through 2D echocardiography after DOX-injection and the improvement after PAB procedure. No alteration was detected through histopatological and molecular tests.

On the other hand, we were able to prove the PAB-induced cardiac proliferation on healthy young rats. The great acknoweledgement is that cardiac proliferation was detected in the left ventricle, as a direct consequence of the mechanical ventricular-ventricular interatction (VVI) driven by PAB.

These preliminary, but promising results may subsequently be confirmed by more research and investigations regarding the involved molecular transduction mechanisms.

Supplemental tables of the collected data on RedCAP

Table 29: Demographic and operative Data

Confidential

Demographic And Operative Data

Record ID	_____ (Please provide number in progressive order of insertion: i.e. 001)
patient ID	_____ (Provide an ID: initial firstname, last name, dateofbirth(dd/mm/yyyy):esample: John Smith, JS10092009)
Date of Birth (dd/mm/yyyy)	_____
Gender	◯ M ◯ F
Weight at PAB (kg)	_____
Associated congenital heart disease (CHD)	◯ Yes ◯ No
Define associated CHD	_____ (Please describe briefly which CHD is associated)
Etiology of end stage heart failure (ESHF)	☐ Dilative cardiomiopathy, idiopathic ☐ Acute viral myocarditis ☐ Chronic viral myocarditis ☐ Spongy myocardium ☐ Genetic Dilative cardiomiopathy ☐ Post ischemia ☐ CHD ☐ Autoimmune ☐ Other (Select the type of ESHF cause that the patient has (select all that apply).)
Etilogy of ESHF -- Other Description	_____ (Please provide further details.)
Endomyocardial biopsy	◯ Yes ◯ No (Please, tick yes if the EMB was performed prior to PAB)
Endomyocardial biopsy results	_____ (Please, describe the diagnosis made at EMB)

Is the patient affected by a genetic syndrome?	☐ DiGeorge ☐ Down ☐ Kartagener ☐ none ☐ other
Echocardiography assessment on admission	◯ Yes ◯ No (If yes, please fill the Echocardio form)
Cardiac MRI assessment on admission	◯ Yes ◯ No (If yes, please fill the Cardiac MRI form)
Date of PAB	_____
Age at PAB	_____ (in days)
Associated surgical procedure	_____ (Please, dscribe if some other surgical procedure was performed together with the PAB.)
PAB peak-gradient at the end of procedure (mm Hg)	_____ (Please, provide the RV-PA pressure gradient at the end of the PAB procedure)
Leftward interventricular septum displacement after PAB	◯ Yes ◯ No (Please specify that after PAB, intraoperative echo was showing a leftward displacement of the IVS)
Was MR reduced after PAB?	◯ Yes ◯ No
Delayed chest closure?	◯ Yes ◯ No
When the chest was closed?	◯ within 24 hours ◯ within 48 hours ◯ within 72 hours ◯ after 72 hours
Has the patient undergone any procedure before PAB?	◯ no ◯ yes, ECMO ◯ yes other: specify
Other procedure prior to PAB	_____ (Please provide further details.)

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Date of prior procedure	_____ (Note the date at which the procedure prior to PAB occured)
Was the patient listed for heart transplant before PAB?	◯ Yes ◯ No
INTERMACS level	◯ 1. Critical cardiogenic shock ◯ 2. Progressive decline ◯ 3. Stable but inotrope dependent ◯ 4. Resing symptoms ◯ 5. Exertion intolerance ◯ 6. Exertion limitation ◯ 7. Advaced NYHA class III ◯ 8. Unknown
Preoperative intubation?	◯ Yes ◯ No
Preoperative infusion of levosimendan?	◯ Yes ◯ No
Did cardiopulmonary bypass occur during the cardiac surgery?	◯ Yes ◯ No
Was cardioplegic arrest used?	◯ Yes ◯ No
How long did cardioplegic arrest last?	_____ (in minutes)
Were there postoperative complications after PAB?	◯ Yes ◯ No
Which postoperative complications occurred?	☐ LCO sdr ☐ bleeding requiring reoperation ☐ AKI (acute kidney injury) ☐ chilothorax ☐ pleural effusion requiring chest tubes stay > 3 days ☐ infection requiring antibiotic therapy for more than 7 days ☐ arrhythmia: bradi ☐ arrhythmia: tachy ☐ Cardiac arrest ☐ Any abdominal problem ☐ any cerebrovascular/neurological with complete resolution ☐ any cerebrovascular/neurological with residual deficit ☐ respiratory failure requiring IPPV > 3 days ☐ other
Other postoperative complication	_____ (describe other postoperative complication)

