Improving care for children with congenital heart disease by cardiovascular biomarker profiling and advanced non-invasive cardiac imaging techniques.

Clausen, Henning

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By cardiovascular biomarker profiling and advanced non-invasive cardiac imaging techniques

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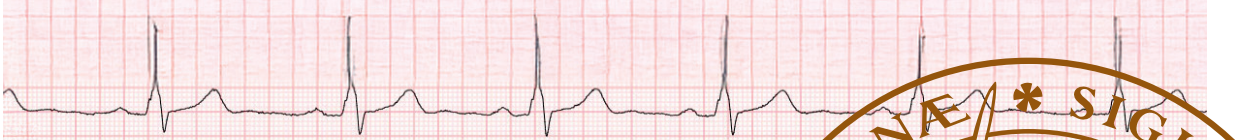

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Improving care for children with congenital heart disease

By cardiovascular biomarker profiling and advanced non-invasive cardiac imaging techniques.

Henning Clausen

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 5th of September 2025 at 13.00 hrs at Belfragesalen, Biomedical Centre (BMC), Sölvegatan 19 in Lund.

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Title and subtitle: Improving care for children with congenital heart disease: By cardiovascular biomarker profiling and advanced non-invasive cardiac imaging techniques.

Abstract:

Background: Congenital heart disease (CHD) is the most common organ anomaly in humans affecting approximately 1:125 newborns worldwide. Early diagnosis enables postnatal stabilisation and may improve outcomes, especially in critical CHD, in which the circulation is dependent on patency of the arterial duct. Detection of CHD in newborns remains incomplete and current screening programs do not aim to detect less critical but common CHD types, such as atrial septal defects (ASD). Blood-based biomarkers may not only improve early diagnosis in newborns with critical CHD, but also in children with other common types of CHD, such as ASD. Direct comparison of blood biomarkers and advanced non-invasive cardiac magnetic resonance (CMR) imaging before and after ASD treatment in children, should allow for improved assessment of the complex cardiovascular remodelling process.

Aims: Develop dried blood spot (DBS) assays for amino-terminal prohormone of brain natriuretic peptide (NT-proBNP) and interleukin-1 receptor-like 1 (IL-1RL1) to detect primarily high-risk CHD lesions, in which cardiac surgery is needed during infancy. Compare IL-1RL1 and CMR findings in children with ASD to assess a possible link between blood biomarker levels and cardiac imaging results. Explore intracardiac kinetic energy (KE) levels in paediatric ASD before and after treatment, using this CHD lesion as a model of a right-sided volume loading condition, to evaluate KE during the remodelling process.

Methods: Case-control study settings to assess DBS assay performance of NT-proBNP and IL-1RL1 in newborns with CHD to improve early diagnoses. Evaluate IL-1RL1 in paediatric ASD cases before and after treatment versus controls, with direct comparison of blood levels to CMR findings, including intraventricular KE levels.

Results: The developed DBS NT-proBNP test compared well to standard venous blood assay (correlation *r*=0.93; Bland-Alman bias±SD: -0.02±0.16; LoA: -0.32 to 0.30). The novel IL-1RL1 assay worked well when comparing DBS to venous blood analyses (correlation r=0.83; Bland-Altman bias±SD:1.00±0.17; LoA0.67 to 1.33). NT-proBNP by DBS analysis alone could identify CHD cases well (ROC: AUC=0.87) Combined NT-proBNP and IL-1RL1 analyses showed improved screening results (ROC: AUC=0.95). IL-1RL1 in newborns with ASD detected cases reasonably well (ROC: AUC=0.77). Levels of KE in children with ASD correlated with volumetric changes of the right and left ventricle following ASD closure. IL-1RL1 decreased in children after ASD treatment from a median [IQR] of 38.9 [22.2-57.6] to 34.1 [23.7-46.4] ng/ml; (p=0.04). Blood concentrations of IL-1RL1 correlated with decreases in right ventricular stoke volumes (r=0.43) and right ventricular systolic peak KE levels on CMR (r=0.50).

Conclusions: The novel DBS assays for NT-proBNP and IL-1RL1 improved early postnatal diagnoses of CHD in this retrospective study setting. In children with ASD, prospective evaluation by advanced non-invasive cardiac imaging, including CMR assessment, the volumetric and KE changes following ASD treatment could be linked to corresponding IL-1RL1 blood levels. Findings reflect previously unrecognized relationships between IL-1RL1 blood levels and cardiac imaging results.

Key words: Atrial septal defect, cardiac magnetic resonance, congenital heart disease, interleukin-1 receptor-like 1, kinetic energy, natriuretic peptide.

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For the love of my heart

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1 List of publications

The following publications form parts of this thesis:

Study I.

Clausen H, Norén E, Valtonen S, Koivu A, Sairanen M, Liuba P. Evaluation of Circulating Cardiovascular Biomarker Levels for Early Detection of Congenital Heart Disease in Newborns in Sweden. *JAMA Netw Open*. 2020 Dec 1;3(12):e2027561. doi: 10.1001/jamanetworkopen.2020.27561.

Study II.

Clausen H, Friberg E, Lannering K, Koivu A, Sairanen M, Mellander M, Liuba P. Newborn Screening for High-Risk Congenital Heart Disease by Dried Blood Spot Biomarker Analysis. *JAMA Netw Open*. 2024 Jun 3;7(6):e2418097. doi: 10.1001/jamanetworkopen.2024.18097.

Study III.

Clausen H, Friberg E, Sairanen M, Sjöberg P, Liuba P. Interleukin-1 receptor-like 1 is elevated in children with atrial septal defects after birth and decreases after treatment. *Manuscript submitted to Pediatric Cardiology 21st May 2025*.

Study IV.

Clausen H, Friberg E, Arvidsson P, Liuba P, Sjöberg P. Cardiac kinetic energy in children with atrial septal defects: a case-control study. *Manuscript*.

The following publications are related to the research addressed by this thesis:

1.

Sjöberg P, **Clausen H**, Arheden H, Liuba P, Hedström E. Atrial septal defect closure in children at young age is beneficial for left ventricular function. Eur Heart J Imaging Methods Pract. 2024 Jun 8;2(1):qyae058. doi: 10.1093/ehjimp/qyae058.

2.

Sjöberg P, **Clausen H**, Arheden H, Steding-Ehrenborg K, Liuba P, Hedström E. Left Ventricular Diastolic Function in Children with Atrial Septal Defects Improves After Closure by Means of Increased Hydraulic Force. Pediatr Cardiol. 2025 Jun;46(5):1194-1201. doi: 10.1007/s00246-024-03534-5. Epub 2024 Jun 11.

2 Abstract

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What is known about this topic?

It is important to diagnose congenital heart disease in newborns to prevent sudden cardiovascular collapse in the early postnatal period for those affected by critical lesions, in which the circulation is depending on patency of the arterial duct. There is incomplete detection of congenital heart disease in newborns using current screening methods, e.g. for those with coarctation of the aorta or those with common, non-critical types of lesions such as atrial septal defects.

Emerging evidence for the usefulness of blood-based biomarkers in newborns and older children with various types of heart disease suggests a link between circulating biomarkers and the cardiovascular disease status. Limited paediatric data is available on such blood biomarkers assessed in parallel with cardiac magnetic resonance imaging in congenital heart disease.

Kinetic energy within the heart can be measured by advanced non-invasive cardiac magnetic resonance imaging, providing new insights into cardiac physiology. There is a paucity of data on ventricular kinetic energy in children with congenital heart disease with no published studies on atrial septal defects and no data on the relationship between kinetic energy levels and blood-based circulating biomarkers in children before and after atrial septal defect closure.

What is new and noteworthy about this research?

Dried blood spot analyses of the circulating, cardiovascular biomarkers amino-terminal prohormone of brain natriuretic peptide (NT-proBNP) and interleukin-1 receptor-like 1 (IL-1RL1) is feasible in newborns using minimal blood amounts. Screening for congenital heart disease in newborns using dried blood spot analyses of NT-proBNP and IL-1RL1 profiles showed high test accuracy and could identify previously unrecognized cases in the retrospective case-control study setting described in this thesis.

Using the common congenital heart disease lesion of atrial septal defects (ASD) in children as a model of a right-sided volume loading condition, advanced non-invasive cardiac imaging by magnetic resonance was used to evaluate novel aspects of the complex cardiac remodelling process following ASD treatment by using ventricular kinetic energy (KE) measures in an explorative study setting. Results of KE could then be compared to IL-1RL1 blood-based biomarker levels, obtained at the same visit, to evaluate links between these in a prospective case-control study setting.

Kinetic energy levels of the right ventricle reduced in line with volumetric reductions of right ventricular volumes, and increased for the left ventricle with concomitant increases of left ventricular volumes after ASD treatment. Circulating blood-based IL-1RL1 biomarker levels decreased in concordance with reductions of peak systolic right ventricular kinetic energy in children after ASD closure.

The elucidated association between IL-1RL1 levels and peak systolic right ventricular kinetic energy levels suggests a possible physiological link between the blood-based biomarker levels and correlating right ventricular KE levels in this setting, which warrants further study to determine how combined assessment of IL-1RL1 and KE could assist in the clinical decision-making process prior to ASD closure and how these biomarkers could be used to additionally guide clinicians during follow-up of ASD patients by assessing the complex remodelling process of the heart and thereby helping to achieve optimal outcomes for patients.

3 Abbreviations

ASD	atrial septal defect
AUC	area under curve
bpm	beats per minute
BSA	body surface area
CHD	congenital heart disease
CI	confidence interval
CMR	cardiac magnetic resonance
CO	cardiac output
CT	computed tomography
DBS	dried blood spot
DELFIA	dissociation-enhanced Lanthanide fluorescent immunoassay
DoM	difference of means
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EF	ejection fraction
ESV	end-systolic volume
EVD	end-diastolic volume
GSP	genetic screening processor
HR	heart rate
IL-1RL1	interleukin-1 receptor-like 1
IQR	interquartile range
LoA	limits of agreement
LV	left ventricular
MoD	mean of differences

N/A	not applicable
N/S	not statistically significant
NT-proBNP	amino-terminal prohormone of brain natriuretic peptide
POX	pulse oximetry
Qp	pulmonary blood flow
Qs	systemic blood flow
ROC	receiver operating characteristics
RV	right ventricular
SD	standard deviation
SEM	standard error of mean
95%CI	95% confidence interval

4 Introduction

4.1 Congenital heart disease in children

4.1.1 Background and rationale for this research

The term congenital heart disease (CHD) covers a wide range of malformations affecting the heart and large blood vessels, with its origins before birth. As a group, CHD represents the most common organ anomaly seen in humans and affects approximately 1 per 125 newborns worldwide [1-3]. There is currently no primary prevention for this group of diseases and its exact causes during early human development remain often unknown. Maternal risk factors, such as advanced age, diabetes, smoking, thyroid hormone imbalances, inborn errors of metabolism in the form of phenylketonuria, as well as consanguinity have been described and studied in certain cohorts [4-8]. Progress has been made in delineating some genetic origins of certain types of CHD, but only a minority of defects, comprising approximately 20-30% of all cases, can currently be explained by genetics [9].

For medical practitioners, the large variety of CHD and the corresponding multitude of clinical signs and symptoms have been a challenge from the outstart and it took more than a few decades to move from post mortem analyses to clinical treatments, largely enabled by the technological advances of medicine since the early 1930's [10]. Since the pioneering work of Helen Brooke Taussig, who helped to established the field of Paediatric Cardiology, diagnostic progress was made using primarily electrocardiograms, x-ray investigations and blood tests until the 1950's [11]. With the application of ultrasound, to image the heart and its function in the 1950-60's, the medical knowledge about heart disease became more widespread as such new cardiac imaging modalities became more accessible [12, 13]. Subsequent advances in the applicability of echocardiography and its implementation into day-to-day clinical practice, also benefited children born with CHD since its first reported usage in infants and children in the 1970's, with continued refinement of echocardiography since then [14-16]. There are numerous ongoing research initiatives exploring the boundaries of what is achievable with modern echocardiography using advanced techniques, such as three-dimensional imaging and speckle tracking imaging [17].

In addition to this, cardiac magnetic resonance imaging (CMR) has enabled more detailed assessments of the heart and its function after establishing its clinical usefulness in both adults and children [18, 19]. In this context, foetal CMR is currently an evolving area to improve the antenatal risk stratification of infants with complex CHD [20]. With advances of such cardiac imaging techniques to assess the heart's structure and function, improvement to clinical outcomes have been demonstrated and benefitted children born with CHD [21, 22].

Aside from this, blood-based biomarkers of heart disease have been explored and their usefulness is established in clinical services for early diagnosis and prognostication of myocardial infarction and heart failure in adults, i.e. by using troponins, and brain natriuretic peptide with its related metabolites [23, 24]. In this context, the amino-terminal pro-hormone of brain natriuretic peptide (NT-proBNP) has been advocated as one of the more robust surrogate markers of adverse outcomes in trials concerning adult heart failure [25]. Evidence from smaller studies in children with CHD, congestive heart failure, pulmonary hypertension and heart transplantation has shown a similar picture [26-30].

In children no effective treatment to prevent the occurrence of CHD in most cases has led to research efforts that focus on the early diagnosis of CHD to prevent sudden cardiovascular deterioration and death in the young. This has been particularly important for newborns suffering from so called critical CHD, in which the circulation is completely dependent on patency of the arterial duct after birth. As part of the transition from foetal to postnatal life, the arterial duct normally closes within the first few days of life, with slight variations dependent on gestational age, mode of delivery, and sex of the baby [31]. In this scenario, ductal closure can be prevented by administration of prostaglandin E1 or E2 and this can stabilize the circulation temporarily until cardiac surgery or cardiac catheter-based intervention can be provided [32]. Because such critical CHD affects approx. one per 500 newborns worldwide, it is paramount to recognize such potentially life-threatening CHD as early as possible to prevent adverse outcomes in these cases [33]. This has led to improved antenatal detection of critical and severe forms of CHD using maternal ultrasound screening with currently more than half of critical cases being diagnosed before birth. This gives time for parental counselling and delivery planning to improve perinatal outcomes [34]. Further improvements to detect some types of critical CHD by pulse oximetry (POX) screening postnatally have also been made [35, 36]. In terms of research efforts, these critical CHD cases have been prioritized over the last decades because they require urgent medical intervention directly after birth, mostly in the form of open heart surgery [37].

Neonatal and infant heart surgery requires highly specialized clinical services to improve outcomes with demonstrated benefits after centralization of clinical care for infants and children with the most complex forms of CHD [38, 39]. With these advances in the medical field, more than 97% of those children born with CHD, who require some type of heart surgery, can be expected to survive nowadays [40]. With

improved survival other challenges have emerged, i.e. providing life-long follow-up care and ensuring results translate into meaningful person-centred outcomes [41, 42].

The global burden of CHD will likely increase in the foreseeable future as more young survivors from the current surgical era reach adulthood and this will demand new approaches to monitor CHD via dedicated adults services that offer specialised care for these individuals with various types of CHD over their whole lifespan [43-45]. This makes research in this area more important than ever to identify early diagnostic biomarkers of CHD, and to establish new blood-based and cardiac imaging markers to guide risk assessment and clinical decision-making over the long run.

4.2 Blood-based biomarkers in congenital heart disease

4.2.1 General considerations influencing choice of circulating, blood-based cardiovascular biomarkers in children with congenital heart disease

For this research project, blood-based biomarkers, with published evidence showing a link between quantifiable blood levels and corresponding cardiovascular pathophysiology, were considered. Ideally, there should be clear causality between blood concentrations of such biomarkers and observed changes to the circulation in children of various age, and with a wide spectrum of CHD lesions.

Because part of this research focused on blood-based biomarker analysis using dried blood spot (DBS) samples in newborns, like those used in worldwide newborn screening programs, such circulating biomarkers to detect CHD in the early postnatal period would need to be measurable with high accuracy in very small samples, utilizing approximately 3 microliters of blood per biomarker analysis. Using such DBS samples, one cannot differentiate the origin of measured biomarkers levels in terms of plasma or corpuscular cell origin. In other words, concentrations of circulating biomarkers would have to be largely unaffected by intracellular levels, chiefly those level in erythrocytes, as this would otherwise impair interpretation of findings. Such circulating biomarkers should also react quickly to changes in the postnatal circulation, within hours or a few days of birth, and should additionally indicate cardiovascular pathophysiology before irreversible cardiac damage occurs. They would also need to represent a spectrum of pathophysiological changes, that are expected to occur in a wide range of CHD lesions in newborns, e.g. pressure loading of the heart, or alterations to normal pulmonary blood flow.

Furthermore, the chosen biomarkers should predominately represent cardiovascular changes rather than other general pathophysiological processes, such as systemic inflammation. For repeat testing and confirmation of DBS analysis results, the chosen biomarkers should be easily accessible through standard clinical laboratory services using venous blood samples in the future. Finally, to enable future transition of research findings into daily clinical care, the evaluated blood-based biomarker assays should be available at a reasonable cost, which would otherwise make such tests, marketed under expensive patent licenses, unsustainable in resource-limited healthcare settings.

4.2.2 Specific considerations influencing the choice of circulating, blood-based cardiovascular biomarkers

Children born with CHD suffer from a variety of structural and functional lesions affecting the heart and circulation [2]. The associated pathophysiological consequences depend on the type of lesion and age of the child, especially during infancy, making it challenging to recognize CHD with its variable clinical signs and symptoms in the young [46].

Heart failure in children with CHD has been broadly defined as the clinical syndrome negatively impacting the heart's pumping function or causing imbalances in venous and arterial flows leading to volume or pressure loading of the heart and circulatory system [47, 48]. Given this broad definition with different pathophysiological causes and consequences, it is hardly surprising that there has been a wide range of studied blood-based biomarkers, which have been associated with CHD and heart failure in children [49, 50]. The role of such blood markers in children has been largely based on previous work in adults with structurally normal hearts and mostly left ventricular dysfunction [51]. Because natriuretic peptides and their metabolites have been causally linked to the underlying pathophysiology in adult heart failure, the clinical use of amino-terminal prohormone of brain natriuretic peptide (NT-proBNP) has been advocated in international adult guidelines. In adults with clinically suspected heart failure, NT-proBNP blood levels >300 pg/ml, have been advocated as screening cut-offs to suggest such a diagnosis and refer for further cardiac evaluation [52]. As another example, research into the role of cardiac troponins and high-sensitivity C-reactive protein levels have led to the successful implementations of circulating blood-based biomarker into daily clinical practice for the treatment of suspected myocardial ischaemia in adults [53].

In contrast to large adult studies, research on the role of circulating biomarkers in children with CHD has been based on smaller reports with their clinical applicability subject to ongoing research efforts in various types and stages of CHD [54, 55]. Because of the continuously widening research field discovering novel

cardiovascular biomarkers in this context, there is a need for further paediatric research to establish clinically meaningful cut-offs for individual cardiovascular biomarkers, depending on the type of CHD, sex and age of the child, and the specific clinical question at hand [56, 57].

Considering these aspects, the most suitable circulating biomarker for the proposed research became NT-proBNP, because it fulfils most of the above-named criteria. It is measurable in high enough plasma concentrations to be suitable for DBS analysis without interference from intracellular concentrations in erythrocytes. Its levels change relatively quickly in response to a range of pathophysiological changes within the circulation before irreversible damage to the heart has occurred and the test can be confirmed using standard clinical laboratory services in the studied setting at a reasonable cost. Similarly, the soluble form of the emerging cardiovascular biomarker interleukin-1 receptor-like 1 (IL-1RL1; formerly known by the alias ST2) was chosen for DBS analysis in newborns and children even though there is currently incomplete knowledge regarding the exact pathophysiological role of soluble IL-1RL1 in children with CHD. To address this situation, a common CHD lesion was chosen to assess IL-1RL1 levels in children and results were compared to cardiac magnetic resonance (CMR) findings. Circulating, blood-based biomarker levels in these children were assessed before and after atrial septal defect treatment and results directly compared to advanced, non-invasive CMR imaging findings.

4.2.3 The circulating biomarker amino-terminal prohormone of brain natriuretic peptide (NT-proBNP)

The role of natriuretic peptides, and its metabolite NT-proBNP, has been studied in children with CHD and reference values in infants outside the neonatal period and in older children have been proposed [26, 58-60]. Because there have been no previous studies on the diagnostic usefulness of assessing NT-proBNP in newborns with CHD by analysing concentrations in DBS samples, as those obtained during routine newborn screening programs, we set out to evaluate this in a group of children with known CHD compared to controls.

4.2.4 The circulating biomarker soluble interleukin-1 receptor-like 1 (IL-1RL1)

This research focused on the soluble form of IL-1RL1 measured in blood samples. The change in nomenclature from ST2 to IL-1RL1 has been implemented to reduce confusion between different types of proteins with different physiological functions. The studied IL-1RL1 belongs to the interleukin 1 receptor family encoded by its gene on chromosome 2 [61]. In its soluble form, IL-1RL1 acts as a decoy receptor

for interleukin 33 and it should not be confused with the suppression of tumorigenicity 2 protein encoded on chromosome 11 [62, 63]. It is currently not a routinely established biomarker of heart disease in standard laboratory services, but has been studied in predominately adult settings with promising results and could be established at reasonable assay costs as there is no patent related to this biomarker [64]. In particular, the soluble form of IL-1RL1 has been studied in adults with acute myocardial infarction and decompensated heart failure and elevated blood levels may predict adverse cardiovascular outcomes, independent of natriuretic peptides [65, 66]. In paediatric and adult cohorts with CHD, elevated IL-1RL1 levels have been associated with adverse outcomes following open heart surgery, albeit without specific explanations to the exact pathophysiological mechanism underpinning these findings [67, 68].

