

ROADMAP TO SOLVING SINGLE VENTRICLE HEART DISEASE

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About Us

ABOUT ADDITIONAL VENTURES

Additional Ventures is a purpose-driven organization leveraging evidence-based research and deep subject matter expertise to make an outsized impact. Additional Ventures is comprised of multiple entities that make grants and/or investments in service of our mission to shape a healthier, more equitable world, including Additional Ventures Foundation, Additional Ventures LLC, and a donor advised fund of the Silicon Valley Community Foundation.

ABOUT ADDITIONAL VENTURES FOUNDATION

Additional Ventures Foundation is a nonprofit 501(c)(3) that aims to accelerate research progress and improve clinical care for individuals born with single ventricle heart defects so that they have a normal duration and quality of life. Although one in one hundred children are born with a congenital heart defect, there are limited options for those with the most complex forms, including single ventricle. For these individuals, there is no cure.

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Introduction

Single ventricle (SV) heart disease care is at a pivotal moment. Enormous progress has been observed over the past half century – rerouting pathways for circulation, radically improving survival. However, much more is needed.

It is time to envision the next phase of SV care, one that is rooted in a fundamental understanding of both etiology and risk, built to overcome the complications and comorbidities that disrupt patient’s quality and duration of life, and focused on providing alternative solutions that deliver a functional rather than palliative solution. Achieving such a lofty goal will require collaboration not just at the scientific or clinical level, but rather, scientists, clinicians, engineers, patients, families, and funders must align themselves along with a living roadmap to solve this disease.

After recognizing the need for such a roadmap, a group of diverse investigators, clinicians, funders, and regulators met regularly over multiple months with the primary focus of developing targeted research programs to overcome the most pressing challenges in the SV field for three specific focus areas:

- Understanding the underlying etiology of SV heart disease,
- Managing and overcoming complications and comorbidities associated with SV heart disease and the Fontan circulation, and
- Developing functional cures for SV patients, including those living with the Fontan circulation.

After much discussion and debate, each team presented targeted research programs at a virtual workshop to a larger audience. Each program addressed key knowledge gaps in the group’s focus area through specific plans that included both the broad and directed research avenues needed to solve these gaps, and defined the infrastructure, timescale, and capital required to implement the program. Finally, the findings were examined, synthesized, and restructured to incorporate the outputs of the workshop and preceding conversations and research. The result was the emergence of four key areas the field must address to understand and overcome single ventricle:

- I. Poor understanding of disease etiology, risk, and prevention;
- II. Limited focus on the underlying biology of outcomes;
- III. Suboptimal ability to address clinical sequelae in patients, today; and
- IV. Treatments are palliative, not curative.

From this analysis, the resulting roadmap was crafted, representing a harmonization of the individual teams’ efforts and outputs, and is intended to outline concrete recommendations to accelerate progress in a collaborative, team-driven approach over the next decade. The following recommendations are approved for release by the participants of the workshop, intended to be a launching point to serve as a living document that will be revisited and refined as scientific and clinical understanding evolves, and as progress is made.

Single Ventricle Heart Disease: An Overview

In human hearts, four distinct chambers form during development: two atria and two ventricles. However, in some individuals, one ventricle does not form properly and is either smaller, underdeveloped, or is missing a valve. Such conditions are referred to as single ventricle (SV) heart defects, which encompass a spectrum of diagnoses including, but not limited to:

- Hypoplastic Left Heart Syndrome
- Tricuspid Atresia
- Double Inlet Left Ventricle
- Double Outlet Right Ventricle
- Single Left Ventricle
- Pulmonary Atresia with Intact Ventricular Septum
- Unbalanced Atrioventricular Canal

Despite research efforts, little is known about the underlying etiology of SV heart disease, although mounting evidence suggests that the basis is multi-factorial and is comprised of genetic, epigenetic, and/or environmental contributions. Studies have demonstrated that SV is a genetically complex disease that is heterogeneous in both etiology and in presentation. Additionally, extra-genetic factors, such as environment and epigenetic patterning, are also emerging as important factors for disease risk, progression, and outcomes. However, beyond prenatal diagnosis from routine screening, nothing yet can be done to alter the course of disease *in utero* or inform outcomes.

Clinically, the path for such patients has changed dramatically, as fifty years ago, a diagnosis of SV was considered universally fatal. After a series of surgical innovations were introduced into practice, the survival and health outcomes for SV patients were drastically improved. In current practice, a child born with SV typically undergoes two or three, staged surgical procedures typically, beginning with a Norwood procedure or shunt, followed by the Glenn procedure, and finally, ending with the Fontan procedure. While the specific surgical path is dependent on the diagnosis and physiology of the child, the overall goal is to create a new parallel flow pattern that compensates for the dysfunctional ventricle, allowing all venous blood returning from the body to go directly to the lungs for oxygenation. As such, clinical innovation has rerouted a pathway for survival in these patients.

Indeed, the survival rate post-Fontan surgery is rising, with excellent transplantation-free outcomes in current surgical era (5-year survival of 95%, 10-year survival of 91% and an estimated 30-year survival of 85%). Yet, these survival milestones are undermined by significant comorbidities and complications in individuals that have undergone the Fontan procedure (known as having a “Fontan circulation”), leading to premature morbidity and mortality, and a poor quality of life.

Damaged ventricles are layered on top of intrinsic abnormalities in other organs that have an increased likelihood of failure, at least partly attributed to the altered hemodynamics of the cardiovascular system as a consequence of the Fontan circulation. These issues often worsen over time, reducing both the quality and duration of life – a condition sometimes referred to as “Fontan circulatory syndrome”.