Length of ICU stay	_____ (in days)
Intubation time after PAB	◯ < or = 24 hrs ◯ 24 -72 hrs ◯ > 72 hrs
Early death	◯ Yes ◯ No (within 30 days from operation of in the same admission)
Date of early death	_____
Cause of early death	_____ (please , describe briefly the main cause of death)
Early reintervention	◯ Yes ◯ No
Which early reintervention was necessary?	☐ surgical PAB tightening ☐ delayed chest closure ☐ ECMO ☐ VAD ☐ Transplant ☐ other
describe other reintervention	_____
Date of early reintervention	_____
Other early reintervention	_____ (Please provide further details on the characteristics of the early reintervention)
Date of Discharge (D/C) home	_____
Home therapy	☐ none ☐ ACE-inhibitors or ARB ☐ beta blockers ☐ diuretics ☐ warfarin ☐ aspirin or antiaggregation therapy ☐ other
Other home therapy	_____ (Please provide further details.)

NYHA/Ross class at D/C	◯ I ◯ II ◯ III ◯ IV
PrePAB BNP	_____
Was echocardiogram performed at discharge?	◯ Yes ◯ No (If yes, please fill the Echocardio form)
PAB peak-pressure gradient at D/C (mm Hg)	_____
Other information	_____ (Please provide other info if considered important)

Table 30: Last Follow-up data

Confidential

Last Follow Up

Record ID	_____ (Please provide number in progressive order of insertion: i.e. 001)
Date of Follow up	_____
Length of Follow up	_____
Late death	◯ Yes ◯ No
Date of late death	_____
Cause of late death	_____ (please , describe briefly the main cause of death)
Has the patient experienced any adverse event (AE) other than death, during follow up?	◯ Yes ◯ No
Late AE	◯ surgical ◯ non surgical
Late surgical AE	◯ Heart transplant ◯ VAD ◯ other (Please select which applies)
Other late surgical AE	_____ (please, briefly describe the OTHER surgical AE that has occurred to the patient)
Date of surgical AE	_____ (Please, consider the first surgical AE)
Late NON Surgical AE	☐ none ☐ arrhythmia (tachy) ☐ Arrhythmia (brady) ☐ renal failure ☐ progressive congestive heart failure ☐ neurological event ☐ sudden cardiac death (SCD) ☐ cardiac arrest/aborted SCD ☐ other
Other NON surgical AE	_____

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Therapy at last FU	☐ ACE Inhibitors or ARB ☐ diuretics, ☐ beta blockers ☐ Antiaggregation ☐ other (Select which meds is the patient taking at the FU time.)
Has the patient required PAB dilation during this FU?	◯ no ◯ yes, once ◯ yes, more than once ◯ yes other
Other PAB dilation	_____
Date of first PAB dilation	_____ (Please, consider the first PAB dilation if more than once)
is the patient on Sinus Rhythm at last FU?	◯ Yes ◯ No
Which arrhythmia?	_____ (Please provide further details.)
NYHA/Ross class	◯ I ◯ II ◯ III ◯ IV (tick the appropriate)
Has the patient required Levosimendan infusion therapy after PAB?	◯ Yes ◯ No
Cycles of levosimendan	☐ one ☐ two ☐ more than 2
Timing of levosimendan cycle	◯ within 1 month form discharge ◯ within 3 months from discharge ◯ within 6 months form discharge ◯ after 6 months form discharge
Has the patient had a CMRI at Follow up?	◯ Yes ◯ No (If yes, please fill the Cardiac MRI form)
Date of CMRI	_____

Table 31: Echocardiographic data

Confidential

ECHO controls

Record ID	_____ (Please provide number in progressive order of insertion: i.e. 001)
Date of echocardiogram	_____
LVEF	_____ (%)
LVEF (visual estimation)	◯ < 20% ◯ 20-30% ◯ 30-45% ◯ 45-55% ◯ >55%
PAB peak-pressure gradient at CW Doppler	_____ (mmHg)
Mitral regurgitation	◯ Yes ◯ No
MV regurgitation grade	◯ none ◯ Mild ◯ mild-moderate ◯ moderate ◯ Severe
LV dilation (visual estimation)	◯ no ◯ mild ◯ moderate ◯ severe
LV dimensions (M-mode, mm)	_____ (Please provide LVEDD at M Mode)
LVEDV (Simpson rule, ml/m2)	_____
TAPSE (mm)	_____ (mm)
Tricuspid valve regurgitation	◯ Absent ◯ Mild ◯ mild-Moderate ◯ Moderate ◯ Severe
Right ventricular pressure Calculation (mm Hg) if TR	_____
Images or video	(Please provide upload any image or video u may think can be of help)
Additional informations	_____