For adult cardiac patients there is currently published evidence suggesting IL-1RL1 is released by cardiomyocytes after mechanical stress as well as from the lung's vasculature, when pulmonary congestion occurs during decompensating heart failure [69, 70]. Given this pathophysiological background of IL-1RL1, and because children with ASD are exposed to increased pulmonary blood flow as well as increased mechanically myocardial stress due to the volume loading of the right ventricle, we chose to evaluate IL-1RL1 in this group of patients. Prior to this research, there were no studies on IL-1RL1 in paediatric ASD and no established blood assays to measure this biomarker in newborns using DBS samples.

4.2.5 Development of dried blood spot biomarker assays in newborns with congenital heart disease

The principle of using dried blood spot (DBS) samples for quantification of metabolites in newborns was first pioneered by the early newborn screening programs for phenylketonuria using so-called Guthrie cards [71]. This effective screening method dramatically changed the outlook for children affected by this rare inheritable disease, that could be ameliorated by early provision of dietary adjustments to prevent the toxic effects of excessive phenylalanine on the brain in infants [72]. The test became an established part of newborn screening programs worldwide and many tests have since been added to these programs, depending on regional and national public healthcare priorities and available resources. This may even extend into whole exome sequencing using DBS analysis in the future [73]. The overall number of children identified by DBS newborn screening programs depends on the prevalence of the respective diseases in the population and in the Swedish context the number of new cases identified by the multi-assay program over a 10-year-period from 2010-2019 was 311, with approx. 115,000 to 120,000 individuals screened annually. This translates roughly to three new cases per 10,000 newborns diagnosed with one of the types of disease included in the current Swedish newborn screening program [74]. In comparison to this, CHD affects 8 per 1,000

newborns overall and the subgroup of potentially life-threatening, critical CHD would account for approximately 2 per 1,000 newborns in this healthcare setting. One must bear in mind that a potential addition of screening for CHD to any newborn screening program would increase its annual case load and any such system would need to be readied for this challenge.

To utilize existing DBS screening programs in newborns, special laboratory assays would need to be developed for the quantification of substrates that need to be reliably measured by using minimal amounts of approximately three microliters of blood from standardized filter paper cards [75]. When accessing stored DBS for analysis of such blood substrates, consideration should also be given to degradation of metabolites over time, which has been evaluated using biobank samples from cooled storage facilities [76, 77].

4.3 Advanced non-invasive imaging in congenital heart disease using cardiac magnetic resonance

This research used cardiac magnetic resonance (CMR) as a mode of advanced, non-invasive imaging in children with CHD. In contrast to computed tomography (CT), as another advanced cross-sectional imaging modality commonly applied in clinical care, CMR does provide anatomical information as well as detailed information on cardiac function, while avoiding exposure to radiation and often reducing the need for intravenous contrast agents. This makes it a suitable examination technique to explore emerging imaging markers of cardiovascular physiology, that could assist functional assessment of the heart before and after treatment of congenital heart disease in children [78].

4.3.1 Cardiac magnetic resonance imaging

With the advance of modern magnetic resonance imaging scanners over the last few decades, CMR has become an invaluable source of diagnostic and prognostic information for adults and children suffering from various types of heart disease and this has resulted in international guidelines on the acquisition and reporting of CMR findings in children with CHD [79-81]. In paediatric CHD, assessment of ventricular volumes and cardiac function is highly accurate and reproducible with reliable quantification of blood flows, e.g. to evaluate the systemic and pulmonary circulation [82, 83]. Application of CMR in children with atrial septal defect (ASD) allows for precise assessment of atrial shunt flows using CMR [84].

When planning paediatric CMR studies, one should consider the fact that CMR requires longer examinations compared to CT. Additionally, with 1.5 Tesla

magnetic field strengths scanners most widely used in clinical practice, this may pose limitations in terms of temporal and spatial image resolution when examining young children, who have smaller hearts and faster heart rates [85]. To improve compliance during relatively long CMR scanning protocols in children, sedation using intranasal dexmedetomidine has been safely and successfully applied, which was offered to children during this research as per established local clinical practice guidelines [86].

4.3.2 Kinetic energy of the heart

Kinetic energy (KE) of the heart can be assessed by advanced CMR imaging using three-dimensional data acquired over the entire cardiac cycle, so-called "4D-flow". This offers new insights into the intracardiac energy levels in health and pathological states with published guidelines on 4D-flow acquisition and its application in children with CHD [87-89]. Based on the principles of physics, KE is the product of ½ mass multiplied by velocity^2 (J). In the cardiac context, this translates into a calculation of ½ blood volume × predefined blood density × blood velocity^2 which can be measured using 4D-flow sequences [90].

At rest, KE makes up approx. 1% of the total energy expenditure of the heart, but increases approximately 10-fold in modelled physical activity, which may make it an important factor contributing to increased functional demands of the heart during physical exercise [91]. Its levels increase, particularly for the right ventricle, in healthy adults during exercise, and the measurable KE levels at rest may serve as a surrogate marker of the underlying physiological processes affecting cardiac function [91]. Cardiac KE levels may even give novel insight into the currently not recognizable stages of evolving cardiac failure in adults, although KE should not be seen as a substitute for standard functional assessment markers, such as ejection fraction [92-94]. Measurements of atrial and ventricular KE levels have been described in adults with various degrees of left ventricular dysfunction [91, 95, 96]. Sex- and age-related KE changes have also been reported in adults and normal references have been proposed [97-99].

There is currently limited published CMR data on the role of KE in children or adults with CHD [100-104]. Data from smaller paediatric studies in patients with tetralogy of Fallot and single ventricle physiology have shown altered KE patterns suggesting disturbances in intracardiac energetics that may be seen as potential precursors of evolving ventricular dysfunction, although there is currently a paucity of published evidence linking KE levels to various degrees of heart failure [100, 101, 105-111]. No studies have assessed KE levels in children with ASD and this research set out to describe the left and right ventricular changes to KE in paediatric ASD before treatment, as well as six to twelve months after treatment using 4D-flow assessments. Describing possible changes to intracardiac KE levels in this common type of CHD over time could link KE closer to standard measures of

cardiac function in children with CHD, as well as blood-based biomarkers of cardiovascular health.

Measurements of KE are dependent on temporal and spatial location within the cardiac chambers and this explorative study aimed to characterize KE levels in the systolic ejection phase and diastolic filling phase by studying peak KE levels in these respective cardiac phases. Absolute levels of KE depend on the volume of blood pumped during the cardiac cycle and the intracardiac velocities within these volumes. Assessing these combined changes to intraventricular KE should lead to new insights into cardiac physiology in children with ASD by assessing blood flow characteristics that may precede overt worsening or improvement of heart function, as indicated by previous data in predominately adults patients with altered ventricular volumes and function, in whom changes to KE may even indicate an increased risk for thrombus formation within the left ventricle following myocardial infarction [92, 100, 110, 112, 113]. The clinical applicability of KE in children with CHD as a novel marker of subtle changes to heart function remains to be shown and this study will contribute KE data to closer link the physiological changes seen in children with ASD after defect closure to standard CMR measures of cardiac function as well as blood-based biomarker levels of IL-1RL1.

4.4 Cardiac imaging and blood-based biomarkers in congenital heart disease

Visualization of cardiac structures and function has been at the forefront of medical research to diagnose disease, assess treatments and outcomes. Making the "invisible" visible has been the key to understanding human cardiovascular macro- and microanatomy and has helped to elucidate the underlying pathophysiological principles [114-116]. The addition of blood-based biomarkers during the assessment of cardiovascular disease has cast a new light on the body's adaptations in health and heart disease and has led to new discoveries. This has helped to rapidly and efficiently diagnose clinically important cardiovascular health problems, e.g. potentially life-threatening myocardial ischaemia by measuring cardiac troponins, as reflected by international cardiology guidelines [53, 117-119].

4.4.1 Combined cardiac imaging and blood-based biomarkers in children with congenital heart disease

Advances in medical imaging and blood-based biomarker discoveries have enabled novel assessments for children with CHD [55]. The transition from foetal to postnatal life makes babies, born with structural anomalies of the heart and blood vessels, potentially vulnerable to rapid changes within the circulatory system [120].

As an example of combining blood tests with cardiac imaging findings, such combined assessments in a paediatric cohort study have been shown to enable risk stratification of CHD patients and to predict outcomes flowing heart surgery [50, 121]. Because of the age-related differences to the pathophysiology in various types of CHD, and the clinical complexity posed by this, it has been challenging to establish unifying biomarker profiles that would be applicable to all children, who suffer from a wide array of heart diseases [49].

With the multitude of CHD lesions, the spectrum of potentially useful blood-based biomarkers has been constantly expanding over time, but there currently is limited published data on normal reference ranges for children of various age for such biomarkers as well as incomplete information on the exact pathophysiological roles of some of these emerging markers of heart disease in the young [122-127]. In the absence of clear-cut causative relationships between blood levels and cardiovascular changes, as depicted by advanced cardiac imaging modalities, it is important to better understand the physiological principles guiding the most promising of these blood-based biomarkers in children.

To evaluate this further a model of a common and clinically important type of CHD, such as ASD in children, would allow for lesion-specific evaluation of pathophysiological changes with validation of blood test results against cardiac imaging finding, which assess the heart's anatomy and function over time. So far, such progress has been made for some rarer, and more complex types of CHD affecting children, such as single ventricle lesions or tetralogy of Fallot [128-131]. No previous study has explored the combined assessment of CMR and the blood-based circulating biomarker IL-1RL1 in children with ASD before and after treatment and this research set out to describe this relationship further.

4.4.2 Cardiac magnetic resonance and interleukin-1 receptor-like 1 in children with atrial septal defects

The rationale for this part of the presented research project was based on the fact, that ASD represents an important CHD lesion in children, but ASD can be clinically challenging to recognize in the young. As an isolated defect, and when diagnosed and treated in good time, it leads to a favourable long-term prognosis in children and adults [132-138]. For this research, ASD would also function as a CHD model of right heart volume loading with increased pulmonary blood flow.

Right ventricular volume load is also seen in more complex CHD, such as postoperative tetralogy of Fallot with significant pulmonary regurgitation [139-141]. In contrast to such complex CHD lesions, ASD patients would be expected to undergo remodelling of the heart towards near normal right ventricular sizes following successful ASD closure within the first year after treatment, as demonstrated by CMR and echocardiography in previous studies [142-144].

Furthermore, in ASD cases there would be no other factors affecting ventricular volumes and function, such as pulmonary regurgitation. Combining assessments of advanced cardiac imaging, using CMR techniques, with blood-based biomarkers that react to changes of ASD pathophysiology with increased blood volumes being pumped through the pulmonary circulation, would improve our understanding of this CHD lesion in children and would potentially make a more tailored, efficient diagnostic and prognostic evaluation possible in the future.

As one of the emerging blood biomarkers of cardiovascular disease, the soluble form of IL-1RL1 has been described to be elevated in various types of pulmonary pathology [68, 145-148]. As mentioned above, children with ASD have altered pulmonary blood flows and studying these before and after successful ASD treatment would allow for evaluation of IL-1RL1 blood levels in parallel with CMR. This in turn should provide new insights into the cardiovascular changes occurring during the expected remodelling process in ASD patients. Because no studies have previously investigated a possible link between CMR imaging findings and IL-1RL1 blood levels, this research aimed to characterize this relationship in a cohort of children with ASD in comparison to controls.

5 Aims

5.1 Study I

This study's aim was to develop of novel, fully automated DBS assay for quantification of the circulating cardiovascular biomarker NT-proBNP and study its usefulness to detect CHD in newborns.

5.2 Study II

The aim of this study was to develop a novel, fully automated DBS assay for quantification of the emerging circulating cardiovascular biomarker IL-1RL1 and combine its analysis with the established NT-proBNP assay from Study I to assess the combined biomarkers' usefulness in screening for CHD in newborns.

5.3 Study III

Using ASD as a common CHD lesion, representing right heart volume conditions, this study aimed to describe levels of the circulating cardiovascular blood-based biomarker IL-1RL1 in relation to advanced, non-invasive imaging findings from CMR in asymptomatic newborns with ASD and in older children before and after ASD closure to elucidate possible associations between blood-based biomarker levels and cardiac imaging findings.

5.4 Study IV

This study aimed to describe the changes to intraventricular cardiac KE using advanced, non-invasive CMR imaging in children with ASD before and after treatment, as well as explore the relationship between KE levels and the circulating cardiovascular blood biomarker IL-1RL1 in these children.

6 Material and methods

6.1 Study designs

This research comprised several observational study designs with paediatric cases and controls. Enrolment of newborn controls for the study of circulating biomarker analysed from DBS samples was prospective, while CHD cases were recruited retrospectively. Cases were identified from existing electronic medical records using the diagnostic codes Q20 through to Q28 as per the *International Statistical Classification of Disease and Related Health Problems; Tenth Revision (ICD-10).* All study participants' parental guardians provided written, informed consent and the children's assent was sought, whenever possible. We estimated study power using standard methods and calculations were based on published data where available to ensure research efforts would lead to achievable and meaningful outcomes [149, 150]. To minimize bias and confounding factors, we applied the Equator Network's reporting guidelines on Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) and the Standards for Reporting of Diagnostic Accuracy Studies (STARD) [151, 152]. After ethical approval and registration of the proposed research via the Medical Faculty at Lund University in Lund (Sweden), active study participants' enrolments and assessments were carried out between August 2019 and December 2024.

Although comprehensive efforts were made to plan for possible study delays from the outset, none of the participating researchers could envision the implication the worldwide viral SARS-CoV-2 pandemic would have on the conduct of this clinical research [153]. Due to public health recommendations, minimizing travel for large parts of the population and imposing severe restrictions on non-essential clinical visits to healthcare facilities during the pandemic in 2020-2021, delays in study recruitment arose as only limited numbers of patients became eligible for initial study participation and no controls could be examined during the height of the pandemic.

6.2 Study I: Dried blood spot analysis of single circulating biomarker to improve diagnostic care in newborns with congenital heart disease.

During this first study phase, eligible newborns were those born within the Swedish healthcare region of Jönköping. All participants were prospectively recruited by approaching families postnatally on the local baby unit or during follow-up visits to maternity services for routine check-ups within the first week of life. Babies were recruited chronologically in order of their birth dates and the research team had no influence on scheduled appointments. Recruitment was restricted to daytime hours on normal working days due to limitations of research resources. Additional DBS and venous EDTA blood samples were obtained at the time of routine DBS sampling as part of the Swedish Newborn Screening Program, normally performed between day two to five of life. Blood sampling for research purposes was timed to this clinical routine to minimize the need for extra blood tests in newborns. All eligible controls had to be born at term, had to be clinically well without the need for medications, and had no requirements of medical intervention at the time of study participation, e.g. no hyperbilirubinaemia treatment. We collected predefined perinatal data on gestational age, birth weight, as well as age at the time of blood sampling. All controls were followed up to the age of one year using medical records to ensure no signs of CHD evolved during infancy outside the neonatal period. Study cases of CHD were identified through local paediatric cardiology services using non-random methods. Children and families were approached in the chronological order of their scheduled clinical assessment and asked to participate. Children less than 18 years at the time of study enrolment, who had been diagnosed with CHD, were eligible. The research team had no influence on scheduled appointments, which had been based on the child's clinical needs for follow-up. All cases underwent echocardiography as per standard paediatric guidelines by one experienced paediatric echocardiographer [154-156]. Diagnoses were checked against existing electronic patient records, including operating notes from previous cardiac surgery, where appropriate. Whenever available and documented in neonatal files, we obtained perinatal data in these children with CHD from electronic healthcare records using the same criteria as in controls to match cases and controls.

After study enrolment, DBS samples for CHD cases were retrieved from the national newborn screening program's biobank in Sweden and stored for subsequent analyses. Storage of DBS samples at this national biobank had been maintained at +4 degrees Celsius in a dedicated air-conditioned cooling area with controlled air humidity, which has been specifically designed for long-term DBS sample storage. All acquired DBS samples for this research were subsequently stored using cooled facilities until batch analyses, comparable to the national biobank's conditions.

EDTA blood samples from newborn controls were stored at -80 degrees Celsius for later batch analyses. Laboratory staff were blinded to clinical data at the time of analyses. A novel, fully automated DBS assay for the analysis of NT-proBNP was developed as illustrated in *figure 1* and as described in the published study results [157]. The DBS test performance was then assessed in controls versus CHD cases to evaluate its usefulness in terms of distinguishing these two groups using the standard statistical methods as described below.

6.3 Study II: Dried blood spot analyses of combined circulating biomarkers to improve diagnostic care in newborns with congenital heart disease.

Eligible for DBS analyses in this part of the research project were those children within the scope of the Swedish healthcare system who had undergone cardiac surgery in Sweden, i.e. at Gothenburg's or Lund's university hospitals during infancy. We evaluated whether the combined tests of NT-proBNP and IL-1RL1 in newborns could be used to screen for so-called "high-risk CHD" compared to controls. "High-risk CHD" was defined as those types of CHD, which required open heart surgery during infancy based on current clinical treatment guidelines, including duct-dependent, critical types of CHD.

Children, aged less than 18 years, were asked to participate. We identified possible study subjects through existing electronic healthcare records and approached these based on their birth dates, starting with those ones born most recently first. Potential study subjects had to be excluded if there were no stored DBS samples at the newborn screening laboratory's national biobank in Sweden, or if insufficient residual DBS amounts were available for the proposed biomarker analyses. For all DBS samples retrieved from biobank storage, the same predefined criteria and sample handling condition were applied as those described in the above methods section for Study I.

In addition to the evaluated NT-proBNP assay developed during Study I, we developed a further DBS assay for the blood-based biomarker interleukin-1 receptor-like 1 (IL-1RL1), with assay details described in the published results of Study II [158]. Laboratory staff were blinded to all clinical data at time of analyses and results were analysed using clinical criteria collected from existing electronic healthcare records in CHD cases and controls as outlined in study I. Standard statistical methods were applied for data analyses after completion of biomarker assessments.

6.4 Study III: Cardiac magnetic resonance imaging combined with blood-based biomarker analysis in children with congenital heart disease.

To link blood-based biomarkers to advanced non-invasive cardiac imaging, this study was set up and focused on children with ASD before and after defect closure, while comparing these to paediatric controls. By using ASD as a condition of right heart volume loading with increased pulmonary blood flow, this common type of CHD was chosen to assess the cardiac remodelling process before and after ASD treatment in children in this case-control study setting.

Newborn controls were enrolled prospectively at time of routine DBS sampling at day two to five of life with paired EDTA blood samples taken as described in Study I. These blood samples were stored for subsequent batch analyses.

Cases with ASD were identified retrospectively after their diagnoses had been confirmed during cardiac assessment outside the neonatal period, i.e. during later echocardiographic assessment in childhood. All newborn ASD cases had been asymptomatic after birth.

Paediatric cases with ASD, including those with partial anomalous pulmonary venous drainage, were eligible for study participation. Study participants had to be less than 18 years old at enrolment and had been referred for ASD treatment to the Children's Heart Centre in Lund (Sweden). We recruited additional paediatric controls for matched comparison to ASD cases before and after ASD defect closure. These were recruited after local study advertisement.

We verified all cardiac diagnoses against electronic healthcare records based on ICD-10 codes as outlined above in Study I. Enrolment followed chronological order of planned admissions for ASD treatment using non-random methodology. All ASD cases were planned to undergo elective ASD treatments at the studied Children's Heart Centre in Lund, following local joint cardiothoracic conference reviews. Recommendations for ASD closure followed published clinical guidelines [159]. Such guidelines suggest that paediatric ASD treatment is reasonable after clinical judgement within dedicated paediatric cardiology services in cases with signs of right-sided volume loading of the heart, chiefly based on standardized paediatric echocardiographic imaging, and when there is clinical assumption of an important atrial shunt, as defined by a pulmonary to aorta flow ratio (Qp:Qs) >1.5 [160].

In these children, we collected predefined clinical data, including body size measurements and blood pressures as well as ECG recordings, and performed standardized paediatric echocardiography. Venous EDTA blood samples were taken at the same visit as CMR imaging in all recruited controls and ASD cases before treatment. Repeat assessments, using the same investigations as above, were

performed in ASD cases six to twelve months after successful defect closure. In conjunction with CMR, sedation in form as intranasal dexmedetomidine at a dose of two to three µg/kg (Dexdor©, Orion Pharma, Espoo, Finland) was offered to children on an individual basis, i.e. in cases where this was deemed necessary to help the child comply with CMR examination. Children with medical contraindications to CMR, or those non-compliant without general anaesthesia were excluded.

As described in the methods section of Study I-II, stored DBS blood samples were retrieved from biobanks. Batched analyses were performed for DBS and EDTA blood samples, with laboratory staff being blinded to clinical data. Assay methods for NT-proBNP and IL-1RL1 followed those described in Study I-II, which have previously been published [158]. Paired DBS and EDTA blood samples to measure IL-1RL1 in newborn controls were directly compared, when available. The recruited ASD cases only had stored DBS samples from the newborn period and no matched EDTA blood samples for comparison.

Magnetic resonance imaging was performed using a 1.5 Tesla scanner (Aera, Siemens Healthineers, Erlangen, Germany). Standard, balanced steady-state free precession short-axis cine images covering the whole heart were acquired with retrospective ECG gating. Two-dimensional free-breathing through-plane phase-contrast flow measurements were performed in the ascending aorta and main pulmonary artery in all participants to quantify effective stroke volumes and estimate atrial shunt ratios. After image acquisition an experienced CMR examiner reviewed CMR sequences to ensure adequate examination quality was available for postprocessing of data. All CMR volume measurements were indexed to body mass index (BSA) using Mosteller's method. The CMR data was analysed using Segment software, version 4.0 R11026 (Medviso AB, Lund, Sweden). Statistical analyses of blood-based biomarkers and CMR imaging findings were carried out as described in the statistic section for Study III.

6.5 Study IV: Cardiac kinetic energy assessed by magnetic resonance combined with blood-based biomarker analysis in children with congenital heart disease.

To explore the relationship of blood-based biomarker levels and advance non-invasive cardiac imaging results, using measurements of intraventricular kinetic energy levels, this case-control study set out to evaluate these measures in children with ASD before and after treatment.