Table 32: CMRI data

Confidential

Pulmonary artery banding for myocardial rehabilitation in infants
Page 1

Cardiac MRI controls

Record ID	_____ (Please provide number in progressive order of insertion: i.e. 001)
patient ID	_____ (Provide an ID: initial firstname, last name, dateofbirth(dd/mm/yyyy):esample: John Smith, JS10092009)
Date of Cardiac MRI assessment	_____
MRI: LV ED volume (ml/m2)	_____ (please provide LV end diastolic volume at CMRI)
MRI: LV ES volume (ml/m2)	_____ (please provide LV end Systolic volume at CMRI)
MRI: LVEF(%)	_____ (please provide LVEF)
MRI: LV Stroke volume (ml/m2)	_____ (please provide Stroke volume at admission for ESHF)
MRI: LV cardiac output (l/min)	_____ (please provide CO at admission for ESHF)
MRI: LV cardiac index (l/min/m2)	_____ (please provide CI at admission for ESHF)
MRI: LV cardiac mass (g/m2)	_____ (please provide cardiac mass at admission for ESHF)
MRI: RVEF (%)	_____ (please provide RVEF)
MRI: RV ED volume (ml/m2)	_____ (please provide RV end diastolic volume at CMRI)

MRI: RV ES volume (ml/m2)	_____ (please provide RV end Systolic volume at CMRI)
MRI: RV Stroke volume (ml/m2)	_____ (please provide Stroke volume at admission for ESHF)
MRI: RV cardiac output (l/min)	_____ (please provide CO at admission for ESHF)
MRI:RV cardiac index (l/min/m2)	_____ (please provide CI at admission for ESHF)
MRI: RV cardiac mass (g/m2)	_____ (please provide cardiac mass at admission for ESHF)
Presence of myocardial oedema	◯ Yes ◯ No
Presence of myocardial fibrosis (LGE)	◯ Yes ◯ No
Site of fibrosis	◯ fibrosis in RV ◯ fibrosis in LV ◯ fibrosis in both
Which type of fibrosis?	◯ Subendocardial ◯ Intramyocardial ◯ transmyocardial
Other information to describe?	_____
MRI images or Video	(Please upload any image or video of MRI that can be of help)

BIBLIOGRAPHY

1. Hsu DT, Pearson GD. Heart Failure in Children. :8.

2. Towbin JA, Lowe AM, Colan SD, Sleeper LA, Orav EJ, Clunie S, et al. Incidence, causes, and outcomes of dilated cardiomyopathy in children. JAMA. 18 ottobre 2006;296(15):1867–76.

3. Bottner PC, Fernandez TC, Valenzuela ML, Romero PC. Dilated cardiomyopathy and severe heart failure. An update for pediatricians. Arch Argent Pediatr [Internet]. 1 giugno 2018 [citato 9 maggio 2022];116(3). Disponibile su: http://www.sap.org.ar/docs/publicaciones/archivosarg/2018/v116n3a18e.pdf

4. Lipshultz SE, Lowe AM, Lurie PR, Messere JE. The Incidence of Pediatric Cardiomyopathy in Two Regions of the United States. N Engl J Med. 2003;9.

5. Cohen JA, Almodovar MC. Dilated cardiomyopathy in children: moving beyond traditional pharmacologic therapy. Curr Opin Cardiol. gennaio 2020;35(1):52–7.

6. Sarnoff SJ, Berglund E. Ventricular Function: I. Starling's Law of the Heart Studied by Means of Simultaneous Right and Left Ventricular Function Curves in the Dog. Circulation. maggio 1954;9(5):706–18.

7. Sinagra G, Merlo M, Pinamonti B, curatori. Dilated Cardiomyopathy: From Genetics to Clinical Management [Internet]. Cham: Springer International Publishing; 2019 [citato 10 maggio 2022]. Disponibile su: http://link.springer.com/10.1007/978-3-030-13864-6

8. Wilkinson JD, Landy DC, Colan SD, Towbin JA, Sleeper LA, Orav EJ, et al. The Pediatric Cardiomyopathy Registry and Heart Failure: Key Results from the First 15 Years. Heart Fail Clin. ottobre 2010;6(4):401–13.

9. Daubeney PEF, Nugent AW, Chondros P, Carlin JB, Colan SD, Cheung M, et al. Clinical Features and Outcomes of Childhood Dilated Cardiomyopathy: Results From a National Population-Based Study. Circulation. 12 dicembre 2006;114(24):2671–8.

10. Franklin O, Burch M. Dilated cardiomyopathy in childhood. Images Paediatr Cardiol. 2000;2(1):3–10.

11. Lipshultz SE, Law YM, Asante-Korang A, Austin ED, Dipchand AI, Everitt MD, et al. Cardiomyopathy in Children: Classification and Diagnosis: A

Scientific Statement From the American Heart Association. Circulation. 2 luglio 2019;140(1):e9–68.