All children in this study were prospectively enrolled, i.e. controls by local study advertisement and cases by non-random selection methods when presenting for clinical treatment of ASD. Paediatric cases with the predefined CHD types were approached for study participation in chronological order of planned hospital admissions. Children were scheduled for treatment at the dedicated tertiary centre for the treatment of paediatric CHD in Lund (Sweden) as described for Study III. The research team had no influence on the order of planned admissions prior to approaching families for study participation. The types CHD to be included in Study IV were defined as those leading to right-sided volume loading of the heart due to atrial shunts. This included isolated ASD as well as those atrial defects associated with partial anomalous pulmonary venous return. Children had to be less than 18 years of age at time of study enrolment. Criteria for ASD treatment were based on those outlined in Study III and followed established clinical guidelines. Data were collected using predefined clinical characteristics and all children enrolled in this part of the research, underwent standard paediatric echocardiographic assessments. During assessment visits, clinical examinations were performed, including assessment of weight and height, and non-invasive blood pressure measurements. We recorded ECGs and study participants underwent cardiac magnetic resonance (CMR) as well as blood sampling on the same study visit. Prior to CMR, intranasal sedation to improve compliance with CMR, was offered to participating children and given on a case-by-case basis after discussions with families. For this we used dexmedetomidine at a dose of two to three µg/kg (Dexdor©, Orion Pharma, Espoo, Finland). Sedation routines followed local clinical practice guidelines, based on previously published data [161]. Children with contraindications to CMR, or those non-compliant without general anaesthesia were excluded.

6.5.1 Cardiac magnetic resonance imaging assessing kinetic energy

All CMR exams were performed using a 1.5 Tesla scanner (Aera, Siemens Healthineers, Erlangen, Germany). Standard, balanced steady-state free precession short-axis cine images covering the whole heart were acquired with retrospective ECG gating. Two-dimensional free-breathing through-plane phase-contrast flow measurements were performed in the ascending aorta and main pulmonary artery in all participants to quantify effective stroke volumes and estimate atrial shunt ratios. Additionally, we acquired three-dimensional-flow sequences over the time of the entire cardiac cycle (4D-flow) covering the whole heart with retrospective ECG-triggering and 2x2 acceleration with respiratory navigator gating. Prior to post-processing of CMR data, one experienced CMR examiner scrutinized image quality. Subsequent CMR data were processed using Segment software, version 4.0 R11026 (Medviso AB, Lund, Sweden) [162]. We adjusted CMR measurements to account for variable body sizes in children as per reporting guidelines on CHD in children [79-81]. Mosteller's method was used to calculate body surface area (BSA) [163]. CMR data were analysed by manually outlining right and left ventricular

endocardial borders in all time phases on cine short-axis stacks to assess right and left ventricular end-systolic and end-diastolic volumes. The CMR data from outlined, endocardial right and left ventricular borders, were transferred to 4D-flow datasets and intraventricular KE was calculated for all ventricular voxels and summed for each time phase as previously described [164, 165]. The KE was calculated within each intraventricular voxel obtained on CMR as follows:

$$KE = \frac{1}{2} volume\ of\ blood\ (ml) * blood\ density\ \left(1.06 \frac{g}{ml}\right) * blood\ velocity^2\ (\frac{m}{s})^2$$

Peak KE in systole and diastole were defined as the maximal measured KE values in the respective cardiac phases. The KE results were displayed graphically and analysed as previously described [100]. Resulting KE findings were adjusted for normal, growth-related changes and ventricular volumes and cardiac function in this paediatric population were indexed to account for variable body sizes by using indexed end-diastolic ventricular volumes, indexed ventricular stroke volumes, as well as cardiac index (CI). Initial CMR assessments in ASD cases were before defect closure and the same children were examined six to twelve months after successful ASD closure. Based on previously published data, follow-up CMR scans and repeat blood sampling was expected to coincide with a largely re-modelled status of the heart following ASD treatment [140, 166, 167]. After data collection, standard statistical tests were used to compare CMR measurements between groups of cases before and after ASD treatment versus controls.

6.5.2 Blood concentrations of interleukin-1 receptor-like 1 compared to kinetic energy derived from cardiac magnetic resonance imaging

Venous blood samples were drawn at the same clinic visits as CMR scans in children before and after ASD closure. This enabled direct comparison of IL-1RL1 blood concentrations versus KE levels to check for linear correlations between blood and cardiac imaging findings. Following study III, that measured correlations of IL-1RL1 findings and right ventricular stroke volumes before ASD closure, we evaluated how KE levels, particularly those measured within the right ventricle, correlated with IL-1RL1 blood biomarker levels in this paediatric ASD cohort.

6.6 Ethical aspects

Ethical considerations around this research were reviewed and approved by the national Swedish Ethical Review Authority prior to enrolment of study participants

(DNR-Nr.: 2019 05490). The project was registered on the public *Clinical Trials* website before commencement (NCT 04667455). We followed the Declaration of Helsinki in its current format at the time of the study [168, 169].

While conducting research in children, considerations were given to various age groups with a tailored approach used during the consent process, that explained the purpose and content of the study to enable age-appropriate adaptation of the consent process. A child-friendly approach during the conduct of the study was envisioned by conducting all study parts within paediatric healthcare settings. Study III-IV's CMR imaging and blood-sampling were performed at the Children's Heart Centre in Lund with this in mind. This study setting allowed the research group to carry out Study III and IV within this specialized healthcare facility, accustomed to the needs of children with CHD.

Specific ethical considerations were given to families from non-native language backgrounds, who could not communicate in Swedish or English and interpretation services were offered whenever possible to enable study participation. As some of the participating families had to travel relatively long distances for several hours to attend research follow-up appointments, we ensured that examination times accommodated for such travel requirements and aimed to reimburse families for their direct travel expenses.

Ethical controversies around the usage of residual DBS samples, that were primarily acquired for the purpose of newborn screening programs, have been described in other healthcare settings and families are generally informed about the possibility of future medical research being conducted on these samples, although no specifics about these future studies can be given at the time of DBS sampling in the newborn period [170, 171]. For this research, the legally required biobank agreements we established, before DBS samples were retrieved from the newborn screening laboratory's biobank in Sweden to address such concerns and all participating families provided written, informed consent for this specific research project before DBS samples were requested. Furthermore, this research aimed to minimize the amount of additional venous blood sampling in children and offered local anaesthetic to the skin prior to this, as blood sampling is often perceived as a negative experience in children during hospital stays.

Although the ethical committee approved the planned study design and written, informed consent was obtained from all study participants and their guardians to retrieve DBS samples for analysis of circulating biomarkers using approximately one whole DBS, this could not be practically achieved due to restrictions imposed by the Swedish national screening laboratory's steering committee. In line with the committee's request, the research group agreed to exclude stored DBS sample analyses from this project for those cases, who had previously tested positive for one of the specific diseases, that are routinely tested for by the national newborn screening program. Likewise, the amount of DBS available for this research was

restricted to a maximum of ½ DBS by the Swedish national screening laboratory to allow for adequate residual DBS amounts per individual to remain in biobank storage, which should facilitate other future biomedical research endeavours. This limited DBS analyses to the two blood-based biomarkers NT-proBNP and IL1RL1 in our study setting.

We kept CMR examinations as short as possible, because children might otherwise not have complied with this imaging modality, which required them to lie still on their backs for approx. 30-40 minutes. As per local clinical practice guidelines, we offered intranasal sedation prior to CMR. This was based on an individual assessment of all study participants and after discussion with families and their children. To accommodate for the child's individual needs and wishes, we offered children participation in all parts of the study from the outset, which included their clinical examinations with non-invasive blood pressure measurements, standard ECG recordings, transthoracic echocardiographic examination, CMR scans and blood sampling. This individualized approach was balanced against the study's need to have as complete data as possible for meaningful data analyses at its conclusion.

There was an additional ethical discussion around the best possible allocation of limited healthcare resources in the light of the recent viral SARS-CoV2 pandemic, which stretched the limits of current healthcare system on an unprecedented, global scale [172, 173]. It appears paramount to have meaningful, and cost-effective medical care in place for children with chronic disease, such as CHD. This research aimed to provide more efficient, diagnostic tests, as well as novel follow-up information for children affected by their life-long heart condition to address this [174, 175].

6.7 Statistical analyses

Case-to-control recruitment followed a 3:1 ratio for Study I and subsequently 2:1 ratio for study II-IV. Because there were no published studies in children with CHD assessing the blood-based biomarkers NT-proBNP or IL-1RL1, we estimated power calculations (80% power, $\alpha = 0.05$) on clinical judgement, aiming to detect differences in biomarker levels between patients and controls in at least 30% of cases. Likewise, in the absence of published data on intracardiac KE levels in children with ASD, power calculations were estimated to detect KE differences between children with ASD before treatment and controls in a minimum of 30% cases [176, 177].

We applied standard statistical analytic methods using predominately SPSS version 28 (IBM Corp., Armonk, New York, USA) and GraphPad Prism version 10.2.1 (Boston, Massachusetts, USA). Additionally, TIBCO Spotfire, version 7.11.1 (TIBCO Software Inc) and R, version 3.5.1, and version 4.2.2 (R Foundation for

Statistical Computing) were used during Study I-II. We used Shapiro-Wilk tests for normality and visually checked data distribution to select appropriate parametric and non-parametric tests for group comparisons. Data was logarithmically transformed to achieve a more normal distribution prior to analysis where necessary. Diagnostic test comparisons were based on correlations and Bland-Altman agreement analyses. Receiver operating characteristic (ROC) curve analysis was used to assess diagnostic test accuracy. Descriptive data summaries were provided and results expressed in whole numbers (percentage). For parametrically distributed data, we reported mean ± standard deviation (SD) with mean of differences (MoD) ± SD when using paired parametric tests. For non-parametric data, results were stated with median and interquartile range [IQR] and difference of means (DoM) ± SD given for comparisons using unpaired tests. A 2-sided p-value <0.05 was considered as statistically significant.

6.7.1 Study I

There were no prior studies on DBS assessment of NT-proBNP in newborns with CHD. We, therefore, estimated the numbers need to be enrolled at a 1:3 case-to-control ratio and the calculated number of controls at 85 with 28 cases to achieve 80% power with $\alpha=0.05$. A total of 113 newborns needed to be recruited for adequate study power. The NT-proBNP data were logarithmically transformed to achieve more symmetrical distribution. Pearson's linear correlation and Bland-Altman analyses were used to compare test performance between DBS versus EDTA blood assays for NT-proBNP. We performed ROC curve analysis to assess the novel DBS NT-proBNP test in terms of detecting any type of CHD cases in this pilot phase. Additional ROC analyses were used to evaluate the combined value of DBS NT-proBNP results together with available POX screening results in this cohort.

6.7.2 Study II

Enrolment was on a 2:1 case-to-control ratio and because there were no published data on the studied biomarkers using DBS in children with CHD, power calculations were based on clinical experience estimating the necessary recruitment numbers. The aim was to minimize false-positive results among controls and false-negative results among cases for the proposed screening test of combining NT-proBNP and IL-1RL1. To achieve 80% power with $\alpha=0.05$, we calculated the number of necessary cases to be 175 and controls 88, giving a total of 263 study participants.

Dried blood spot versus EDTA plasma levels of IL-1RL1 were compared by Pearson's linear correlation and Bland-Altman agreement plots and data logarithmically transformed as necessary. A logistic regression approach was used to analyse biomarker results by evaluating logarithmically transformed NT-proBNP

and IL-1RL1 measurements [178]. We performed ROC curve analyses to assess combined DBS test performances to identify high-risk CHD.

6.7.3 Study III

Study power calculation methods followed those outlined for Study I-II. A planned recruitment ratio of cases-to-controls was set at 2:1. With no published studies in children with ASD comparing IL-1RL1 blood-levels to CMR imaging findings, we estimated power calculations with 80% power and α=0.05. Our goal was to detect differences in IL-1RL1 levels and CMR measures between children with ASD and controls in at least 30% of cases. This required a minimum of 18 ASD cases and nine paediatric controls. We analysed group data of children before and after ASD treatment versus paediatrics controls using Students t-tests and Mann-Whitney-U tests.

For enrolled newborns, we estimated study power calculations as outlined above in Study I-II and compared DBS results of IL-1RL1 in 20 ASD cases versus 105 controls, who were available from combined recruitment efforts of Study I-III. Pearson's linear correlation and Bland-Altman agreement plots were used to assess DBS and EDTA assays of IL-1RL1 in controls, in whom both samples had been taken at the time of routine DBS sampling as part of the newborn screening program in the first week of life.

6.7.4 Study IV

Case-to-control recruitment followed a 2:1 ratio. As there were no published data on children with ASD assessing KE, we estimated power calculations (80% power, α=0.05) on clinical judgement, aiming to detect KE differences between cases versus controls in 30%, which required enrolment of 18 cases and nine controls [176, 177]. Paired Students t-tests and Wilcoxon tests were used when comparing ASD cases on initial and follow-up visits. To compare ASD groups before and after treatment versus controls, we used unpaired Students t-tests as well as Mann-Whitney-U tests. Linear correlation analyses using Pearson's method were applied to assess relationships between IL-1RL1 blood concentrations and measured KE levels in children before and after ASD closure.

7 Results

7.1 Study I: Dried blood spot analysis of single circulating biomarker to improve diagnostic care in newborns with congenital heart disease.

7.1.1 Congenital heart disease in the studied setting

The baseline incidences of CHD were reviewed within the regional healthcare region of Jönköping (Sweden) during 2019-2020. There was a total of 4071 registered deliveries at the single, participating maternity service. This represented approximately half of all births in the entire healthcare region, in which 102 new cases of CHD were diagnosed over the study's two-year-period. That amounted to an annual CHD incidence of approx. 1.3%. Similar national incidences of CHD were seen over this period in Sweden, as published through the Swedish Registry of Congenital Heart Disease (Swedcon). National changes to CHD prevalences over the last few decades have since been published for Sweden and the number of CHD cases has slightly increased over time and has been attributed to advances in medical care, with improved survival after neonatal and paediatric cardiac surgery with a corresponding 30-day mortality of approx. 2% [179]. Prenatal detection of CHD by maternal ultrasound screening has improved over the last decade in Sweden with more than half of severe types of CHD diagnosed before birth [34]. This prenatal detection has varied depending on the type of CHD, with >80% of single ventricles identified, whereas coarctation of the aorta has remained a challenge with less than 20% of cases identified before birth. The combined rate of prenatal CHD diagnoses in the three years directly preceding Study I was 18/30 newborn cases (55%), which included single ventricles, atrioventricular septal defects, lesions with large ventricular septal defect and an overriding aorta, such as Fallot's tetralogy, transposition of the great arteries, severe aortic valve disease, heterotaxy lesions, and Ebstein's anomaly. In 8/34 (24%) of newborns this affected maternal delivery planning with antenatal referral to the dedicated tertiary paediatric heart centre in Lund (Sweden). Postnatal screening for CHD using POX was implemented soon after published results from a Swedish study, approx. one decade before the initiation of Study I [35]. Following this, over the first 5.5 years of the regional POX screening program, eight new cases of critical CHD were diagnosed based on POX results, one critical CHD case was

missed and later diagnosed on follow-up during infancy, and two postnatal deaths occurred in unrecognized cases. The absolute number of critical CHD cases identified by POX screening in this setting was relatively low following this change to routine clinical care and the numbers of echocardiography needed to diagnose critical CHD was six, when not considering other important heart disease, such as persistent pulmonary hypertension of the newborns.

7.1.2 Newborn controls and cases with congenital heart disease

A total of 115 newborns (81 controls; 34 CHD cases) were enrolled in this first study. In 4/84 (5%) initially recruited controls, families declined to participate in additional EDTA blood sampling taken in parallel to DBS samples or phlebotomy staff could not obtain sufficient EDTA blood samples for biomarker analyses. These controls had to be excluded from direct NT-proBNP assay comparisons, i.e. EDTA venous blood versus DBS samples. Three newborns in the control group were found to have minor CHD types, i.e. small muscular ventricular septal defect, and patency of the arterial duct. None of these required cardiac intervention on follow-up. Amongst recruited CHD cases, 19/34 (56%) were classified as having critical CHD with the circulation dependent on patency of the arterial duct following birth. *Table 1* gives an overview of the types of CHD lesions enrolled in this study. Study participants had been born at term with mean±SD gestational age at 39.6±1.4 weeks. In total, there were 63 (55%) males and 52 (45%) females. The percentage of boys within the CHD group was slightly higher compared with controls, 23/34 (68%) versus 40/81 (49%); (p=0.11). We observed a higher percentage of Caesarean sections amongst CHD cases, but this was statistically not different to controls with 7/34 (21%) versus 7/81 (9%); (p=0.23). Neither of these perinatal factors affected outcome measures to predict CHD by DBS analyses using NT-proBNP.

7.1.3 Dried blood spot and venous blood tests for amino-terminal prohormone of brain natriuretic peptide (NT-proBNP)

A new, fully automated DBS assay for quantification of NT-proBNP was developed as described in detail within the subsequent publication of results with *figure 1* providing an illustrative overview of the analysis process [157]. Comparing log-transformed DBS versus EDTA venous blood test data showed a linear correlation amongst 80 paired control samples with r=0.93. Bland-Altman test agreement analysis of NT-proBNP data showed a bias±SD of -0.02±0.16; LoA: -0.32 – 0.30; *figure 2*. In controls, the median [IQR] levels of NT-proBNP decreased between 2 to 4 days of life from 3.921 [2.946-5.475] ng/ml to 0.608 [0.438-0.802] ng/ml. Median [IQR] DBS values were 1.900 (1.100-4.000) in controls versus 17.240 (4.735-26.940) ng/ml in cases. Group comparison revealed higher NT-proBNP values measured by DBS analysis in CHD cases compared to controls ($p<0.05$).

Using a NT-proBNP cut-off value >12.000 ng/mL to detect CHD, three asymptomatic newborns within the control group who had minor CHD in form of a small muscular ventricular septal defect and two cases with a small patent ductus arteriosus were identified. This cut-off could also identify 6/9 (67%) non-critical CHD cases who required heart surgery during the first six months of life. In contrast, 2/11 (18%) of postnatally asymptomatic critical CHD cases had NT-proBNP levels <12.000 ng/ml. These two presented later with symptoms of heart failure and were diagnosed with coarctation of the aorta after the neonatal period. Both these cases with coarctation underwent surgical management and survived.

Overall, the level of NT-proBNP alone could identify 24/34 (71%) of all enrolled CHD cases and 13/19 (68%) of critical CHD when using a cut-off at 12.000 ng/ml. Corresponding ROC curve analysis of the NT-proBNP assay on its own to diagnose CHD showed an area under the curve (AUC) of 0.87 with a 95% confidence interval (95%CI) of 0.79-0.95; *figure 3 (black curve)*. When considering different time points of DBS sampling within the first week of life, ROC curve analysis of NT-proBNP levels to diagnose CHD revealed an AUC of 0.83 for day 2 (n = 50), 0.92 for day 3 (n = 44), and 0.96 for blood sampling on day 4 (n = 18).

When controls were matched to cases, who had been born less than one year before DBS analysis, with shorter storage times of DBS samples, the overall AUC±SE improved to 0.96±0.03 (95%CI: 0.91-1.00). This revised ROC curve analysis suggested an optimized NT-proBNP cut-off for the detection of any CHD in the studied cohort at a level of 8.550 ng/ml.

Combing DBS assay results with postnatal POX screening improved overall detection of CHD to 82% (28/34 cases) and detection of critical CHD improved to 89% (17/19 cases). Combination of DBS NT-proBNP values and abnormal POX screening results achieved an AUC of 0.93 (95%CI: 0.87-0.99) on ROC curve analysis; *figure 3 (grey curve)*.

7.2 Study II: Dried blood spot analyses of combined circulating biomarkers to improve diagnostic care in newborns with congenital heart disease.

In this case-control study involving newborns with CHD and matched controls, we investigated whether combined DBS biomarker analyses of NT-proBNP and IL-1RL1 could be used to screen for “high-risk CHD” and developed an additional new DBS assay for IL-1RL1 to test this. “High-risk CHD” was defined as those CHD cases requiring cardiac surgery during infancy, including critical cases with duct-dependent lesions. The study was performed in Sweden from August 2019 to June 2023.

7.2.1 Newborn controls and cases with congenital heart disease

Children with known CHD, who were younger than 18 years, were approached for participation. Controls were recruited from Study I, using the described methods as outlined above. Of 342 children in total, 313 (92%) comprised the final case-control cohort for biomarker analyses. In 217/237 (92%) cases and 96/105 (91%) controls stored DBS samples were available and the provided DBS amounts were sufficient for biomarker analyses. Amongst the 237 CHD cases, 188 (79%) met the definition for high-risk CHD. The dominant lesion seen amongst these high-risk CHD cases was coarctation of the aorta, with or without hypoplasia of the transverse aortic arch, followed by shunt lesions (e.g. atrioventricular and ventricular septal defects); transposition of the great arteries; severe pulmonary or aortic stenoses; single ventricle lesions and other complex biventricular lesions, such as Fallot's tetralogy and heterotaxy disorders (*table 2*). All participants had been born between January 2005 to June 2023. Cases and controls were matched in terms of sex, gestational age, and birth weights with no significant differences observed. There were 181 (58%) males in the cohort. The overall mean±SD gestational age was 39.4±1.3 weeks and birth weight showed a mean±SD of 3499±486 grams.

7.2.2 Combined dried blood spot analyses of amino-terminal prohormone of brain natriuretic peptide and interleukin-1 receptor-like 1 biomarkers

The required DBS samples were retrieved from national biobank storage and had been taken at a median [IQR] of 2 [2-3] days of life, in line with Swedish newborn screening routines that normally aim for DBS sampling in term babies between 2-5 days of age. The applied DBS assay for NT-proBNP was developed during Study I and its methods have been published [157]. Amongst 217 cases, the median [IQR] for NT-pro-BNP levels was 25.5 [11.6-44.3] ng/ml while IL-1RL1 levels showed a median [IQR] of 13.4 [7.4-21.2] ng/ml. To assess the novel assay of IL-1RL1, biomarker concentrations were measured in DBS and EDTA venous blood samples using 82 controls, who had both samples taken at the same point in time. Log-transformed data of IL-1RL1 showed a Pearson's linear correlation between DBS and EDTA blood results with r=0.83 ($p<0.001$) and Bland-Altman analysis showed good test agreement with bias mean±SD of 1.00±0.17; LoA: 0.67 – 1.33; *figure 4*.