12. Schranz D, Recla S, Malcic I, Kerst G, Mini N, Akintuerk H. Pulmonary artery banding in dilative cardiomyopathy of young children: review and protocol based on the current knowledge. Transl Pediatr. aprile 2019;8(2):151–60.

13. Schultheiss HP, Fairweather D, Caforio ALP, Escher F, Hershberger RE, Lipshultz SE, et al. Dilated cardiomyopathy. Nat Rev Dis Primer. 9 maggio 2019;5(1):1–19.

14. Lee TM, Hsu DT, Kantor P, Towbin JA, Ware SM, Colan SD, et al. Pediatric Cardiomyopathies. Circ Res. 15 settembre 2017;121(7):855–73.

15. Rossano JW, Shaddy RE. Update on Pharmacological Heart Failure Therapies in Children: Do Adult Medications Work in Children and if Not, Why Not? Circulation. 4 febbraio 2014;129(5):607–12.

16. Schranz D, Voelkel NF. "Nihilism" of chronic heart failure therapy in children and why effective therapy is withheld. Eur J Pediatr. aprile 2016;175(4):445–55.

17. Kirk R, Dipchand AI, Rosenthal DN, Addonizio L, Burch M, Chrisant M, et al. The International Society for Heart and Lung Transplantation Guidelines for the management of pediatric heart failure: Executive summary. J Heart Lung Transplant. settembre 2014;33(9):888–909.

18. Hussey AD, Weintraub RG. Drug Treatment of Heart Failure in Children: Focus on Recent Recommendations from the ISHLT Guidelines for the Management of Pediatric Heart Failure. Pediatr Drugs. aprile 2016;18(2):89–99.

19. Miyamoto SD, Stauffer BL, Nakano S, Sobus R, Nunley K, Nelson P, et al. Beta-adrenergic adaptation in paediatric idiopathic dilated cardiomyopathy. Eur Heart J. gennaio 2014;35(1):33–41.

20. Miyamoto SD, Stauffer BL, Polk J, Medway A, Friedrich M, Haubold K, et al. Gene expression and β-adrenergic signaling are altered in hypoplastic left heart syndrome. J Heart Lung Transplant Off Publ Int Soc Heart Transplant. agosto 2014;33(8):785–93.

21. Shin YR, Park YH, Park HK. Pediatric Ventricular Assist Device. Korean Circ J. 3 luglio 2019;49(8):678–90.

22. Fraser CD, Jaquiss RDB, Rosenthal DN, Humpl T, Canter CE, Blackstone EH, et al. Prospective Trial of a Pediatric Ventricular Assist Device. N Engl J Med. 9 agosto 2012;367(6):532–41.

23. Almond CS, Morales DL, Blackstone EH, Turrentine MW, Imamura M, Massicotte MP, et al. Berlin Heart EXCOR pediatric ventricular assist device for bridge to heart transplantation in US children. Circulation. 23 aprile 2013;127(16):1702–11.

24. Rossano JW, VanderPluym CJ, Peng DM, Hollander SA, Maeda K, Adachi I, et al. Fifth Annual Pediatric Interagency Registry for Mechanical Circulatory Support (Pedimacs) Report. Ann Thorac Surg. dicembre 2021;112(6):1763–74.

25. INTERMACS Manual of Operations and Procedures. 2014;49.

26. de By TMMH, Mohacsi P, Gummert J, Bushnaq H, Krabatsch T, Gustafsson F, et al. The European Registry for Patients with Mechanical Circulatory Support (EUROMACS): first annual report. Eur J Cardiothorac Surg. maggio 2015;47(5):770–7.

27. O'Connor MJ, Lorts A, Davies RR, Fynn-Thompson F, Joong A, Maeda K, et al. Early experience with the HeartMate 3 continuous-flow ventricular assist device in pediatric patients and patients with congenital heart disease: A multicenter registry analysis. J Heart Lung Transplant. giugno 2020;39(6):573–9.

28. Baldwin JT, Adachi I, Teal J, Almond CA, Jaquiss RD, Massicotte MP, et al. Closing in on the PumpKIN Trial of the Jarvik 2015 Ventricular Assist Device. Semin Thorac Cardiovasc Surg Pediatr Card Surg Annu. gennaio 2017;20:9–15.

29. Hollander SA, Chen S, Murray JM, Lin A, McBrearty E, Almond CS, et al. Rehospitalization Patterns in Pediatric Outpatients with Continuous-Flow VADs. ASAIO J Am Soc Artif Intern Organs 1992. agosto 2017;63(4):476–81.

30. Dipchand AI. Current state of pediatric cardiac transplantation. Ann Cardiothorac Surg. gennaio 2018;7(1):31–55.

31. Barnes A, Gibson W. Pediatric heart transplant. Semin Pediatr Surg. 1 aprile 2021;30(2):151039.

32. Rossano JW, Cherikh WS, Chambers DC, Goldfarb S, Khush K, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: Twentieth Pediatric Heart Transplantation Report-2017; Focus Theme: Allograft ischemic time. J Heart Lung Transplant Off Publ Int Soc Heart Transplant. ottobre 2017;36(10):1060–9.

33. Dipchand AI, Laks JA. Pediatric heart transplantation: long-term outcomes. Indian J Thorac Cardiovasc Surg. agosto 2020;36(Suppl 2):175–89.

34. Kawaguchi AT, Bergsland J, Ishibashi-Ueda H, Ujiie T, Shimura S, Koide S, et al. Partial Left Ventriculectomy in Patients with Dilated Failing Ventricle. J Card Surg. settembre 1998;13(5):335–42.

35. Friedberg MK, Roche SL, Balasingam M, Stephenson E, Slorach C, Fackoury C, et al. Evaluation of Mechanical Dyssynchrony in Children With Idiopathic Dilated Cardiomyopathy and Associated Clinical Outcomes. Am J Cardiol. aprile 2008;101(8):1191–5.

36. Fadley AA, Manea WA, Fayyadh MA, Ahmadi MA. Cardiac resynchronization therapy in children with dilated cardiomyopathy: A case series. J Saudi Heart Assoc. aprile 2013;25(2):115.

37. Michel-Behnke I, Pavo I, Recla S, Khalil M, Jux C, Schranz D. Regenerative therapies in young hearts with structural or congenital heart disease. Transl Pediatr. aprile 2019;8(2):140–50.

38. Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park SY, et al. Cardiomyocyte proliferation contributes to heart growth in young humans. Proc Natl Acad Sci U S A. 22 gennaio 2013;110(4):1446–51.

39. Rupp S, Bauer J, Tonn T, Schächinger V, Dimmeler S, Zeiher AM, et al. Intracoronary administration of autologous bone marrow-derived progenitor cells in a critically ill two-yr-old child with dilated cardiomyopathy. Pediatr Transplant. 2009;13(5):620–3.

40. Lacis A, Erglis A. Intramyocardial administration of autologous bone marrow mononuclear cells in a critically ill child with dilated cardiomyopathy. Cardiol Young. febbraio 2011;21(1):110–2.

41. Bergmane I, Lacis A, Lubaua I, Jakobsons E, Erglis A. Follow-up of the patients after stem cell transplantation for pediatric dilated cardiomyopathy. Pediatr Transplant. maggio 2013;17(3):266–70.

42. Rivas J, Menéndez JJ, Arrieta R, Alves J, Romero MP, García-Guereta L, et al. [Usefulness of intracoronary therapy with progenitor cells in patients with dilated cardiomyopathy: Bridge or alternative to heart transplantation?]. An Pediatr Barc Spain 2003. aprile 2011;74(4):218–25.

43. Olguntürk R, Kula S, Sucak GT, Ozdoğan ME, Erer D, Saygili A. Peripheric stem cell transplantation in children with dilated cardiomyopathy: preliminary report of first two cases. Pediatr Transplant. marzo 2010;14(2):257–60.

44. Rupp S, Jux C, Bönig H, Bauer J, Tonn T, Seifried E, et al. Intracoronary bone marrow cell application for terminal heart failure in children. Cardiol Young. ottobre 2012;22(5):558–63.

45. Zeinaloo A akbar, Zanjani KS, Khosroshahi AG. Further follow up of the cardiomyopathic patient treated by intracoronary administration of autologous mesenchymal stem cells. Pediatr Transplant. giugno 2011;15(4):442.

46. Burkhart HM, Qureshi MY, Peral SC, O'Leary PW, Olson TM, Cetta F, et al. Regenerative therapy for hypoplastic left heart syndrome: first report of intraoperative intramyocardial injection of autologous umbilical-cord blood-derived cells. J Thorac Cardiovasc Surg. marzo 2015;149(3):e35-37.

47. Agasthi P, Graziano JN. Pulmonary Artery Banding. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 [citato 15 maggio 2022]. Disponibile su: http://www.ncbi.nlm.nih.gov/books/NBK560838/

48. Muller WH, Danimann JF. The treatment of certain congenital malformations of the heart by the creation of pulmonic stenosis to reduce pulmonary hypertension and excessive pulmonary blood flow; a preliminary report. Surg Gynecol Obstet. agosto 1952;95(2):213–9.

49. Stark J, Aberdeen E, Waterston DJ, Bonham-Carter RE, Tynan M. Pulmonary artery constriction (banding): a report of 146 cases. Surgery. maggio 1969;65(5):808–18.

50. Hunt CE, Formanek G, Levine MA, Castaneda A, Moller JH. Banding of the pulmonary artery. Results in 111 children. Circulation. marzo 1971;43(3):395–406.