7.2.3 Detection of high-risk congenital heart disease including coarctation of the aorta

The combined DBS tests' ability to detect any high-risk CHD amongst 188 cases compared to 96 controls was assessed by ROC curve analysis, which showed an AUC of 0.95 (95%CI: 0.93 to 0.98); *figure 5*. Biomarker analyses for NT-proBNP

alone showed an AUC for all high-risk cases of 0.94 (95%CI: 0.91 to 0.97). For IL-1 RL1 on its own, this showed an AUC of 0.90 (95%CI: 0.86 to 0.93). Thirty-six of 188 high-risk CHD cases (19%) were initially not recognized by standardized prenatal or postnatal screening methods, in including POX. Of these, 31/36 (86%) could be diagnosed by combined NT-proBNP and IL-1RL1 testing.

In a subgroup of 70 coarctations amongst the 188 high-risk cases, who could initially not be recognized by postnatal POX screening and clinical examination, comparison with 86 controls showed that detection using NT-proBNP levels alone had an AUC of 0.96 (95%CI: 0.95 to 0.99) with IL-1RL1 alone showing an AUC of 0.91 (95%CI: 0.86 to 0.96). Combined biomarker test performance to detect these initially asymptomatic newborns with evolving coarctation showed the highest AUC of 0.97 (95% CI: 0.94 to 0.99); *figure 6*.

Test performance assessment of combined NT-proBNP and IL-1 RL1 analyses to detect any high-risk CHD lesion showed 93.0% test accuracy (sensitivity: 93.6%; specificity: 91.8%). The positive predictive value of identifying high-risk CHD in this cohort was 95.7%.

7.3 Study III: Cardiac magnetic resonance imaging combined with blood-based biomarker analysis in children with congenital heart disease.

To link the above-mentioned blood-based biomarkers to cardiac CMR imaging findings, this case-control study was conducted at the Children's Heart Centre for treatment of paediatric CHD in Lund (Sweden) during the period of October 2020 to January 2024. Participants were under the age of 18 years at study enrolment.

Levels of IL-1RL1 were assessed in 20 asymptomatic newborns with ASD and compared to 105 newborn controls using DBS samples to see how blood biomarker levels differed between groups and how this could help to establish an early ASD diagnosis in the first week of life. In 80 newborn controls, time-matched blood samples were available for direct comparison of IL-1 RL1 levels to evaluate test agreement on venous blood samples versus the novel DBS assay.

In an older group of children with ASD and matched controls, venous IL-1RL1 blood concentrations were measured and directly compared to cardiac magnetic resonance (CMR), performed on the same clinic visit to explore a possible link between blood-based biomarker levels and cardiac imaging findings. These blood and CMR investigations could be successfully performed in 23/25 (92%) paediatric ASD cases before treatment and 19/25 (76%) of these after successful ASD

treatment. Results were compared to 16/17 (94%) age-matched controls in which blood and CMR tests were also available from the same visit.

7.3.1 Clinical characteristics of newborn controls and cases

Recruited newborn controls had been born at a mean±SD of 39.7±1.3 weeks gestation with mean±SD birth weight of 3489±476 grams. Blood sampling in these occurred at mean±SD of 2.7±0.7 (95%CI: 2.5-2.8) days of age. All newborn controls were clinically well and not on any medications at the time of DBS sampling. There were limited perinatal data on ASD cases, who were recruited retrospectively with some born outside the studied healthcare region. All ASD cases had been asymptomatic following birth at term and not required any neonatal care. In these, DBS sampling had occurred on day two to three of life in line with the Swedish newborn screening guidelines and parts of stored DBS samples were retrieved from the national biobank. There were 7/20 (35.0%) male ASD cases compared to 53/105 (50.5%) male controls (p=0.04).

7.3.2 Clinical characteristics of paediatric controls and cases

Table 3 summarizes clinical characteristics for this study part in paediatric controls and children referred for ASD treatment. Venous blood sampling was timed to CMR investigations. Sufficient blood samples were taken in 16/17 (94.1%) controls and 23/25 (92.0%) ASD cases before and 19/23 (82.6%) after treatment. Cases were followed up at a mean±SD of 7.7±1.6 (95%CI: 6.9-8.5) months. There was no difference between these ASD cases and paediatric controls with regards to age, sex, and body sizes. An isolated ASD was seen in 21/23 (91.3%) of cases with two (8.7%) having additional partial anomalous pulmonary venous drainage. Defect closure was achieved using ASD-devices during cardiac catheterization in 17/23 (73.9%), and by cardiac surgery in 6/23 (26.1%). All children had sinus rhythm on ECG and normotensive blood pressures. There were no additional valvular lesions or signs of pulmonary hypertension on echocardiography. None of study participants were on cardiovascular medications nor had known genetic syndromes, obesity, diabetes, pulmonary or inflammatory diseases at the time of their assessments.

7.3.3 Amino-terminal prohormone of brain natriuretic peptide in newborn controls and atrial septal defect cases

Dried blood spot analysis of NT-proBNP was available for twenty newborns with ASD and for 93 controls. Analyses showed no difference between groups, even after

logarithmic transformation of data, with respective median [IQR] 1.20 [0.70-2.51] in ASD cases versus 1.10 [0.70-1.90] ng/ml in controls (p=0.48); *figure 7*.

7.3.4 Dried blood spot concentrations of interleukin-1 receptor-like 1 in newborns with atrial septal defects and controls

No sex differences in IL-1RL1 levels assessed by DBS analysis were seen between 53 boys versus 52 girls in the newborn control group; (p=0.99), and no sex differences were seen amongst newborn ASD cases either (p=0.25). *Table 4* summarizes DBS concentrations of IL-1RL1. The measured IL-1RL1 levels were higher in ASD cases with a median [IQR] 6.45 [5.15-12.70] compared to 3.80 [2.20-5.70] ng/ml in controls; DoM±SEM 5.94±1.09 (95%CI: 3.78-8.10); (p<0.01); *figure 8*. The corresponding ROC curve analysis using DBS IL-1RL1 results to detect ASD in newborns showed an AUC±SD=0.77±0.06 (95%CI: 0.66-0.89); asymptotic p<0.01; *figure 9*. *Table 5* gives an overview of how different cut-offs affected test performance. As an example, IL-1RL1 >4.28 ng/ml reached a sensitivity of 90% with specificity of 62% (likelihood ratio: 2.36). A higher cut-off at 6.03 ng/ml, showed corresponding 60% sensitivity and 80% specificity (likelihood ratio: 3.00).

7.3.5 Venous blood interleukin-1 receptor-like 1 concentrations in children with atrial septal defects and controls

In controls, venous blood EDTA levels of IL-1RL1 were similar in newborns with a median [IQR] 30.56 [21.12-44.31] compared to older paediatric controls with 34.75 [24.25-55.68] ng/ml; (p=0.16). In cases referred for ASD closure after infancy, a decrease in IL-1RL1 levels after ASD treatment was observed. Venous blood concentrations of IL-1 RL1 reduced from a median [IQR] of 38.90 [22.20-57.60] beforehand to 34.10 [23.73-46.35] ng/ml after ASD treatment, MoD±SD 6.13±12.08 (95%CI: 0.47-11.78); (p=0.04); *figure 10*.

7.3.6 Cardiac magnetic resonance imaging in children with atrial septal defects before and after defect closure versus controls

Table 6 summarizes CMR findings. Overall, shunt volumes were successfully treated in all ASD cases. Right ventricular volumes reduced in line with this, while a slight increase left ventricular volumes was seen. Heart functions in cases remained within normal ranges for children. Before ASD treatment, atrial shunt ratios (Qp:Qs) were clinically important with a mean±SD of 2.1±0.7, which normalized afterwards to 1.0±0.1 (p<0.01). Following ASD treatment these ratios were comparable to controls, who showed Qp:Qs at 1.01 [0.94-1.05]; (p=0.39).

Left ventricular assessment:

Left ventricular ejection fraction (LV EF; %) remained within normal range in cases before and after treatment. Mean±SD of LV EF was 58.83±5.36 (95%CI. 56.51-61.14) before ASD closure and afterwards 59.82±6.07 (95%CI: 56.71-62.97); (p=0.64).

Amongst cases, cardiac index (CI, l/min/m^2) increased from a mean±SD of 3.22±0.59 (95%CI:2.96-3.48) before to 3.77±0.81 (95%CI: 3.38-4.16) after ASD closure with MoD±SD 0.54±0.90 (95%CI: 0.11-0.97); (p<0.01).

Indexed left ventricular stroke volumes (LV SVi; ml/m^2) were lower before ASD treatment with a mean±SD 39.2±6.6 (95%CI: 36.3-42.1) compared to controls 49.7±8.6 with DoM±SEM 10.5±2.5 (95%CI: 5.35-15.69); (p<0.01). After ASD closure LV SVi were within the range of controls and showed a mean±SD of 43.93±11.63 (95%CI: 37.95-49.91) on follow-up (p=0.85).

Indexed left ventricular end-diastolic volumes (LV EDVi; ml/m^2) increased from a mean±SD of 65.5±10.3 before ASD closure to 78.3±18.3 afterwards with MoD±SD 12.5±15.0 (95%CI: 4.8-20.2) ml/m^2; (p<0.01).

Indexed left ventricular end-systolic volumes (LV ESVi; ml/m^2) increased after ASD closure from mean±SD 26.3±5.5 to 34.4±12.6 with MoD±SD 7.5±9.7 (95%CI: 2.6-12.5); (p<0.01).

Right ventricular assessment:

Right ventricular ejection fraction (RV EF; %) remained within normal ranges, but reduced after ASD treatment from a mean±SD of 57.4±5.1 (95%CI: 55.2-59.7) to 51.2±12.8 (95%CI: 44.6-57.7) with MoD±SD 6.5±12.5 (95%CI: 0.1-12.9); (p<0.05).

Indexed right ventricular stroke volumes (RV SVi; ml/m^2) decreased in ASD cases from a mean±SD of 76.7±19.8 (95%CI: 67.9-85.5) to 47.1±12.7 (95%CI: 40.6-53.7) with MoD±SD 36.2±47.0 (95%CI: 12.0-60.4); (p<0.01); *figure 10.*

Indexed right ventricular end-diastolic volumes (RV EDVi; ml/m^2) reduced from mean±SD 134.5±38.0 (95%CI: 117.6-151.3) to 96.0±21.6 (95%CI: 84.9-107.1) ml/m^2 with MoD±SD 42.7±32.8 (95%CI: 26.1-59.2); (p<0.01).

Indexed right ventricular end-systolic volumes in ml/m^2 (RV ESV; ml/m^2) reduced from a mean±SD of 58.0±20.8 (95%CI: 48.7-67.2) to 48.7±17.1 (95%CI: 39.9-57.5) with MoD±SD 10.8±14.3 (95%CI: 3.5-18.1); (p<0.01).

7.3.7 Relationship between interleukin-1 receptor-like 1 and cardiac magnetic resonance imaging in children with atrial septal defects

When assessing for linear correlations between IL-1RL1 concentrations and CMR measurements prior to ASD treatment, left ventricular function (LV EF) and volumes showed low correlations. Pearson's *r* was -0.08 for LV EF, 0.07 for LV end-diastolic volumes, 0.13 for LV stroke volumes, and 0.14 for LV end-systolic volumes. Correlation between IL-1RL1 and Qp:Qs before ASD treatment showed r=0.22.

Modest linear correlations were seen between IL1-RL1 and right ventricular function (RV EF) and volumes before treatment. Pearson's *r* was 0.30 for correlation with indexed RV end-diastolic volumes, 0.39 for indexed end-systolic volumes as well as RV EF.

The single CMR parameter correlating best with IL-1 RL1 blood levels was indexed RV stroke volume, which showed r=0.43 before ASD treatment and r=0.37 afterwards; *figure 11*. Furthermore, ROC curve analysis of DBS blood IL-1RL1 concentrations in newborns with ASD and controls to predict indexed RV stroke volumes greater than 55 ml/m^2 before ASD closure showed an AUC±SD of 0.77±0.09 (95%CI: 0.59-0.95); (asymptotic p<0.01); *figure 12.*

7.4 Study IV: Cardiac kinetic energy assessed by magnetic resonance combined with blood-based biomarker analysis in children with congenital heart disease.

To explore advanced 4D-flow CMR measures of KE with blood-based biomarker levels of IL-1RL1, this case-control study was performed in Sweden during January 2020 and December 2024 by using CMR in children with ASD before and after treatment versus matched controls. All enrolled children were assessed via the Children's Heart Centre in Lund.

When conducting tests of normality for CMR data in cases and controls, Shapiro-Wilk test showed slightly larger spread of diastolic KE measurements within the left ventricle in ASD patients before treatment, suggesting a trend towards non-parametric data distribution. Visual assessment of data distribution showed no obvious skewedness. We analysed KE data using both parametric and non-parametric tests, i.e. paired Students t-tests and Wilcoxon tests and unpaired Students t-tests, Mann-Whitney-U tests to compare groups of ASD patients before and after treatment with controls. Results were similar using both parametric and

non-parametric tests, i.e. the choice of test did not affect overall statistical significance and the following results are presented using parametric test results only.

A graphical summary of measured KE levels over the whole cardiac cycle in ASD cases before defect closure is presented in *figure 13* for the left ventricle and right ventricle. *Figure 14* illustrates KE findings in ASD cases after defect closure and *figure 15* demonstrates KE results in controls.

7.4.1 Clinical characteristics of atrial septal defect cases and controls

Clinical characteristics of children assessed by 4D-flow CMR to measure KE levels are summarized in *table 7*. No differences were seen between cases and controls regarding sex; p=0.10, age; p=0.18, or BSA; p=0.27. Cases underwent follow-up CMR assessment at 7.7±1.6 (95%CI: 6.9-8.5) months after ASD treatment. The BSA (m^2) in cases was not significantly different from controls on follow-up (p=0.86), but increased in cases with normal growth from 1.15±0.44 (95%CI: 0.97-1.33) to 1.30±0.44 (95%cI: 1.10-1.51); (p<0.01). Likewise, height (cm) increased significantly in cases from a mean+/-SD of 135.5±25.8 (95%CI:124.8-146.1) beforehand to afterwards 144.5±23.4 (95%CI: 133.5-155.5); (p<0.01). All children had sinus rhythm on ECG. There were no arrhythmias seen during at the study assessment points. Heart rates per minute (HR) were 75±10 (95%CI: 71-80) in controls and 86±13 (95%CI: 80-91) in cases before treatment; (p<0.01). After ASD treatment, HR in cases were comparable to controls; (p=0.22).

No genetic syndromes were seen amongst study participants and none of the cases was on cardiovascular drug therapy, nor had other known problems potentially affecting cardiovascular health, e.g. arterial hypertension, ventricular dysfunction, pulmonary hypertension, or diabetes mellitus. Defect closure was achieved using ASD-devices during cardiac catheterization in 17/23 (73.9%), and by cardiac surgery in 6/23 (26.1%). Two of six (33.3%) surgical cases had additional partial anomalous pulmonary venous drainage. Amongst cases, 20/23 (87.0%) were CMR-compliant with sufficient CMR data for KE analyses and 18/20 (90.0%) underwent successful follow-up scans with two of the 20 opting out of repeat CMR assessment. Sedation during CMR was not required for any of the twelve controls, and enabled scanning in 4/20 (20.0%) of initial ASD cases, and in 2/18 (11.1%) on follow-up.

7.4.2 Systolic and diastolic peak left ventricular kinetic energy in children with atrial septal defects before and after defect closure

Table 8 summarizes left and right ventricular KE findings in children with ASD before and after defect closure versus matched paediatric controls.

Systolic peak left ventricular kinetic energy:

Systolic peak left ventricular (LV) KE (mJ) increased significantly in children after ASD closure. Before ASD closure this showed a mean±SD of 2.17±1.57 (95%CI: 1.49-2.85) and after ASD closure 2.80±1.92 (95%CI: 1.78-3.83) with a MoD±SD 0.67±0.89 (95%CI: 0.20-1.14); ($p<0.01$); *figure 16 (left panel)*. Overall, these KE levels in ASD cases were within the range seen in controls, who showed systolic peak LV KE of 3.27±2.98 (95%CI: 1.47-5.08); (ASD cases before treatment vs. controls: p=0.15 / ASD cases after treatment vs. controls: p=0.61).

Systolic peak left ventricular kinetic energy indexed for end-diastolic volumes and stroke volumes:

To account for potential volumetric changes within the left ventricle, indexing systolic peak LV KE values for corresponding end-diastolic and stroke volumes was performed. Systolic peak LV KE indexed for end-diastolic volume pre-treatment showed means+/-SD 25.04+/-10.86 (95%CI: 20.35-29.74) and post-treatment 27.07+/-14.01 (95%CI: 19.60-34.53); (p=0.42). Controls showed 29.33+/-12.39 (95%CI. 21.84-36.82). Pre- / post-treatment levels were not different to controls (ASD cases before treatment vs. controls: p=0.29 / ASD cases after treatment vs. controls: p=0.65).

Systolic peak LV KE indexed for stroke volume in ASD cases before defect closure showed a mean+/-SD of 41.53+/-17.18 (95%CI: 34.10-48.96) and post-treatment 48.51+/-24.13 (95%CI: 35.65-61.37); (p=0.30). Controls showed 49.97+/-21.26 (95%CI: 37.13-62.82). Pre- / post-treatment level were not different to controls (ASD cases before treatment vs. controls: p=0.20 / ASD cases after treatment vs. controls: p=0.87).

Results demonstrated that the observed changes to systolic peak LV KE levels in children before and after ASD closure were influenced by changes to LV volumes.

Systolic peak left ventricular kinetic energy indexed for cardiac index:

To correct for possible changes to CI, indexing of systolic peak LV KE for this parameter was additionally performed. Systolic peak LV KE indexed for CI in cases before ASD closure showed a mean+/-SD of 0.76+/-0.62 (95%CI: 0.49-1.03) and after ASD closure 0.75+/-0.52 (95%CI: 0.48-1.02); (p=0.79). Controls showed 0.84+/-0.60 (95%CI: 0.48-1.20). Pre- / post-treatment levels were not different to controls; (ASD cases before treatment vs. controls: p=0.73 / ASD cases after treatment vs. controls: p=0.68).

These results showed that changes to CI influenced the observed changes to systolic peak LV KE in children before and after ASD closure, which is in line with the volumetric LV indices described above.

Diastolic peak left ventricular kinetic energy:

Overall, diastolic peak LV KE remained essentially unchanged in children before and after ASD treatment. Diastolic peak LV KE levels in cases before ASD closure showed a mean±SD of 2.77+/-2.14 (95%CI:1.85-3.70) and this increased only slightly after ASD treatment to a mean+/-SD of 3.83+/-2.94 (95%CI: 2.25-5.40), with results not reaching statistically significance; (p=0.10); *figure: 16 (right panel)*. There was no difference between pre- / post-treatment cases and controls. Controls showed a mean+/-SD of 3.41+/-2.82 (95%CI: 2.45-4.47); (ASD cases before treatment vs. controls: p =0.22 / ASD cases after treatment vs. controls p=0.90).

Diastolic peak left ventricular kinetic energy indexed for end-diastolic volumes and stroke volumes:

When indexing LV diastolic peak KE to corresponding end-diastolic volumes or LV stroke volumes, there were no significant differences between groups; non-parametric tests yielded comparable results to parametric ones for all diastolic KE measurements. Diastolic peak LV KE indexed for end-diastolic volumes before ASD closure showed a mean+/-SD of 32.96+/-18.10 (95%CI: 25.13-40.79) and after ASD closure 33.62+/-13.94 (95%CI: 26.19-41.05); (p=0.82). Controls showed 36.20+/-18.91 (95%CI: 24.77-47.62). Pre- / post-treatment comparison of cases versus controls showed no differences (ASD cases before treatment vs. controls: p=0.62 / ASD cases after treatment vs. controls: p=0.68).

Diastolic peak LV KE indexed for LV stroke volume before ASD closure showed a mean+/-SD of 55.28+/-30.96 (95%CI: 41.90-68.67) compared to after ASD closure 60.18+/-26.10 (95%CI: 46.27-74.09); (p=0.92). Controls showed 62.28+/-33.72 (95%CI: 41.90-82.65) with no statistically significant differences seen between pre- and post-treatment cases and controls (ASD cases before treatment vs. controls: p=0.53 / ASD cases after treatment vs. controls p=0.85).

These indexed diastolic peak KE results indicate that the diastolic KE levels were also related to volumetric changes and indexing KE levels did not unmask any potential KE differences in children before and after ASD closure.

Diastolic peak left ventricular kinetic energy indexed for cardiac index:

When considering CI potentially influencing diastolic peak LV KE levels, indexing for this showed that there were no statistically significant differences between cases before and after ASD treatment versus controls.

Diastolic peak LV KE indexed for CI in cases before ASD closure showed a mean+/-SD of 1.02+/-0.92 (95%CI: 0.62-1.42) and after ASD closure 1.06+/-0.89 (95%CI: 0.60-1.51); (p=0.78). Controls showed 1.00+/-0.56 (95%CI: 0.66-1.34). Levels in controls were not different to those seen in cases before and after ASD closure (ASD cases before treatment vs. controls: p=0.96 / ASD cases after treatment vs. controls: p=0.85).

Correcting for possible changes in CI did not alter initial findings for diastolic peak LV KE in children with ASD before and after treatment versus controls, that showed KE levels in ASD cases within the range of those seen in controls and no KE differences in cases after ASD defect closure.

7.4.3 Systolic and diastolic peak right ventricular kinetic energy in children with atrial septal defects before and after defect closure

Kinetic energy of the right ventricle:

Absolute changes to right ventricular KE levels in children before and after ASD treatment are illustrated by *figure 17* and showed significant KE reductions after ASD closure.

Systolic peak right ventricular kinetic energy:

Overall, systolic peak right ventricular (RV) KE decreased markedly in patients after ASD treatment to levels seen in controls. Systolic peak RV KE levels in pre-treatment cases showed a mean+/-SD of 5.47+/-5.25 (95%CI: 3.12-7.78) and post-treatment 3.56+/-2.59 (95%CI: 2.19-4.94); ($p<0.01$); *figure 17 (left panel)*. Controls showed 3.59+/-2.12 (95%CI: 2.31-4.87). Pre- / post-treatment levels were within the range of controls (ASD cases before treatment vs. controls: p=0.23 / ASD cases after treatment vs. controls: p=0.97).

Systolic peak right ventricular kinetic energy indexed for end-diastolic volumes and stroke volumes:

After indexing RV systolic peak KE for corresponding end-diastolic volumes and stroke volumes there were no statistically significant differences amongst groups. Systolic peak RV KE levels indexed for end-diastolic RV volume in pre-treatment cases showed mean+/-SD 21.08+/-13.53 (95%CI: 15.23-26.92 and post-treatment 19.07+/-10.88 (95%CI: 13.04-25.09); (p=0.42). Controls showed 15.76+/-5.54 (95%CI: 12.57-18.96). Pre- / post-treatment levels were not different to controls (ASD cases before treatment vs. controls: p=0.17 / ASD cases after treatment vs. controls: p=0.32).

Systolic peak RV KE levels indexed for stroke volume in pre-treatment cases showed mean+/-SD 39.70+/-24.30 (95%CI: 29.19-50.21) and post-treatment 35.09+/-19.81 (95%CI: 24.12-46.06); (p=0.38). Controls showed 28.62+/-9.41 (95%CI: 23.18-34.05). Pre- / post-treatment levels were not different to controls; (ASD cases before treatment vs. controls: p=0.11 / ASD cases after treatment vs. controls: p=0.28).

This indicates that the observed changes to KE within the right ventricle were related to volumetric changes.

Systolic peak right ventricular kinetic energy indexed for cardiac index:

Systolic peak RV KE levels indexed for CI were significantly higher before treatment of ASD compared to controls and dropped to normal levels afterwards. Systolic peak RV KE levels indexed for CI in pre-treatment cases showed mean+/-SD 1.64+/-1.65 (95%CI: 0.91-2.37) and post-treatment 0.60+/-0.44 (95%CI: 0.37-0.82); ($p<0.01$). Controls showed 0.45+/-0.19 (95%CI: 0.33-0.56). Pre-treatment levels were higher than in controls; ($p=0.01$). Post-treatment levels were not different from controls ($p=0.26$).

This shows that changes to CI, largely a measure of LV performance, influenced KE levels within the right ventricle, albeit to a lesser degree than the above-mentioned volumetric RV changes.

Diastolic peak right ventricular kinetic energy:

Overall, diastolic peak RV KE decreased significantly after ASD treatment in children to those levels seen in controls. Diastolic peak RV KE levels in pre-treatment cases showed a mean+/-SD of 4.54+/-4.63 (95%CI: 2.49-6.59) and post-treatment 2.15+/-1.58 (95%CI: 1.30-2.99); ($p<0.01$); *figure 17 (right panel).* Controls showed 1.71+/0.83 (95%CI: 1.20-2.21). Pre-treatment levels were significantly higher than in controls; ($p=0.04$). Post-treatment levels were comparable to controls; ($p=0.37$).

Diastolic peak right ventricular kinetic energy indexed for end-diastolic volumes and stroke volumes:

Diastolic peak RV KE levels indexed for end-diastolic volume in pre-treatment cases showed mean+/-SD 27.83+/-10.79 (95%CI: 23.16-32.50) and post-treatment 28.55+/-12.61 (95%CI: 21.57-35.54); ($p=0.48$). Controls showed 31.11+/-9.56 (95%CI: 25.59-36.63). Pre- / post-treatments levels were not different to controls (ASD cases before treatment vs. controls: $p=0.36$ / ASD cases after treatment vs. controls: $p=0.55$).

Diastolic peak RV KE levels indexed for stroke volume in pre-treatment cases showed mean+/-SD 53.12+/-19.37 (95%CI: 44.74-61.50) and post-treatment 54.02+/-26.80 (95%CI: 39.18-68.86); ($p=0.48$). Controls showed 56.90+/-18.92 (95%CI: 45.98-67.83). Pre- / post-treatment levels were not different to controls (ASD cases before treatment vs. controls: $p=0.57$ / ASD cases after treatment vs. controls: $p=0.74$).

These volumetric indices showed how reductions of RV KE levels after ASD closure were related to the observed decreases of RV volumes.

Diastolic peak right ventricular kinetic energy indexed for cardiac index:

Diastolic peak RV KE levels indexed for CI showed significant decrease after ASD treatment. Pre-treatment levels for indexed CI were significantly higher than in

controls. Diastolic peak RV KE levels indexed for CI in pre-treatment cases showed mean+/-SD 1.69+/-1.76 (95%CI: 0.92-2.45) and were lower post-treatment 0.75+/-0.37 (95%CI: 0.56-0.93); ($p<0.01$). Controls showed 0.94+/-0.48 (95%CI: 0.65-1.23). Pre- / post-treatment levels were not different from controls (ASD cases before treatment vs. controls: $p=0.15$ / ASD cases after treatment vs. controls: $p=0.22$).

These results indicate that RV KE levels were less influenced by overall CI levels than RV volumetric changes as described above.

7.4.4 Relationship between blood concentrations of interleukin-1 receptor-like 1 and kinetic energy findings in children before and after atrial septal defect closure

Blood concentrations of IL-1RL1 (ng/ml) decreased in children with ASD after defect closure from mean±SD 41.56±21.60 (95%CI: 32.16-50.86) to 34.27±13.64 (95%CI: 27.88-40.65) with a MoD±SD 9.03±10.84 (95%CI: 3.64-14.42), ($p<0.01$); *figure 18*.

When comparing CMR derived data of left and right ventricular peak systolic and diastolic kinetic energy levels with IL-1RL1 blood concentrations in children with ASD before treatment, left ventricular measures showed low linear correlations with left and right KE measurements. However, the one measure correlating best with blood test findings was peak systolic right ventricular KE ($r=0.50$); *figure 19*. This observed relationship between blood levels of IL-1 RL1 and systolic peak RV kinetic energy was maintained after ASD closure, which showed a linear correlation between blood and imaging findings in cases with $r=0.55$; *figure 20.* This correlation was slightly higher than the previously observed finding of IL-1RL1 blood concentrations and RV stroke volumes ($r=0.43$), as described in Study III.

8 Discussion

8.1 Study I

8.1.1 Early diagnosis of congenital heart disease by novel dried blood spot biomarker analysis using amino-terminal prohormone of brain natriuretic peptide (NT-proBNP)

This first case-control study explored the feasibility of diagnosing a range of CHD lesions in newborns using DBS analysis of the blood-based biomarker NT-proBNP. A novel, fully automated assay was developed and tested in a cohort of healthy babies born at term, who served as controls and in whom test comparison with standard laboratory assays for NT-proBNP using venous blood samples showed very good test agreements. In addition to providing reference ranges, timing of blood tests in these controls mimicked clinical practice, in which routine newborn DBS screening in Sweden is usually performed on day two to three of life.

A large proportion of retrospectively recruited cases, with various types of CHD, could be identified by NT-proBNP analysis alone. In combination with known POX results, a combined screening approach to detect primarily critical CHD, proved to be effective, and could even identify previously unrecognized cases in the studied cohort through the novel NT-proBNP analysis.

Because natriuretic peptide production in children reflects only some of the complex adaptive processes affecting the circulatory system in children with heart disease, it is important to realize that various published studies in paediatric cohorts, with different types of heart diseases, have led to slightly different conclusions, all of which should ultimately be explainable by the physiological role that natriuretic peptides play in the studied clinical context [180-184]. In this study the physiological natriuretic peptide response, as reflected by its metabolite NT-proBNP, added diagnostic value in a wide range of clinically important types of CHD amongst newborns and the main goal of this study was achieved by providing a novel DBS assay for NT-proBNP to screen for a large range of CHD types in newborns. The results indicate that even asymptomatic cases after birth may benefit from this screening approach to improve early identification of potentially life-threatening CHD lesions, as such coarctation of the aorta.

8.1.2 Limitations

This study was designed as a single centre, case-control study in Sweden and explored the feasibility of detecting CHD in a wide spectrum of CHD. While controls were recruited prospectively, all cases were enrolled retrospectively after their diagnoses had been established. For these, stored DBS samples were retrieved from cold biobank storage for biomarker analyses. The length of storage time may influence the level of measurable proteins, such as NT-proBNP, and older samples would have been expected to yield lower concentrations of the studied biomarker [77]. Screening a larger CHD cohort would be preferrable in future studies and Study II aimed to address this current limitation. It remains to be seen, how the observed results can be repeated in a prospective study setting, that would need to provide robust and reproducible screening data with meaningful translation of findings into clinical care. A second-tier testing, such as repeat blood biomarker analysis or confirmatory echocardiography would need to be included in such future screening studies. Ultimately, a blood-based screening test for CHD in newborns using DBS samples would depend on acceptable specificity and sensitivity, i.e. a high rate of correctly identifying babies with CHD and a low false positive rate to prevent an unnecessary burden on paediatric echocardiography services, to confirm screening test results and identify the specific CHD lesion in these vulnerable babies. Overall, the use of the circulating biomarker NT-proBNP showed promising results in this study, because it reflects the rapidly evolving, circulatory changes with related changes to natriuretic peptide production in the early postnatal period.

The study focused on assessment in newborns who were born at term and not in need of neonatal intensive care treatment. From previous studies in premature neonates, evidence is emerging that NT-proBNP levels may behave differently in premature neonates compared to those born at term [185, 186]. It is important to note, that the diagnostic focus for using NT-proBNP in previous neonatal studies has predominately been to stratify risks in premature babies with patent arterial duct, even though natriuretic peptide levels are not specific to this type of heart disease, as demonstrated in this study [187-191]. This underscores the need to establish robust reference ranges for NT-proBNP levels in premature neonates as well as in those born at term, especially during the first week of life to ultimately improve diagnostic accuracy and applicability of this study's findings in the future.

8.2 Study II

8.2.1 Dried blood spot analyses to screen for high-risk congenital heart disease in newborns using amino-terminal prohormone of brain natriuretic peptide (NT-proBNP) and interleukin-1 receptor-like 1 biomarkers (IL-1RL1)

Following the results from Study I, that demonstrated the feasibility of screening for CHD by using minimal amounts of DBS samples in newborns, this study aimed to expand the novel screening approach by using a combination of circulating blood-based biomarkers to improve diagnostic screening results.

In this case-control study, cases with CHD were retrospectively identified using electronic healthcare records across two centralized Children's Heart Centres in Gothenburg and Lund (Sweden). These two centres provide cardiothoracic surgery for all types of CHD in children within the country. Prospectively enrolled controls, primarily recruited in Study I, were used for case comparison. We focused our study efforts on so-called "high-risk" cases, defined as CHD lesions requiring cardiac surgery or cardiac catheter intervention during infancy based on current clinical guidelines. This definition encompassed all duct-dependent, critical types of CHD.

A novel, fully automated dried blood spot test to quantify soluble IL-1RL1 concentrations was successfully developed and its usability evaluated in newborn controls and those with high-risk CHD. The encouraging results showed improvement of screening test performance when combing IL-1RL1 with the previously developed NT-proBNP assay from Study I. These results would need to be replicated in larger, prospective trials assessing this novel approach to detect high-risk congenital heart disease in newborns as early as possible.

Because this study focused on the more complex, and potentially life-threatening types of CHD, further studies are also needed to evaluate this screening approach for other types of common congenital heart disease, that do not require cardiac surgery in infancy. Study III aimed to investigate this further by analysing paediatric cases with a common, and clinically important type of CHD, represented by atrial septal defects (ASD), because these children are largely asymptomatic in infancy.

8.2.2 Combined biomarker analyses of amino-terminal prohormone of brain natriuretic peptide and interleukin-1 receptor-like 1 biomarkers

The combination of circulating biomarkers improved overall newborn screening test performance, compared to single NT-proBNP or IL-1RL1 tests alone. This may be explainable by these two blood-based biomarkers reflecting different

pathophysiological aspects of the body's response to CHD. The investigated biomarkers may serve as surrogate markers of e.g. pressure and volume loading conditions or may indicate changes to pulmonary blood flow.

Relying solely on the new DBS tests to identify high-risk CHD cases in the studied cohort, only 2.7% of all cases would have been missed by combined NT-proBNP and IL-1RL1 biomarker analyses. This compares to 19.1% without the applied, novel DBS screening approach using current screening methods in clinical practice, including POX. The presented findings may have prevented clinical deterioration in these previously unidentified infants in the first few days after birth. Future prospective studies should address the question whether this novel approach to improve the detection of important CHD in newborns could not just lead to earlier diagnosis, but also reduce postnatal morbidity and mortality.

8.2.3 Limitations

This was a retrospective, multi-centre study focusing on high-risk CHD cases, who were surgically treated during infancy at the two centralized Children's Heart Centres in Sweden. We did not analyse deceased CHD cases, who had died due to unrecognized CHD during the neonatal period within the studied Swedish healthcare system. Although the number of such cases identified through post-mortem examinations in Sweden would have been likely to be low, some of these cases might have had stored DBS samples available for analyses. Before approaching emotionally traumatized families, who had lost their children, we felt it paramount to prove the biomarkers' usefulness in the chosen national cohort of survivors before considering further expansion of the DBS testing for deceased cases.

We also aimed to account for the sex, gestational age and birth weight of newborns and recorded the day of life on which dried blood sampling occurred. We did not allow blood samples taken after the first week of life to be included in our analysis as the aim of this study was to screen for CHD lesions as early as possible after birth, i.e. at the time of routine newborn screening in Sweden, which is usually conducted on day two to three of life. Additionally, we endeavoured to correct results for clinical factors, such as intensive care support and intravenous prostaglandin E1/E2 administration in babies with critical CHD at the time of DBS sampling, with retrospective medical chart reviews having its inherent methodological limitations and not all clinical data could be ascertained in all critical CHD cases.

8.3 Study III

In this prospective, case-control study the cardiac changes occurring in paediatric ASD patients following defect closure were assessed using cardiac magnetic resonance. Cardiac image findings were then linked to blood levels of the circulating biomarker IL-1RL1 to explore associations between changes to cardiac morphology and function seen on CMR and blood concentrations of IL-1RL1 before and after ASD treatment, which should ultimately reflect on the underlying cardiac remodelling process in this CHD lesion with initial right heart volume loading and increased pulmonary blood flow.

8.3.1 Cardiac magnetic resonance imaging in children with atrial septal defects before and after closure

Cardiac magnetic resonance revealed a significant reduction of right ventricular volumes after ASD closure related to normalization of Qp:Qs on follow-up six to twelve months after successful treatment. Right ventricular end-diastolic volumes reduced to a larger degree compared to end-systolic volumes over this time frame in the studied children, which led to a corresponding slight reduction of EF for the right ventricle. Overall, the observed right ventricular EF in ASD cases remained within the range of controls. These findings may indicate an ongoing RV remodelling process in these children and partial remodelling of the right ventricle after ASD closure has previously been described in adults [140, 142, 192].

Significant increases to left ventricular volumes and cardiac output indexed for body size (CI) were observed in the studied children following ASD closure. These changes were not simply related to normal somatic growth on follow-up as demonstrated by adjusting CMR findings for body sizes. No signs of acute left ventricular diastolic dysfunction were unmasked following ASD closure and such diastolic dysfunction of the left ventricle is rarely encountered in childhood [193]. In contrast to this, adult studies have described left ventricular diastolic dysfunction as a contributing factor to reduced exercise performance, especially in the elderly, after ASD closure [140, 194-196]. How exactly, left ventricular function in the studied cohort improved, remains debatable, although a shift in the interventricular septum during diastole, following reductions of right ventricular volumes after ASD closure, may have improved left ventricular function and its effective size. Additionally, normalization of inflow to the left ventricle following ASD closure should have aided diastolic filling as previously described [197, 198].

8.3.2 Circulating biomarker levels of interleukin-1 receptor-like 1 in asymptomatic newborns with atrial septal defects

During Study III, blood concentrations of IL-1RL1 were measured using minimal amounts from stored DBS samples in children with ASD. These had been asymptomatic following birth, and were subsequently diagnosed with ASD during childhood, i.e. after infancy. While NT-proBNP levels in these newborns were not different between ASD cases and controls, the novel IL-1RL1 assay could differentiate reasonably well between ASD patients and normal controls with ROC curve analysis showing an AUC of 0.77. In this studied population, a cut-off for IL-1RL1 at 6.0 ng/ml, showed 60% sensitivity and 80% specificity with a positive likelihood ratio of 3.0. This indicates a modest improvement of identifying newborns with ASD through a positive IL-1RL1 screening result. Even though the novel IL-1RL1 test using DBS samples was far from perfect in diagnosing ASD cases amongst asymptomatic newborns, one should bear in mind that none of these newborns would have been identified as having ASD without IL-1RL1 testing. Further prospective evaluation of these encouraging findings is warranted to improve screening test performance for newborns with ASD using DBS biomarker analyses.

8.3.3 Linking circulating blood-based biomarker and advanced non-invasive cardiac imaging in children with atrial septal defects

When directly comparing CMR findings in children before and after ASD closure with circulating levels of the blood-based biomarker IL-1RL1, left ventricular CMR measures showed lower correlations compared to right ventricular ones. Right ventricular stroke volumes correlated best with IL-1RL1 findings before and after ASD treatment, albeit with only modest linear correlations (r=0.43). Likewise, ROC curve analysis to predict right ventricular stroke volumes greater than 55 ml/m^2 before ASD closure based on IL-1RL1 concentrations using DBS samples from the newborn period, showed a modest AUC of 0.77. This cut-off at 55 ml/m^2 was chosen to coincide with the upper 95%CI limit in controls and should minimise false positive screening results.

Findings indicate that the pathophysiological role of IL-1RL1 in this paediatric ASD setting is related to increased volume loading conditions affecting the right ventricle and corresponding alterations to the pulmonary circulation. Increases to pulmonary blood flow in ASD patients is a pathophysiological consequence of the atrial shunt flow and may have contributed to higher levels of IL-1RL1 before ASD closure with pulmonary production of IL-1RL1 having been previously described, and this is likely a contributing factor for elevated IL-1RL1 levels in adults with acutely decompensated congestive heart failure and related pulmonary congestion [145, 199]. Whether the observed relationship between RV stroke volumes and IL-1RL1

levels in children before ASD closure could be clinically used to estimate the significance of pulmonary over-circulation in children with ASD would need to be evaluated in future studies. This may in turn lead to a refinement of current clinical treatment guidelines, which is currently largely based on atrial shunt volumes in children.

8.3.4 Limitations

This was a single centre study comprising a relatively small sample size of children with ASD. Because DBS and venous blood assays for quantification of IL-1RL1 have their intrinsic test differences, measured levels using these two different assays, could not be directly compared between newborns and older children with ASD. A larger cohort would be necessary to establish robust test matrix comparisons, which could not be performed due to resource limitations in this study.

Although reduced myocardial strain of the right ventricle has been reported on long-term follow-up of adults after ASD closure, this paediatric study was not designed to assess this on short-term follow-up [197]. Similarly, cardiopulmonary exercise testing in adults following ASD closure has revealed slightly reduced exercise capacities [194-196]. As most children in this study were too young to comply with standard cardiopulmonary exercise testing, this could not be formally assessed.

8.4 Study IV

This prospective, case-control study explored how intraventricular KE levels change in children before and after ASD treatment. Results were compared to controls and KE findings from 4D-flow CMR were additionally compared to blood levels of IL-1RL1 before and after ASD closure to see how peak KE levels changed following this, and whether there could be a link between IL-1RL1 blood levels and KE levels, mirroring aspects of the cardiac remodelling process following ASD treatment.

8.4.1 Cardiac magnetic resonance assessment of ventricular kinetic energy in children with atrial septal defects

Using advanced, non-invasive CMR imaging, KE was successfully measured in children with ASD before, and in the same patients six to twelve months after ASD closure. These results were compared to matched controls with left and right ventricular KE, analysed by peak systolic and peak diastolic levels to represent respective phases of the cardiac cycle. The anticipated number of study participants were recruited for this study and KE could be assessed for the first time in children

with ASD using previously described CMR methods [91]. To account for possible age- and sex-related changes in KE, ASD cases were matched to paediatric controls [97]. Peak systolic left ventricular KE levels in ASD cases increased after defect closure on follow-up and this could be attributed to increases in LV volumes by indexing these KE findings for body-size-adjusted LV volumes and LV stroke volumes. On the other hand, peak diastolic left ventricular KE did not change significantly after ASD closure, even though end-diastolic left ventricular volumes increased significantly. As the product of KE is related to changes in the blood volume and its intrinsic velocity and the LV volumes increased in diastole, the blood pool's velocity in diastole must have decreased to account for this observation. Slightly lower heart rates were observed in cases after ASD closure. Additionally, normalized pulmonary venous blood amounts returning to the left atrium would have aided LV inflow during slightly longer diastolic phases and could explain the observed diastolic peak KE findings.

How increases of systolic peak KE levels within the left ventricle in these children may have influenced the overall heart function remains challenging to predict. The observed findings can be largely explained by volumetric changes of the left ventricle and the described physiological LV adaptations after ASD closure. In the studied cohort, all children had normal left ventricular systolic function, as assessed by EF. An improvement to CI was noted after ASD treatment, which suggests a functional benefit after ASD closure in the young. In fact, improvements to left ventricular diastolic filling and overall left ventricular functional performance have been described in young ASD patients following treatment [198, 200].