51. Thomson NB, Niguidula FN, Hohn A. Correction of complete atrioventricular canal defect in patients with previous pulmonary artery banding. Am J Cardiol. novembre 1966;18(5):769–76.

52. Somerville J, Agnew T, Stark J, Waterston DJ, Aberdeen E, Carter RE, et al. Banding of the pulmonary artery for common atrioventricular canal. Br Heart J. novembre 1967;29(6):816–28.

53. Epstein ML, Moller JH, Amplatz K, Nicoloff DM. Pulmonary artery banding in infants with complete atrioventricular canal. J Thorac Cardiovasc Surg. luglio 1979;78(1):28–31.

54. Tingelstad JB, Lower RR, Howell TR, Eldredge WJ. Pulmonary artery banding in tricuspid atresia without transposed great arteries. Am J Dis Child 1960. maggio 1971;121(5):434–7.

55. McNicholas K, de Leval M, Stark J, Taylor JF, Macartney FJ. Surgical treatment of ventricular septal defect in infancy. Primary repair versus banding of pulmonary artery and later repair. Br Heart J. febbraio 1979;41(2):133–8.

56. Oldham HN, Kakos GS, Jarmakani MM, Sabiston DC. Pulmonary artery banding in infants with complex congenital heart defects. Ann Thorac Surg. aprile 1972;13(4):342–50.

57. Silverman N, Levitsky S, Fisher E, DuBrow I, Hastreiter A, Scagliotti D. Efficacy of pulmonary artery banding in infants with complete atrioventricular canal. Circulation. settembre 1983;68(3 Pt 2):II148-153.

58. Sharma R. Pulmonary artery banding: Rationale and possible indications in the current era. Ann Pediatr Cardiol. gennaio 2012;5(1):40–3.

59. Corno AF. Pulmonary artery banding. Swiss Med Wkly. 3 settembre 2005;135(35–36):515–9.

60. Trusler GA, Mustard WT. A method of banding the pulmonary artery for large isolated ventricular septal defect with and without transposition of the great arteries. Ann Thorac Surg. aprile 1972;13(4):351–5.

61. Horowitz MD, Culpepper WS, Williams LC, Sundgaard-Riise K, Ochsner JL. Pulmonary artery banding: analysis of a 25-year experience. Ann Thorac Surg. settembre 1989;48(3):444–50.

62. Takayama H, Sekiguchi A, Chikada M, Noma M, Ishizawa A, Takamoto S. Mortality of pulmonary artery banding in the current era: recent mortality of PA banding. Ann Thorac Surg. ottobre 2002;74(4):1219–23; discussion 1223-1224.

63. Schranz D, Veldman A, Bartram U, Michel-Behnke I, Bauer J, Akintürk H. Pulmonary artery banding for idiopathic dilative cardiomyopathy: A novel therapeutic strategy using an old surgical procedure. J Thorac Cardiovasc Surg. settembre 2007;134(3):796–7.

64. Schranz D, Akintuerk H, Bailey L, Miera O, Danne F, Kavarana MN, et al. Pulmonary Artery Banding for Functional Regeneration of End-Stage Dilated Cardiomyopathy in Young Children: World Network Report. Circulation. 27 marzo 2018;137(13):1410–2.

65. Recla S, Steinbrenner B, Schranz D. Medical therapy in dilated cardiomyopathy and pulmonary arterial banding in children. J Heart Lung Transplant. ottobre 2013;32(10):1045–6.

66. Di Candia A, Castaldi B, Bordin G, Cerutti A, Reffo E, Biffanti R, et al. Pulmonary Artery Banding for Ventricular Rehabilitation in Infants With Dilated Cardiomyopathy: Early Results in a Single-Center Experience. Front Pediatr. 16 luglio 2020;8:347.

67. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals [Internet]. 8th ed. Washington (DC): National Academies Press (US); 2011 [citato 30 maggio 2022]. (The National Academies Collection: Reports funded by National Institutes of Health). Disponibile su: http://www.ncbi.nlm.nih.gov/books/NBK54050/

68. Vasić M, Lončar-Turukalo T, Tasić T, Matić M, Glumac S, Bajić D, et al. Cardiovascular variability and β-ARs gene expression at two stages of doxorubicin - Induced cardiomyopathy. Toxicol Appl Pharmacol. 1 gennaio 2019;362:43–51.

69. Vida VL, Dedja A, Faggin E, Speggiorin S, Padalino MA, Boccuzzo G, et al. The effects of basic fibroblast growth factor in an animal model of acute mechanically induced right ventricular hypertrophy. Cardiol Young. agosto 2012;22(4):436–42.