Right ventricular assessment of peak systolic and diastolic KE levels showed a marked decrease in measurements following ASD closure. With successful ASD treatment, the related volume loading conditions of the right ventricle ceased and systolic and diastolic RV volumes decreased on follow-up. These RV volume changes were relatively large and influenced measured RV KE levels, as shown by indexing these KE findings for body-size-adjusted RV volumes as well as RV stroke volumes.

Changes to RV KE levels have previously been studied in children with pulmonary regurgitation after operated Fallot's tetralogy, although the pathophysiology leading to alteration in KE would be slightly different to ASD cases. Fallot patients commonly develop enlarged right ventricles over time due to free regurgitation across the pulmonary outflow tract after so called transannular patch surgery, compared to RV volume loading due to atrial shunt flows in ASD patients. The described postoperative volume loading of the right ventricle, with more turbulent flow in the RV outflow tract, should influence KE levels in Fallot cases differently to the observed KE levels in ASD [100]. It remains challenging to draw any firm conclusions on the causative relationship between changes to RV KE levels and the overall heart function in operated Fallot's tetralogy or ASD patients with published data from smaller cohort studies suggesting such a possible link [111, 201]. Any associations between changes

to RV KE levels and RV function in children with ASD would require a clear cause and effect relationship, which cannot be proven by this study. However, the overall aim of the presented study was to explore ventricular KE levels in children with ASD before and after treatment, which has not been reported previously. The observed KE results are in line with expected remodelling of the right ventricle after ASD closure and further study into the pathophysiological mechanisms underpinning the observed KE levels would be necessary to elucidate a stronger causative relationship between KE and ventricular pumping function. This has, so far, only been assessed in adults with ASD using cardiopulmonary exercise testing and standard CMR without KE measurements [140, 142].

In summary, the observed increases to peak systolic LV KE following ASD closure in children were in line with increases of LV volumes. Improvements of CI were noted after ASD closure. Diastolic peak KE remained unchanged with previously published data suggesting improved diastolic filling of the left ventricle following ASD closure in children [198, 200]. Right ventricular KE levels decreased in the studied children following successful ASD closure, as would be expected after abolishment of the atrial shunt and volume reductions within the right ventricle. Published evidence suggests that the reductions of RV volumes in adults after ASD closure may lead to overall improved physical performance, with a prolonged RV remodelling processes evident during follow-up [140, 142]. Linking alterations of observed KE to standard measures of cardiac function, such as EF, remains challenging with a need for further research focusing on this relationship after this explorative study in children with ASD. Overall, findings of left and right ventricular KE in children with ASD were in line with expected pathophysiological adaptations of the heart and volumetric changes after ASD closure.

8.4.2 Blood-based interleukin-1 receptor-like 1 and cardiac kinetic energy levels in children with atrial septal defects

The blood levels of IL-1RL1 correlated reasonably well with systolic peak RV KE levels in children before and after ASD closure with Pearson's correlation before ASD treatment showing r=0.50 and afterwards r=0.55. This correlation was slightly higher than that observed between IL-1RL1 and RV stroke volumes as seen on standard CMR imaging in these paediatric ASD cases and described in Study III. It appears that the peak systolic RV KE relationship with IL-1RL1 blood levels follows the same pattern as that seen for RV stroke volumes.

No previous data exists that profiles this circulating biomarker in children with ASD and compares blood-based IL-1RL1 concentrations with KE findings. Published studies in adults with decompensated heart failure have shown elevated levels of IL-1RL1, which may even predict adverse cardiovascular outcomes [62, 202, 203]. The observed higher levels of IL-1RL1 in the studied children before ASD closure

reduced after treatment and levels were within ranges of those seen in matched controls. The alteration to pulmonary blood flow may explain elevated IL-1RL1 levels in children before ASD treatment, as soluble IL-1RL1 is produced within the pulmonary circulation [145]. In this context it is interesting to note, that the correlation between IL-1RL1 and systolic peak RV KE levels in this study may be related to possible IL-1RL1 production within the lungs before ASD treatment. The pulmonary origin of IL-1RL1 production could theoretically be assessed by sampling systemic venous blood, pulmonary arterial and pulmonary venous blood simultaneously from ASD cases before ASD closure, e.g. during cardiac catheterisation. Differences in levels from paired samples would indicate the part of the circulation contributing more significantly to soluble IL-1RL1 levels than others, provided there would be a large enough difference of biomarker concentrations. Although Study IV was not designed to analyse this, it nevertheless showed some promising, previously unknown findings of IL-1RL1 in children with ASD. The current study achieved its goal of comparing this blood-based biomarker to advanced non-invasive CMR imaging findings of KE.

8.4.3 Limitations

This was a single centre study encompassing a small case-control cohort of children. Even though we aimed to match cases and controls for age, sex, and body size, one should bare this in mind when trying to draw more generalized conclusions from the presented results. There is currently no standardized paediatric reporting guideline on cardiac KE findings, making direct comparisons with other published findings challenging. Although there is emerging evidence about the clinical usefulness of IL-1RL1 in adult patients with decompensated heart failure, there is still a paucity of scientific data to causally link IL-1RL1 concentrations to pathophysiological changes within the heart and pulmonary circulation, especially in children with CHD [145, 199, 204, 205]. Even though this study aimed to highlight the relatively close relationship between KE levels within the right ventricle in systole and IL-1RL1 blood concentrations in children with ASD before and after treatment, these results should be interpreted with caution in the absence of data showing clear causality between blood and cardiac imaging findings.

9 Conclusion

This research used an observational, case-control study approach to show that screening for CHD in newborns using minimal blood amounts from DBS samples is potentially feasible. Throughout the project, novel DBS assays for cardiovascular biomarkers were established and quantification of NT-proBNP and IL-1RL1 levels in newborns could differentiate the most severe types of CHD from controls with high test accuracy.

The new DBS tests could even diagnose most critical CHD cases, which had previously been unrecognized in the newborn period. This included cases of evolving coarctation of the aorta in babies discharged from postnatal maternity services without their diagnoses being suspected.

When studying a group of clinically asymptomatic newborns, who were diagnosed with ASD at a later stage during childhood, higher IL-1RL1 levels on DBS samples were seen compared to controls. IL-1RL1 analyses could help to identify most ASD cases in newborns in the studied setting.

To further evaluate and link blood-based biomarkers to advanced non-invasive cardiac imaging using CMR, a CHD model of right heart volume loading was chosen to assess children with ASD before and after treatment. Using CMR assessment in children with ASD in comparison to matched paediatric controls, studies showed that intracardiac KE in children with ASD is altered and that KE changes after ASD treatment, in line with RV reductions of volumes during the cardiac remodelling process.

Measurements of KE also showed increases to systolic LV KE levels after ASD treatment in line with slight increases to LV volumes and this may have positive implications for longer-term outcomes in these young patients.

The combination of blood-based biomarker analysis for IL-1RL1 and CMR elucidated the association between the blood-based biomarker's decreasing levels after ASD treatment and the observed reductions of RV volumes on CMR.

When comparing peak systolic KE levels of the right ventricle in children with ASD before and after treatment and comparing this to IL-1RL1 levels in the blood, a similar correlation was seen, strengthening this link between RV volumetric changes as well as systolic KE levels of the right ventricle and blood-based biomarker concentrations of IL-1RL1.

The concordant correlation between decreasing IL-1RL1 blood levels and reduction of RV volumes and reductions of RV KE after ASD closure towards normal values, points towards a clinically important relationship and highlights the need for further study of IL-1RL1 and KE to improve our understanding of the pathophysiological mechanisms underpinning the complex cardiac remodelling in children with ASD.

10 Future perspective

Firstly, this research provides new insights into the early stages of CHD in children by elucidating the role of the studied blood-based biomarkers in children with CHD. Because the presented findings illustrate that DBS analyses of circulating cardiovascular biomarkers, such as NT-proBNP and IL-1RL1, is feasible using minimal blood amounts in newborns with various types of CHD, future studies should explore this method's usefulness in a prospective study setting to improve the early postnatal detection of CHD. This could be facilitated by novel, point-of-care testing after birth or by additional centralized testing through existing newborn screening programs, which have been implemented in more than one hundred countries globally. In settings with emerging paediatric cardiac services, identifying newborns with CHD by so called Guthrie card testing could represent an efficient postnatal screening process, albeit with its own challenges of reaching out to affected families in a timely fashion to prevent sudden cardiac collapse in those suffering from critical CHD, in which the circulation is dependent on patency of the arterial duct after birth.

Secondly, even in advanced healthcare settings with comprehensive foetal and neonatal cardiac screening programs, the early postnatal detection of CHD is currently incomplete and would benefit from prospective evaluation of the studied blood-based biomarkers to see how additional biomarker screening, as outlined in this research, can aid the early diagnoses of suspected or unsuspected CHD in newborns.

Thirdly, this research has shown that children with ASD, representing one of the most common types of CHD, may benefit from DBS analysis of IL-1RL1 in the newborn period, as this may recognize asymptomatic cases with reasonable test accuracy. This warrants further evaluation in the foreseeable future to see whether early diagnosis in ASD can benefit clinical management and treatment planning.

Fourthly, the observed associations between IL-1RL1 levels in children with ASD and their corresponding CMR findings, including measurements of ventricular KE, should enable future research initiatives to better link this blood-based biomarker to cardiac changes revealed by magnetic resonance imaging. Because this research focused on ASD as a CHD model of right ventricular volume loading with increased pulmonary blood flow, translation of its findings may aid research into more complex CHD lesions, such as Fallot's tetralogy or single ventricles with

hypoplastic left heart syndrome. Following ASD closure, the right ventricular volume-loading was largely reversible with concomitant normalisation of Qp:Qs. In comparison to more complex types of CHD, interpretations of related cardiac findings are less likely to be influenced by additional pathophysiological factors, such as valvular lesions or ventricular dysfunction. Strengthening the link between circulating biomarkers and advanced non-invasive cardiac imaging, would need to be addressed in future studies that should be designed to establish clear causality between blood-based biomarkers and cardiac imaging findings. Additional studies should also answer the question, how such blood-based biomarkers – together with novel cardiac imaging measures such as KE levels – may represent a surrogate marker for cardiac remodelling in the paediatric and adult setting of CHD, and how this may impact risk stratification for patients as well as long-term treatment outcomes in the clinical context.

Lastly, it is my sincere hope to have inspired others to embark on the windy road of medical research within the field of paediatric cardiology by describing the fascinating and sometimes surprising new discoveries made during this research project. The dedicated, enthusiastic children and their supportive families, who voluntarily took part in this research, and who are heavily dependent on our help, should be reason enough to encourage such endeavours. One should remember that it can also be real fun to work with these children, who shared some laughs and comical moments with the research team, even when it meant to invest some extra and precious time of theirs for the purpose of this research. In the end, without further research initiatives, medicine would be stuck in its status quo forever. So, from the bottom of my heart, I urge the medical reader to make the future brighter for all children who, without any fault of their own, suffer from heart disease. They are certainly worth all the research effort you can image!

11 Populärvetenskaplig sammanfattning (Popular science summary in Swedish)

Forskningsprojektets design baserades på fall-kontrollstudier hos barn för att visa att screening hos nyfödda med medfödda hjärtfel medels blodbaserade biomarkörer är möjlig genom användnnig av minimala blodmängder från torkade blodfläcksprover. Under projektet förlopp etablerades nya blood analyser för kardiovaskulära biomarkörer. Torkade blodfläcksprover hos friska nyfödda och de med allvarliga, medfödda hjärtfel användes för mätning av nivåer av så kallad amino-terminal prohormon av hjärnnatriuretisk peptid (NT-proBNP) samt löslig interleukin-1 receptor-likande 1 peptid (IL-1RL1). Blodbaserade biomarkörer kunde skilja de allvarligaste typerna av medfödda hjärtfel med hög tillförlitlighet från friska kontroller. De nya blodtesterna kunde till och med diagnostisera de flesta fallen av kritiska medfödda hjärtfel, vilka tidigare inte upptäckts i nyföddhetsperioden och som hade skrivits ut från mödravården utan att deras diagnoser misstänktes.

Hos en grupp av asymptomatiska nyfödda, som diagnostiserades med förmaseptumdefekter i ett senare skede under barndomen, såg man högre IL-1RL1-nivåer jämfört med kontrollpersoner och IL-1RL1-analys skulle kunna hjälpa till att identifiera de flesta ASD i framtiden. Med hjälp av avancerad magnetkamerabedömning av hjärtat hos barn med förmaksseptumdefekter före och efter behandling, kunde man demonstrera att kinetisk energi inuti i hjärtat förändras hos barn efter behandling. Nivåer av kinetisk energi ändrade sig i linje med volymreduktioner i vänster och höger kammare. Kombinationen av blodbaserad biomarköranalys för IL-1RL1 och avancerade magnetkameraundersökningar visade ett samband mellan de cirkulerande biomarkörerna och de observerade förändringar av högersidiga kammarvolymer och kinetik energinivåer. Korrelationen mellan minskande IL-1RL1-blodnivåer och minskning av höger kammarvolymer samt kinetisk energi i högerkammare efter stängning av förmakseptumdefekter, signaliserar behovet av ytterligare studier för att förbättra vår förståelse av de patofysiologiska mekanismer som ligger bakom detta.

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Reflecting a bit longer on the years spent researching the current topics, there is a terrible risk that I will miss out to thank someone important and thereby inadvertently causing offence. Please forgive me in this case and accept my warmest thanks to you as my welcomed companion along this journey. What a fantastic road trip it has been!

As I am only human with my own flaws and faults, I may have forgotten to mention a part of my research findings or simply had to choose not to write about it as there always seems to be insufficient space on these pages, or too little time to remember everything. Of course, I take full responsibility for my failings.

One thing has become clearer to me during this work and that is, how messy medicine can be when it comes to scientific research. There are plenty of things to consider and the little variations of human life are not only important to consider, they also make this an adventurous endeavour, which I cherished. I would like to explicitly thank the inspiring children and their enthusiastic and wonderful families, who participated in this research. We could not make any progress in medicine without research and I will always remember the challenges life has thrown at you, who are born with congenital heart disease and who volunteered to get involved in

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Thinking long and hard about this page, that almost everyone seems to read in the hope of catching a glimpse of, perhaps, themselves contributing to the tremendous effort involved in this thesis, I have chosen not to reel off all your names here because you will know in your hearts how much you mean to me. I will always treasure your advice, companionship, kindness, compassion, wisdom, friendship, and love. Instead, to show a little bit of my appreciation for all of you, I would like to share my favourite waffle recipe here with you, because waffles are usually heart-shaped and will make you happy: Take 250 grams of soft, molten butter; 250 grams caster sugar; five eggs and mix with a blender until smooth. Add 500 grams flour; one scooped tea spoon vanilla sugar; two scooped tea spoons backing soda; 500 ml milk and blend again with a mixer. This will make for about 20 waffles. Enjoy the feast!

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15 Figures

15.1 Study I

Figure 1

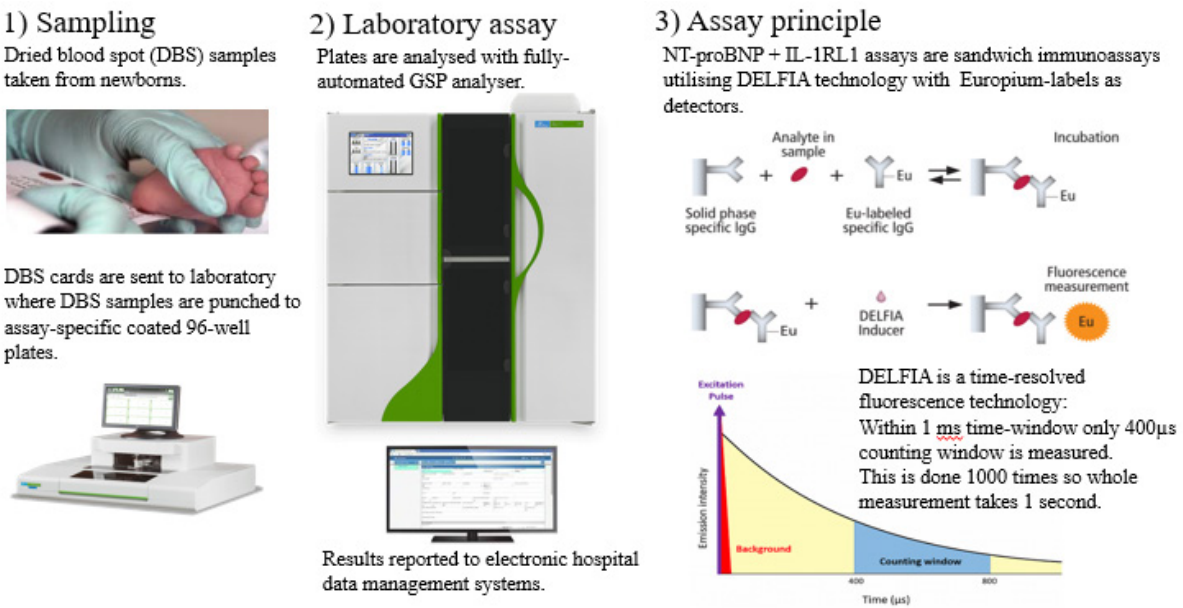
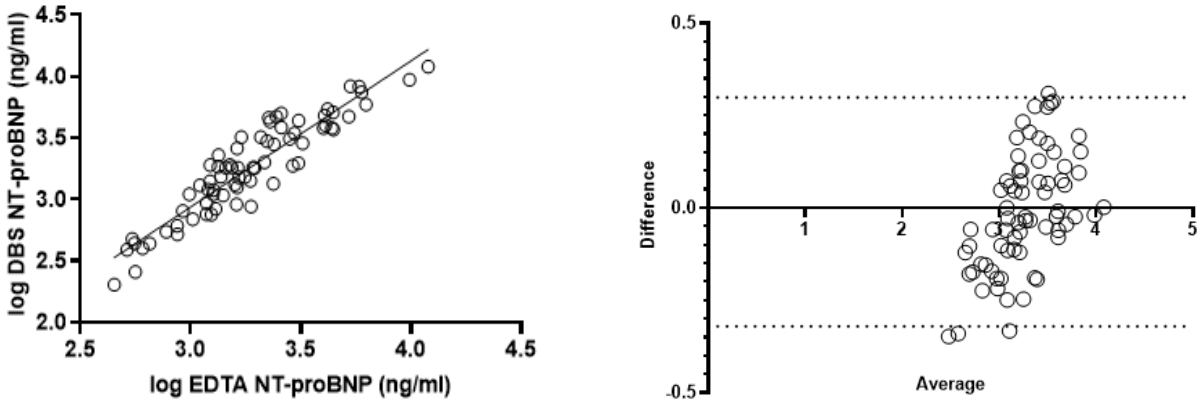


Illustration of dried blood spot analysis using fully-automated assays in newborns. Reproduced with kind permission from Mikko Sairanen, research & development division, Revvity, Turku (Finland). Abbreviations in illustration: DBS = dried blood spot, DELFIA = dissociation-enhanced Lanthanide fluorescent immunoassay, GSP = genetic screening processor, IL-1RL1 = interleukin-1 receptor-like 1, NT-proBNP = amino-terminal prohormone of brain natriuretic peptide.

Figure 2:

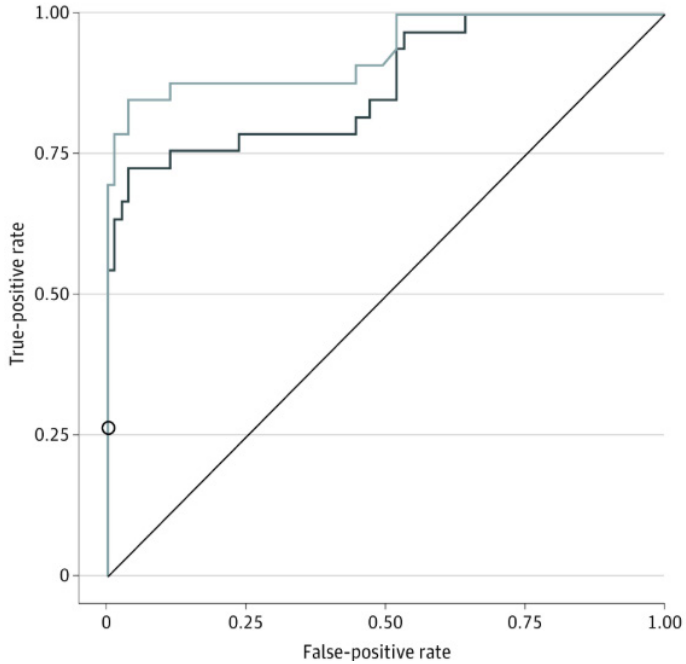


Test comparison of log-transformed NT-proBNP data in newborn controls: EDTA venous blood versus dried blood spot (DBS) assay.

Left panel: Log-transformed NT-proBNP data in controls showing Pearson correlation r=0.93.

Right panel: Log-transformed NT-proBNP Bland-Altman data in controls: bias/SD: -0.02/0.16; LoA: -0.32 – 0.30.