70. O'Connell JL, Romano MMD, Campos Pulici EC, Carvalho EEV, de Souza FR, Tanaka DM, et al. Short-term and long-term models of doxorubicin-induced cardiomyopathy in rats: A comparison of functional and histopathological changes. Exp Toxicol Pathol Off J Ges Toxikol Pathol. 4 aprile 2017;69(4):213–9.

71. Georgiadis N, Tsarouhas K, Rezaee R, Nepka H, Kass G, Dorne J, et al. What is considered cardiotoxicity of anthracyclines in animal studies Corrigendum in /10.3892/or.2020.7717. Oncol Rep. 14 luglio 2020;44(3):798–818.

72. Halliday BP, Gulati A, Ali A, Newsome S, Lota A, Tayal U, et al. Sex- and age-based differences in the natural history and outcome of dilated cardiomyopathy. Eur J Heart Fail. 2018;20(10):1392–400.

73. Kim DH, Choi ES, Kwon BS, Park CS, Cha SG, Baek JS, et al. Development of Cardiac Events and Functional Recovery Prediction Models for Pediatric Dilated Cardiomyopathy. Front Pediatr. 2021;9:736872.

74. Wang PY, Tseng WC, Fu CM, Wu MH, Wang JK, Chen YS, et al. Long-Term Outcomes and Prognosticators of Pediatric Primary Dilated Cardiomyopathy in an Asian Cohort. Front Pediatr. 2021;9:771283.

75. Schmerler P, Jeuthe S, O h-Ici D, Wassilew K, Lauer D, Kaschina E, et al. Mortality and morbidity in different immunization protocols for experimental autoimmune myocarditis in rats. Acta Physiol. aprile 2014;210(4):889–98.

76. Weinberg EO, Schoen FJ, George D, Kagaya Y, Douglas PS, Litwin SE, et al. Angiotensin-converting enzyme inhibition prolongs survival and modifies the transition to heart failure in rats with pressure overload hypertrophy due to ascending aortic stenosis. Circulation. settembre 1994;90(3):1410–22.

77. Schunkert H, Dzau VJ, Tang SS, Hirsch AT, Apstein CS, Lorell BH. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. J Clin Invest. dicembre 1990;86(6):1913–20.

78. Litwin SE, Katz SE, Weinberg EO, Lorell BH, Aurigemma GP, Douglas PS. Serial Echocardiographic-Doppler Assessment of Left Ventricular Geometry and Function in Rats With Pressure-Overload Hypertrophy. Circulation. 15 maggio 1995;91(10):2642–54.

79. Miyamoto MI, del Monte F, Schmidt U, DiSalvo TS, Kang ZB, Matsui T, et al. Adenoviral gene transfer of SERCA2a improves left-ventricular function

in aortic-banded rats in transition to heart failure. Proc Natl Acad Sci U S A. 18 gennaio 2000;97(2):793–8.

80. del Monte F, Williams E, Lebeche D, Schmidt U, Rosenzweig A, Gwathmey JK, et al. Improvement in Survival and Cardiac Metabolism After Gene Transfer of Sarcoplasmic Reticulum Ca2+-ATPase in a Rat Model of Heart Failure. Circulation. 18 settembre 2001;104(12):1424–9.

81. Sengupta P. The Laboratory Rat: Relating Its Age with Human's. Int J Prev Med. 2013;4(6):7.

82. Yerebakan C, Boltze J, Elmontaser H, Yoruker U, Latus H, Khalil M, et al. Effects of pulmonary artery banding in doxorubicin-induced left ventricular cardiomyopathy. J Thorac Cardiovasc Surg. giugno 2019;157(6):2416-2428.e4.

83. Teraoka K, Hirano M, Yamaguchi K, Yamashina A. Progressive cardiac dysfunction in adriamycin-induced cardiomyopathy rats. Eur J Heart Fail. 2000;2(4):373–8.

84. Tokudome T, Mizushige K, Noma T, Manabe K, Murakami K, Tsuji T, et al. Prevention of doxorubicin (adriamycin)-induced cardiomyopathy by simultaneous administration of angiotensin-converting enzyme inhibitor assessed by acoustic densitometry. J Cardiovasc Pharmacol. settembre 2000;36(3):361–8.

85. Wakasugi S, Fischman AJ, Babich JW, Aretz HT, Callahan RJ, Nakaki M, et al. Metaiodobenzylguanidine: evaluation of its potential as a tracer for monitoring doxorubicin cardiomyopathy. J Nucl Med Off Publ Soc Nucl Med. agosto 1993;34(8):1283–6.

86. Schwarz ER, Pollick C, Dow J, Patterson M, Birnbaum Y, Kloner RA. A small animal model of non-ischemic cardiomyopathy and its evaluation by transthoracic echocardiography. Cardiovasc Res. luglio 1998;39(1):216–23.