Figure 3:



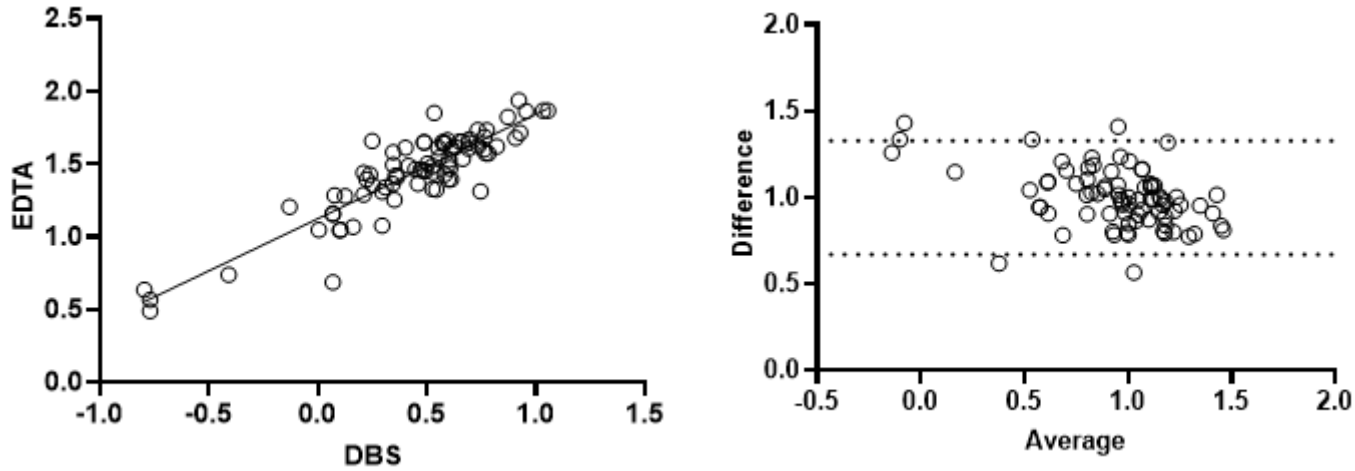
ROC curve analyses of dried blood spot NT-proBNP assay to predict cases of congenital heart disease.

Black curve represents dried blood spot NT-proBNP analysis with cut-off at 12 000 ng/ml: AUC 0.87 (SE: 0.04; 95%CI: 0.79-0.95; asymptotic $p<0.05$).

Grey curve represents pulse oximetry screening results in combination with dried blood spot NT-proBNP analysis: AUC 0.93 (SE: 0.03; 95%CI: 0.87-0.99; asymptotic $p<0.05$).

15.2 Study II

Figure 4:

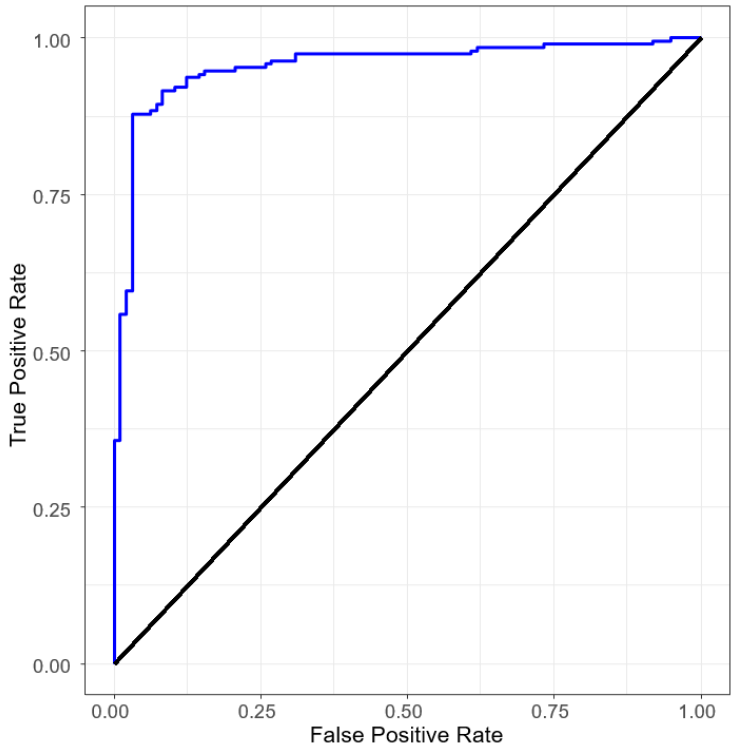


Interleukin-1 receptor-like 1 (IL-1RL1) test comparison in newborn controls using EDTA venous blood and dried blood spot (DBS) assays.

Left panel: Log-transformed IL-1RL1 data in controls showing Pearson's correlation (r=0.83).

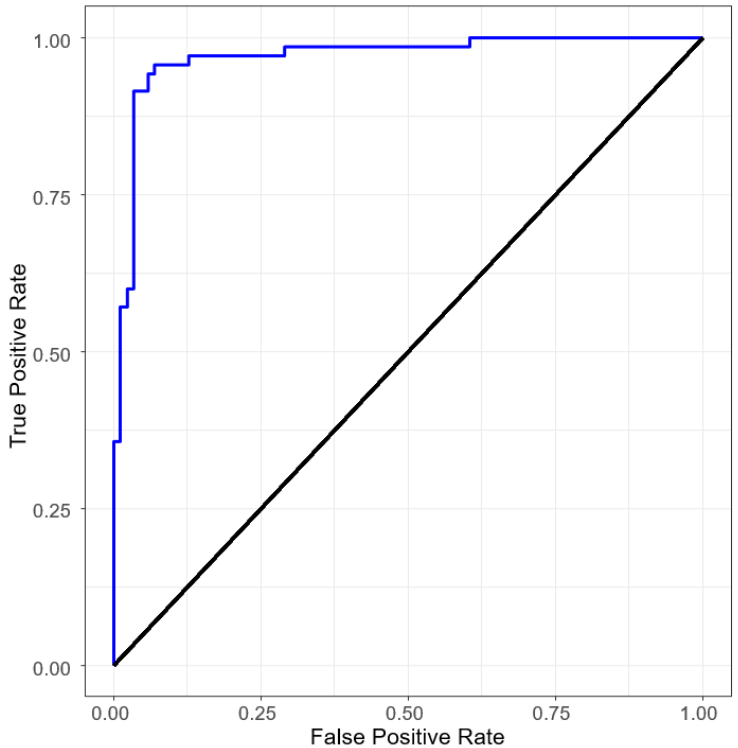
Right panel: Log-transformed IL-1RL1 Bland-Altman data in controls showing bias±SD: 1.00±0.17; LoA: 0.67 – 1.33.

Figure 5:



ROC analysis of combined NT-proBNP and IL-1RL1 dried blood spot test performance to detect any newborn with high-risk congenital heart disease (AUC: 0.95; 95%CI:0.93-0.98).

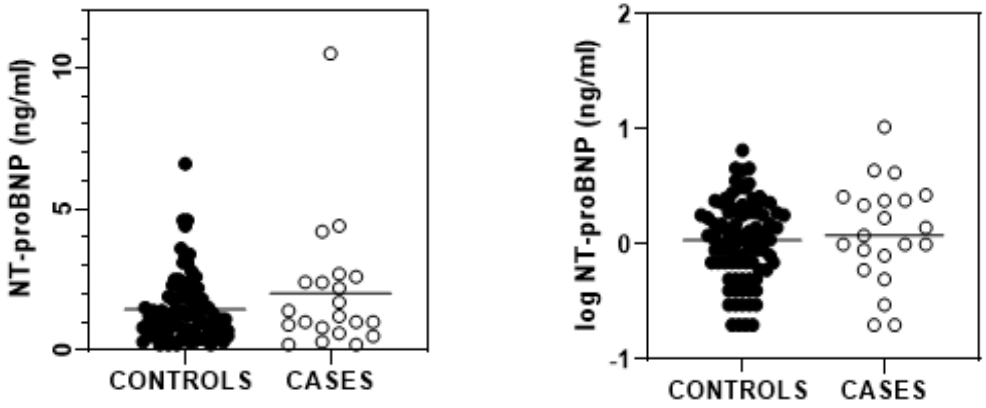
Figure 6:



ROC analysis of combined NT-proBNP and IL-1RL1 dried blood spot test performance to detect postnatally asymptomatic newborns with coarctation of the aorta (AUC: 0.97; 95%CI: 0.94-0.99).

15.3 Study III

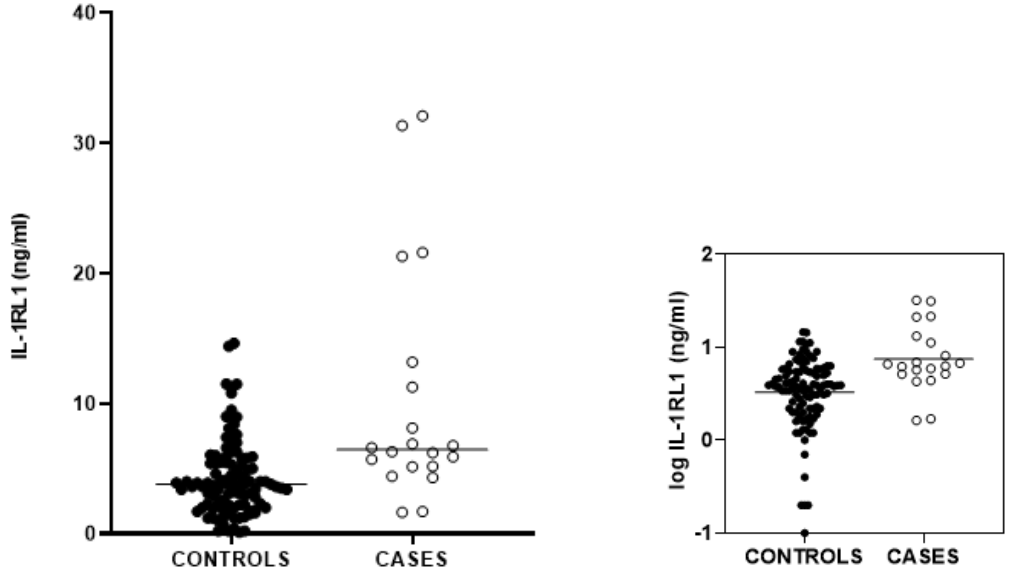
Figure 7:



Left panel: Dried blood spot (DBS) analysis of NT-proBNP in newborns with atrial septal defects (ASD) versus controls (p=0.10).

Right panel: Log-transformed dried blood spot (DBS) analysis of NT-proBNP in newborns with atrial septal defects (ASD) versus controls (p=0.48).

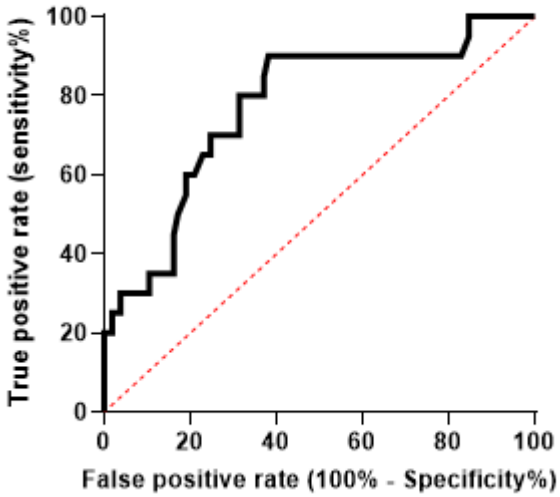
Figure 8:



Left panel: Dried blood spot (DBS) analysis of IL-1RL1 in newborns with atrial septal defects (ASD) versus controls ($p<0.01$).

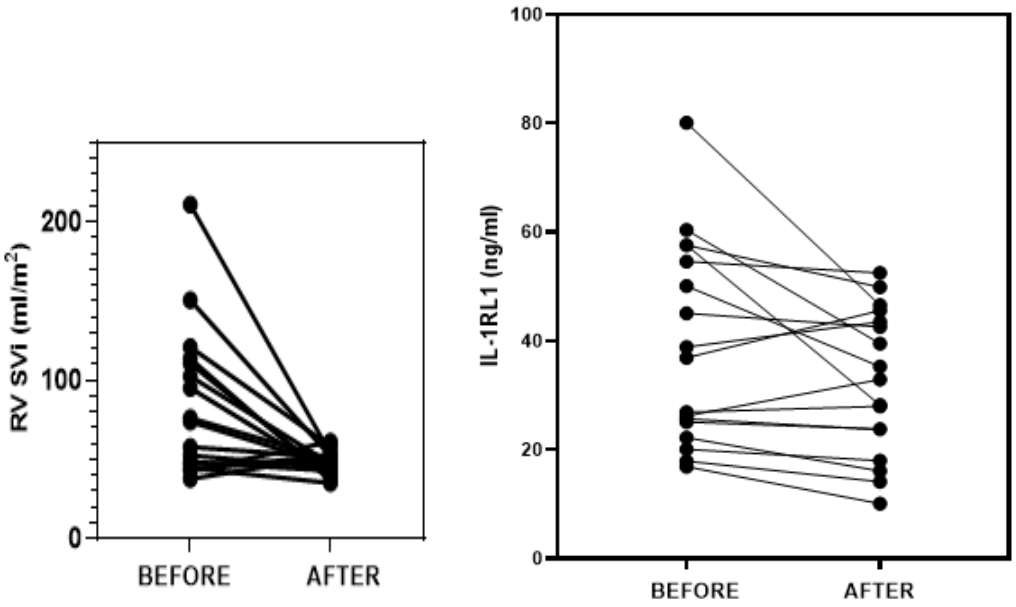
Right panel: Log-transformed dried blood spot (DBS) analysis of IL-1RL1 in newborns with atrial septal defects (ASD) versus controls ($p<0.01$).

Figure 9:



ROC curve: Dried blood spot analysis of IL-1RL1 in newborns to detect atrial septal defects (AUC: 0.77, 95CI: 0.66-0.89).

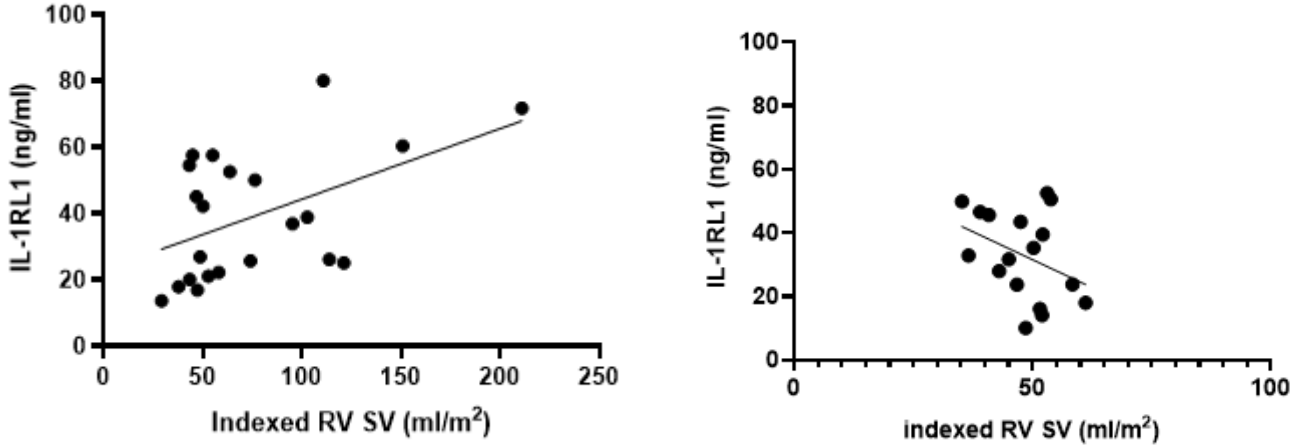
Figure 10:



Left panel: Blood concentrations of IL-1RL1 in children with atrial septal defects before and after treatment ($p=0.04$).

Right panel: Indexed right ventricular stroke volumes (RV SVi) in children with atrial septal defects before and after treatment ($p<0.01$).

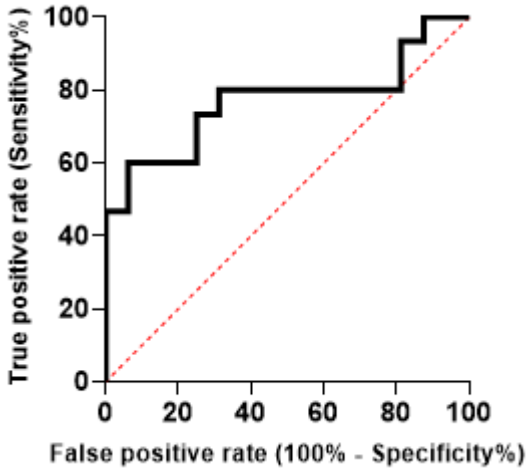
Figure 11:



Left panel: Linear correlation of IL-1RL1 blood concentrations and indexed right ventricular stroke volumes (RV SV) in children with atrial septal defects (AS) before treatment (r=0.49).

Right panel: Linear correlation of IL-1RL1 blood concentrations and indexed right ventricular stroke volumes (RV SV) in children with atrial septal defects (AS) after treatment (r=0.37).

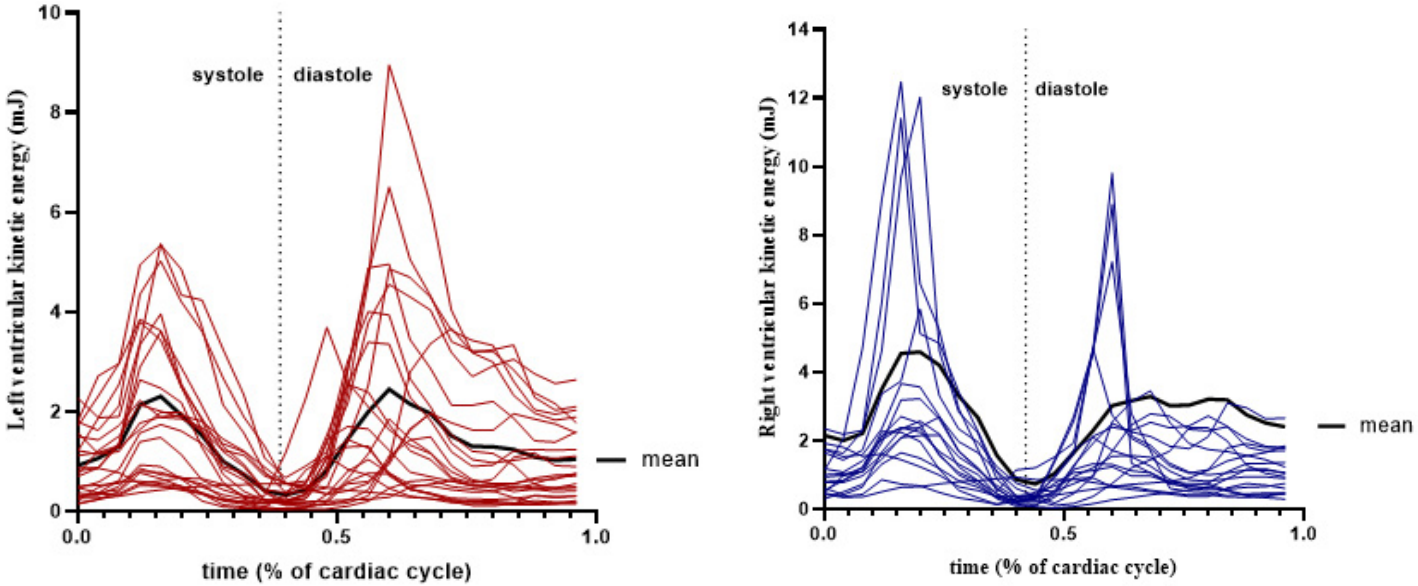
Figure 12:



ROC curve analysis of dried blood spot blood concentrations for IL-1RL1 in children with atrial septal defects (ASD) to predict indexed right ventricular stroke volumes >55 ml/m^2 before treatment in later childhood with AUC±SD=0.77±0.09 (95%CI: 0.59-0.95); asymptotic p<0.01.

15.4 Study IV

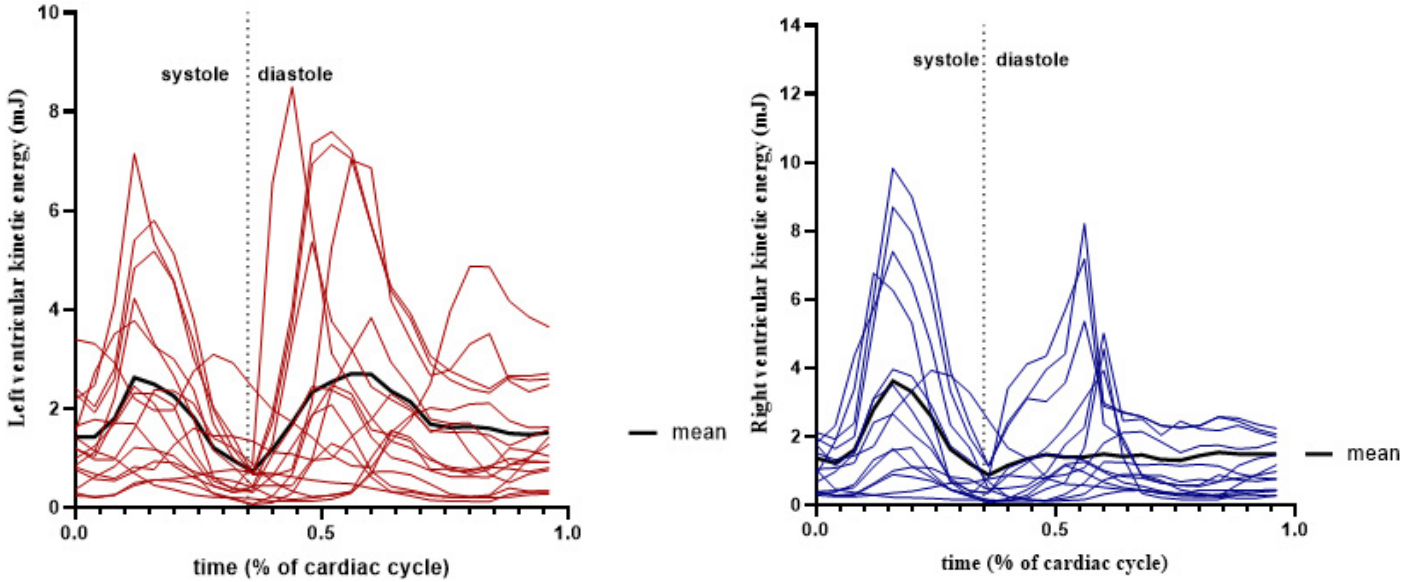
Figure 13:



Kinetic energy (KE) levels over the cardiac cycle in children with atrial septal defects *before* treatment.