87. Maral R, Bourat G, Ducrot R, Fournel J, Ganter P, Julou L, et al. [Toxicologic study and experimental antitumor activity of rubidomycin (13,057 R.P.)]. Pathol Biol. ottobre 1967;15(19):903–8.

88. Cusack BJ, Musser B, Gambliel H, Hadjokas NE, Olson RD. Effect of dexrazoxane on doxorubicin pharmacokinetics in young and old rats. Cancer Chemother Pharmacol. febbraio 2003;51(2):139–46.

89. Moulin M, Piquereau J, Mateo P, Fortin D, Rucker-Martin C, Gressette M, et al. Sexual dimorphism of doxorubicin-mediated cardiotoxicity: potential role of energy metabolism remodeling. Circ Heart Fail. gennaio 2015;8(1):98–108.

90. Chen J, Chemaly ER, Liang LF, LaRocca TJ, Yaniz-Galende E, Hajjar RJ. A new model of congestive heart failure in rats. Am J Physiol Heart Circ Physiol. settembre 2011;301(3):H994-1003.

91. Agnoletti G, Cargnoni A, Agnoletti L, Di Marcello M, Balzarini P, Pasini E, et al. Experimental ischemic cardiomyopathy: insights into remodeling, physiological adaptation, and humoral response. Ann Clin Lab Sci. 2006;36(3):333–40.

92. Watanabe K, Ohta Y, Inoue M, Ma M, Wahed MI, Nakazawa M, et al. Bisoprolol improves survival in rats with heart failure. J Cardiovasc Pharmacol. ottobre 2001;38 Suppl 1:S55-58.

93. Watanabe K, Ohta Y, Nakazawa M, Higuchi H, Hasegawa G, Naito M, et al. Low dose carvedilol inhibits progression of heart failure in rats with dilated cardiomyopathy. Br J Pharmacol. agosto 2000;130(7):1489–95.

94. Maczewski M, Mackiewicz U. Effect of metoprolol and ivabradine on left ventricular remodelling and Ca2+ handling in the post-infarction rat heart. Cardiovasc Res. 1 luglio 2008;79(1):42–51.

95. Rokutan H, Suckow C, von Haehling S, Strassburg S, Bockmeyer B, Doehner W, et al. Furosemide induces mortality in a rat model of chronic heart failure. Int J Cardiol. 20 settembre 2012;160(1):20–5.

96. Mougenot N, Bos R, Médiani O, Lechat P. Captopril-furosemide survival study in experimental heart failure. Fundam Clin Pharmacol. agosto 2005;19(4):457–64.

97. Watanabe K, Sreedhar R, Thandavarayan RA, Karuppagounder V, Giridharan VV, Antony S, et al. Comparative effects of torasemide and furosemide on gap junction proteins and cardiac fibrosis in a rat model of dilated cardiomyopathy. BioFactors Oxf Engl. marzo 2017;43(2):187–94.

98. Arumugam S, Sreedhar R, Karuppagounder V, Harima M, Nakamura M, Suzuki H, et al. Comparative evaluation of torasemide and spironolactone on adverse cardiac remodeling in a rat model of dilated cardiomyopathy. Cardiovasc Ther. ottobre 2017;35(5).

99. Padalino MA, Stellin G, Brawn WJ, Fasoli G, Daliento L, Milanesi O, et al. Arterial switch operation after left ventricular retraining in the adult. Ann Thorac Surg. novembre 2000;70(5):1753–7.

100. Morikawa Y, Heallen T, Leach J, Xiao Y, Martin JF. Dystrophin-glycoprotein complex sequesters Yap to inhibit cardiomyocyte proliferation. Nature. luglio 2017;547(7662):227–31.

101. Heallen T, Morikawa Y, Leach J, Tao G, Willerson JT, Johnson RL, et al. Hippo signaling impedes adult heart regeneration. Dev Camb Engl. dicembre 2013;140(23):4683–90.

102. Morikawa Y, Heallen T, Leach J, Xiao Y, Martin JF. Dystrophin-glycoprotein complex sequesters Yap to inhibit cardiomyocyte proliferation. Nature. 13 luglio 2017;547(7662):227–31.

103. Murphy CO, Pan-Chih null, Gott JP, Guyton RA. Microvascular reactivity after crystalloid, cold blood, and warm blood cardioplegic arrest. Ann Thorac Surg. ottobre 1995;60(4):1021–7.

104. Panciera T, Azzolin L, Cordenonsi M, Piccolo S. Mechanobiology of YAP and TAZ in physiology and disease. Nat Rev Mol Cell Biol. dicembre 2017;18(12):758–70.

105. Heallen T, Zhang M, Wang J, Bonilla-Claudio M, Klysik E, Johnson RL, et al. Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. Science. 22 aprile 2011;332(6028):458–61.