Left panel: left ventricular KE, right panel: right ventricular KE.

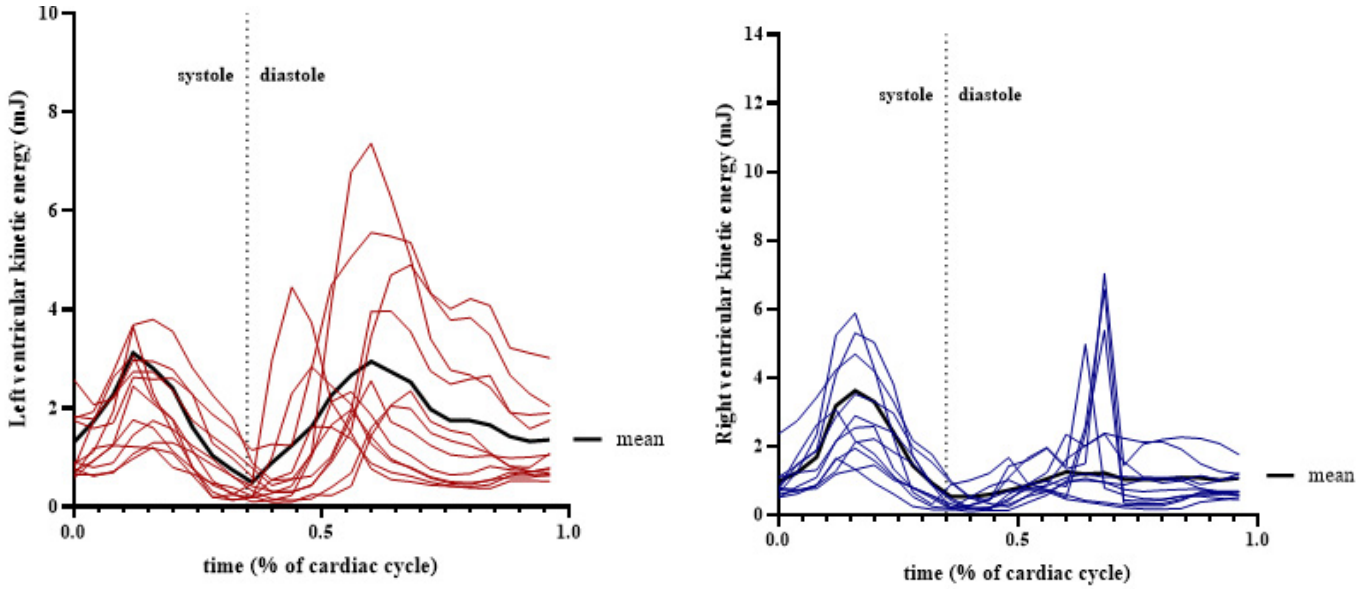
Figure 14:



Kinetic energy (KE) levels over the cardiac cycle in children with atrial septal defects *after* treatment.

Left panel: left ventricular KE, right panel: right ventricular KE.

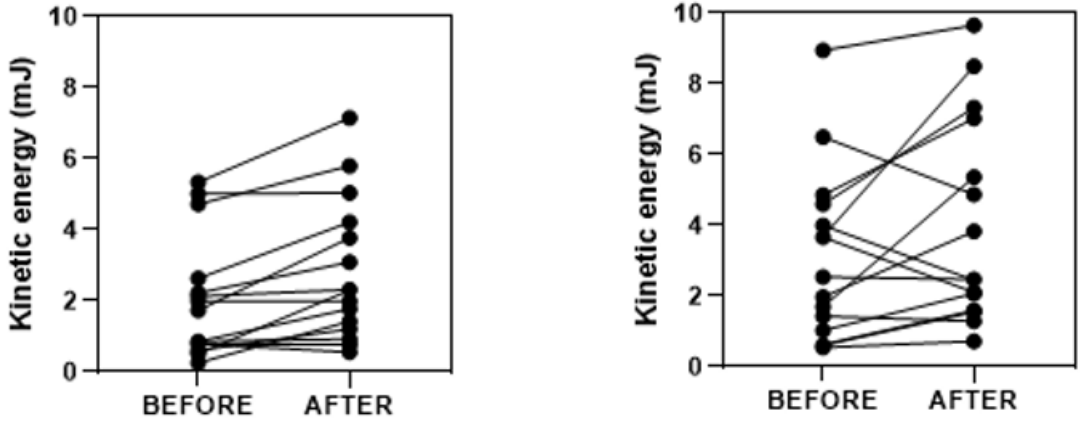
Figure 15:



Kinetic energy (KE) levels over the cardiac cycle in paediatric controls.

Left panel: left ventricular KE, right panel: right ventricular KE.

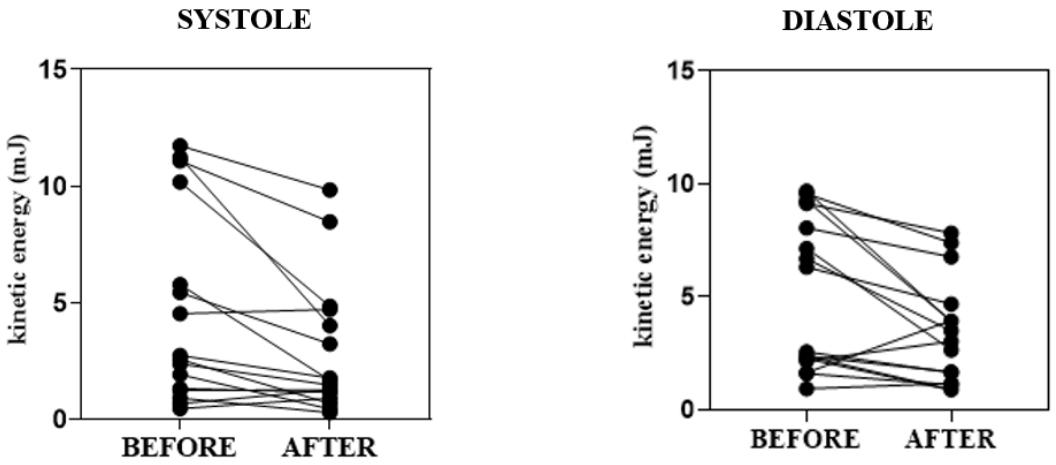
Figure 16:



Left panel: Left ventricular systolic peak kinetic levels in children with atrial septal defects (ASD) before and after defect closure ($p<0.01$).

Right panel: Left ventricular diastolic peak kinetic levels in children with atrial septal defects (ASD) before and after defect closure ($p=0.10$).

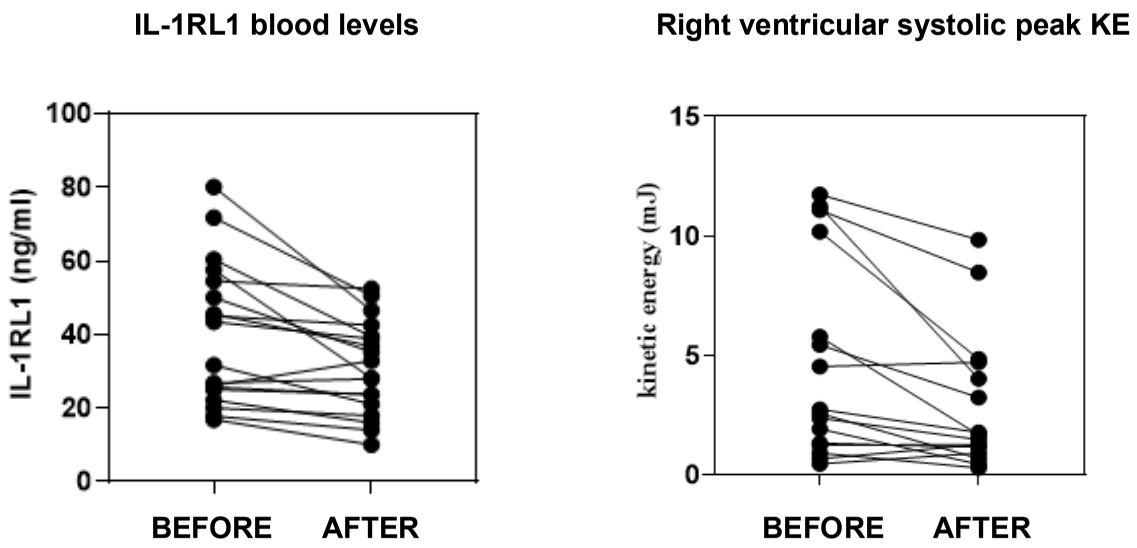
Figure 17:



Left panel: Right ventricular systolic peak kinetic levels in children with atrial septal defects (ASD) before and after defect closure ($p<0.01$).

Right panel: Right ventricular diastolic peak kinetic levels in children with atrial septal defects (ASD) before and after defect closure ($p<0.01$).

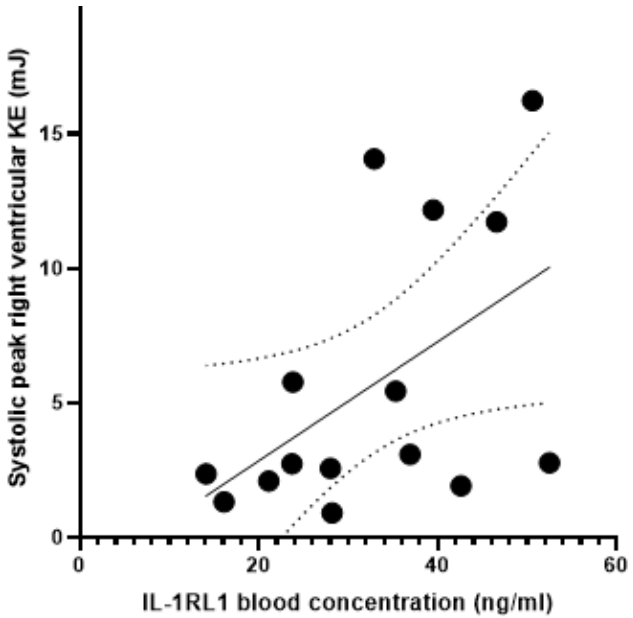
Figure 18:



Left panel: Measurement of IL-1RL1 (ng/ml) in children with ASD before and after treatment ($p<0.01$).

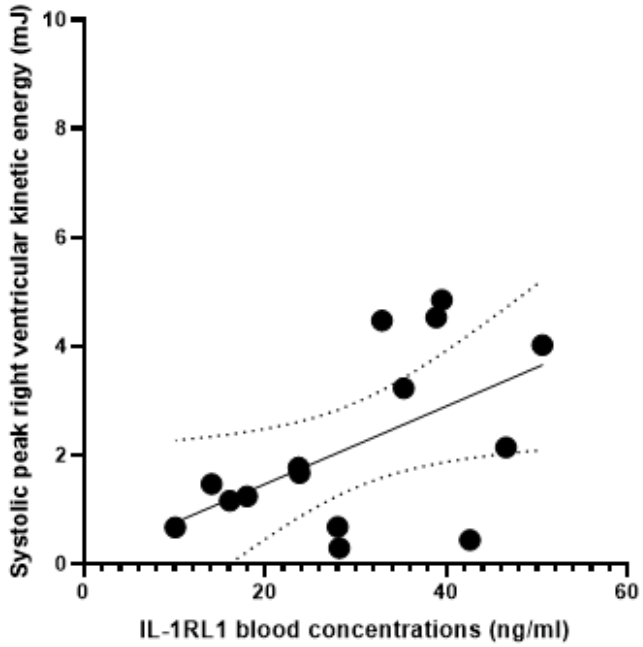
Right panel: Measurement of systolic peak right ventricular kinetic energy (mJ) in children with ASD before and after treatment ($p<0.01$).

Figure 19:



Linear correlation of magnetic resonance derived systolic peak right ventricular kinetic energy levels and blood concentrations of interleukin-1 receptor-like 1 (IL-1RL1) in children *before* closure of atrial septal defects (ASD); r=0.50.

Figure 20:



Linear correlation of magnetic resonance derived systolic peak right ventricular kinetic energy levels and blood concentrations of interleukin-1 receptor-like 1 (IL-1RL1) in children *after* closure of atrial septal defects (ASD); r=0.55.

16 Tables

Table 1: Overview of types of congenital heart disease (CHD) cases

Type of congenital heart disease	Number	Percentage
Coarctation of the aorta with/without hypoplasia of the transverse aortic arch	7	20.6
Atrioventricular septal defects, atrial & ventricular septal defects, patent arterial duct	7	20.6
Transposition of the great arteries with intact ventricular septum	2	5.9
Single ventricle lesions	2	5.9
Critical/severe aortic or pulmonary valve stenosis	5	20.8
Heterotaxy and other complex biventricular lesions	3	8.8
Tetralogy of Fallot and other conotruncal anomalies	6	17.6
Other types of CHD (anomalous left coronary artery from the pulmonary artery, hypertrophic obstructive cardiomyopathy)	2	5.9
All cases	34	100

Table 2: Overview of types of "high-risk" congenital heart disease (CHD) cases

Type of congenital heart disease	Number	Percentage
Coarctation of the aorta with/without hypoplasia of the transverse aortic arch	92	38.8
Atrioventricular septal defects, atrial & ventricular septal defects, patent arterial duct	37	15.6
Transposition of the great arteries with intact ventricular septum	34	12.7
Single ventricle lesions	26	11.0
Critical/severe aortic or pulmonary valve stenosis	18	7.6
Heterotaxy and other complex biventricular lesions	18	7.6
Tetralogy of Fallot	12	50.6
All cases	237	100

Table 3: Clinical characteristics of atrial septal defect (ASD) cases and controls

	ASD cases	Controls	
Sex (male)	7/23 (30.4%)	10/19 (52.6%)	p=0.10
Age (years)	9.08±4.07 (7.40-10.76)	10.58±2.84 (9.21-11.95)	p=0.18
Weight (kg)	36.44±22.43 (27.18-45.70)	40.0±15.0 (32.77-47.23)	p=0.55
Height (cm)	135.5±25.8 (124.8-146.1)	150.9±21.0 (139.2-162.5)	p=0.06
Body surface area (m^2)	1.15±0.44 (0.97-1.33)	1.28±0.32 (1.13-1.43)	p=0.28
Follow-up (months)	7.68±1.61 (6.90-8.46)	N/A	N/A

Table 4: Interleukin-1 receptor-like 1 in children with atrial septal defects versus controls

median [IQR]	**Newborn ASD cases**	**Newborn Controls**	**Paediatric ASD cases before treatment**	**Paediatric ASD cases after treatment**	**Paediatric Controls**	**Statistical significance**
DBS assay IL-1RL1 (ng/ml)	**6.45*** [5.148-12.70]	**3.35*** [1.828-4.608]	N/A	N/A	N/A	***p<0.01**
EDTA assay IL-1RL1 (ng/ml)	N/A	*30.56*** [21.12-44.31]	**38.90^** [22.20-57.60]	**34.10^** [23.73-46.35]	*34.75*** [24.25-55.68]	**^p=0.04 **p=0.16**

Abbreviations in table: ASD = atrial septal defect, DBS = dried blood spot, EDTA = ethylenediaminetetraacetic acid, IL-1RL1 = interleukin-1 receptor-like 1, IQR = interquartile range.

Table 5: Cut-offs for dried blood spot IL-1RL1 analyses in newborns to detect atrial septal defects

IL-1RL1 cut-off (ng/ml)	True positive rate in % (Sensitivity)	95% CI of Sensitivity	True negative rate in % (Specificity)	95% CI of Specificity	False positive rate in % (1-Specificity)	Likelihood ratio
1.685	95	76.39-99.74	15.24	9.603-23.33	84.8	1.121
4.280	90	69.90-98.22	61.9	52.35-70.62	38.1	2.363
4.350	85	63.96-94.76	62.86	53.31-71.49	37.1	2.288
5.045	80	58.40-91.93	68.57	59.17-76.66	31.4	2.545
5.155	75	53.13-88.81	68.57	59.17-76.66	31.4	2.386
5.640	70	48.10-85.45	75.24	66.19-82.51	24.8	2.827
5.885	65	43.29-81-88	77.14	68.24-84.13	21.9	2.844
6.13	60	38.66-78.12	80.95	72.4-87.32	19.0	3.150
6.255	55	34.21-74.18	80.95	72.4-87.32	19.0	2.888
6.450	50	29.93-70.07	82.86	74.52-88.87	17.1	2.917
6.685	45	25.82-65.79	83.81	75.59-89.64	16.2	2.779
6.830	40	21.88-61.34	83.81	75.59-89.64	16.2	2.471
8.090	35	18.12-56.71	89.52	82.21-94.05	10.5	3.341
11.215	30	14.55-51.90	96.19	90.61-98.51	3.8	7.875

Table 6: Cardiac magnetic resonance findings in children with atrial septal defects before and after treatments versus controls

	Paediatric Controls	ASD cases before treatment	ASD cases after treatment	Statistical significance
Atrial shunt ratio (Qp:Qs)	0.99±0.09 (0.94-1.04)	**2.07±0.67*** (1.78-2.36)	**1.01±0.07*** (0.98-1.05)	***p<0.01**
HR at CMR (bpm)	**75.4±9.5**** (70.7-80.1)	**85.7±12.9**** (79.9-91.4)	81.3±17.8 (72.7-89.9)	****p<0.01**
LV EDVi (ml/m^2)	84.8±12.8 (77.4-92.2)	**65.5±10.3***** (60.9-70.1)	**78.3±18.3***** (68.9-87.8)	*****p<0.01**
LV ESVi (ml/m^2)	35.1±6.3 (31.5-38.7)	**26.3±5.4****** (23.9-28.7)	**34.4±12.6****** (27.9-40.9)	******p<0.01**
LV SVi (ml/m^2)	**49.7±8.6^** (44.8-54.7)	**39.2±6.6^** (36.3-42.1)	43.9±11.6 (38.0-49.9)	**^p<0.01**
LV EF (%)	58.6±4.6 (55.9-61.2)	**58.8±5.4** (56.5-61.1)	59.8±6.1 (56.7-63.0)	N/S
CI (l/min/m^2)	**3.76±0.56^^ #** (3.47-4.05)	**3.22±0.59 #** (2.96-3.48)	**3.77±0.81^^** (3.38-4.16)	**^^p<0.01 #p=0.02**
RV EDVi (ml/m^2)	90.0±15.6 (81.4-98.6)	**134.5±38.0##** (117.6-151.3)	**96.0±21.6##** (84.9-107.1)	**##p<0.01**
RV ESVi (ml/m^2)	39.9±9.5 (34.6-45.2)	**58.0±20.8###** (48.7-67.2)	**48.7±17.1###** (39.9-57.5)	**###p<0.01**
RV SVi (ml/m^2)	50.1±8.4 (45.5-54.8)	**76.7±19.8§** (67.9-85.5)	**47.1±12.7§** (40.6-53.7)	**§p<0.01**
RV EF (%)	55.9±5.2 (53.0-58.8)	**57.4±5.1§§** (55.2-59.7)	**51.2±12.8§§** (44.6-57.7)	**§§p=0.04**

Abbreviations in table: ASD = atrial septal defect, CI = cardiac index, CMR = cardiac magnetic resonance, EDVi = indexed end-diastolic volume, EF = ejection fraction, ESVi = indexed end-systolic volume, HR = heart rate, LV = left ventricular, N/S = not statistically significant, Qp = pulmonary flow, Qs = systemic flow, RV = right ventricular, SVi = indexed stroke volume.

Table 7: Clinical characteristics of children with atrial septal defects (ASD) versus controls for kinetic energy (KE) assessment

	ASD before treatment (n=20)	ASD after treatment (n=18)	ASD before vs. after treatment	Controls (n=12)	Controls vs. ASD before treatment	Controls vs. ASD after treatment
Sex male/total	8/20 (40.0%)	8/18 (44.4%)	N/A	7/12 (58.3%)	p=0.10	p=0.15
Age at enrolment (years)	9.1±4.1 (7.4-10.8)	N/A	N/A	10.6±2.8 (9.2-12.0)	p=0.18	N/A
BSA (m^2)	1.15±0.44 (0.97-1.33)	1.30±0.44 (1.10-1.51)	**p<0.01**	1.28±0.32 (1.23-1.43)	p=0.28	p=0.86
Heart rate (bpm)	86±13 (80-91)	81±18 (73-90)	p=0.23	75±10 (71-80)	**p<0.01**	p=0.22

Abbreviations in table: ASD = atrial septal defect, bpm = beats per minute, BSA = body surface area.

Table 8: Kinetic energy in atrial septal defects before and after treatment versus. controls

	ASD before treatment (n=20)	ASD after treatment (n=18)	ASD before vs. after treatment	Controls (n=12)	Controls vs. ASD before treatment	Controls vs. ASD after treatment
Left ventricle						
Systolic peak KE (mJ)	2.2±1.6 (1.5±2.8)	2.8±1.9 (1.8-3.8)	**p<0.01**	3.3±3.0 (1.5-5.1)	p=0.15	p=0.61
Diastolic peak KE (mJ)	2.8±2.1 (1.9-3.7)	3.8±2.9 (2.3-5.4)	p=0.10	3.4±2.8 (2.5-5.5)	p=0.22	p=0.90
Right ventricle:						
Systolic peak KE (mJ)	5.5±5.3 (3.1-7.8)	3.6±2.6 (2.2-4.9)	**p<0.01**	3.6±2.1 (2.3-4.9)	p=0.23	p=0.97
Diastolic peak KE (mJ)	4.5±4.6 (2.5-6.6)	2.2±1.6 (1.3-3.0)	**p<0.01**	1.7±0.8 (1.2-2.2)	**p=0.04**	p=0.37

Abbreviations in table: ASD = atrial septal defect, KE = kinetic energy, LV = left ventricular, RV = right ventricular.