Complications include, but are not limited to:

- Myocardial dysfunction
- Pulmonary vascular dysfunction
- Arrhythmias
- Bleeding and stroke
- AV valve regurgitation and dysfunction
- Protein-losing enteropathy
- Plastic bronchitis
- Lymphatic obstruction
- Liver disease
- Renal failure
- Neurodevelopmental defects
- Psycho-social challenges

While studies to understand the drivers of these complications are underway, they are limited in scope and scale, reducing the ability to draw relevant conclusions about the causes, treatments, and prevention strategies. To some degree, many of the current knowledge gaps are rooted in an incomplete understanding of the disease etiology and epidemiology, but the challenges are further compounded by a lack of clinical tools, including a suboptimal detection timeline, limited diagnostics, a lack of treatment options, and an insufficient interdisciplinary research lens. These solvable challenges preclude the robust and timely identification of risk factors, hinder the potential to stratify this complex population, and limit progress on improvements for disease management, treatment, and therapeutic development.

Currently, heart transplantation is the closest option to a functional cure; however, this procedure faces tremendous challenges that compromise overall quality and duration of life. A combination of extensive organ waiting list time, limited donor supply, immune complications, organ rejection, and mounting complications and comorbidities limit the feasibility of heart transplant as a curative approach. For example, availability of donor hearts is limited, causing donor-recipient matching to be highly selective. This selectivity is further exacerbated by the fact that many Fontan patients are referred too late and are thus ineligible for a transplant. While clinicians are working on ways to avoid transplant for SV patients, the ability to better prognosticate and identify an SV patient with impending failure before it is too late is another important avenue to explore. Therefore, both alternative methodologies and improvements to the referral process are critically important.

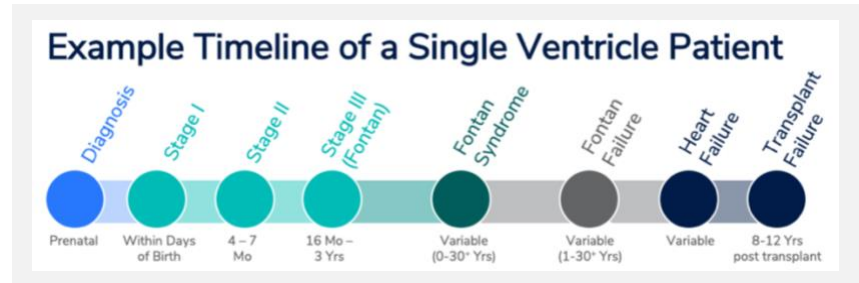


Figure 1. While not all SV patients follow the above trajectory, many follow a similar timeline. After diagnosis, patients experience three staged palliative surgeries that occur within the first several years of life to create a new systemic circulatory flow. However, patients often experience “Fontan Syndrome” which can compound and result in Fontan failure and ultimately, heart failure. If transplanted successfully, a subset will experience transplant failure over the next decade, though outcomes are variable. The order of the nodes in this timeline are not fixed; some patients may never reach Fontan failure, whereas other may receive a transplant before reaching Fontan.

In light of these critical issues, researchers are investigating a variety of novel approaches to functionally cure patients prior to palliation or after the Fontan procedure. To do so, researchers are exploring regenerative medicine solutions to heal or repair an injured heart, mechanical and biological devices to serve as a conduit for flow, and tissue engineering approaches to create *de novo* tissues, ventricles, and even entire organs. If researchers are successful, SV could become a curable disease where patients can expect a normal duration and quality of life. This will only be possible through advancements in multiple discrete fields and improved coordination of current and future research efforts.

Capitalizing on the momentum in the SV field and adjacent genomics, engineering, and regenerative medicine fields through strategic investment in discovery science, infrastructure, and research tools is essential for continued progress. Fundamental questions about the contributing molecular and cellular mechanisms, initiating factors and events, genetic risk, conduits for improved flow, *de novo* organ development, lymphatic and end-organ intervention, and much more remain.

This roadmap aims to define an approach to address the most pressing challenges in the SV field – challenges that are only overcome by supporting meaningful, multidisciplinary collaboration and driving intense focus to selected research themes and hasten discovery. We hope that this roadmap unifies research, clinical, and funding communities to accelerate progress and yields high-impact results.

Roadmap Development

To understand the most pressing challenges and barriers to progress within the single ventricle heart defect community, we interviewed over 100 key opinion leaders, conducted a review of the published literature, and attended relevant conferences. The leaders, literature, and conferences represent a broad diversity of disciplines (including scientific, engineering, clinical, and regulatory) and provided a holistic lens to understand both the scope of the problem and potential avenues of investment. In synthesizing our findings, we organized our exploration around three key areas (more information on each can be found in [Appendix 1-3](#)): i) understanding the underlying etiology of SV heart disease, ii) managing and overcoming complications and comorbidities associated with SV heart disease and the Fontan circulation, and iii) developing functional cures for SV patients, including those living with the Fontan circulation.

We then formed small working groups, one for each focus area, which were comprised of 12-13 investigators across disciplines, and tasked each with designing a holistic targeted research program to address key knowledge and clinical care gaps in their focus area. The programs yielded a broad overview of the projects necessary to close the identified gaps, including the experiments and experimental tools, the rationale behind the projects, and the timescale, and budget required to complete each component. Groups also demonstrated how their program was differentiated from existing efforts and the unique value that would be added to the field.

In the subsequent weeks, the team at Additional Ventures worked to further examine and refine the major focus areas and to consolidate the three individual targeted research programs into a cohesive, central plan. Interestingly, a fourth focus area emerged as fundamental to the goal of understanding and overcoming SV (Figure 2). Below are the resulting four focus areas:

- I. Poor understanding of disease etiology, risk, and prevention
- II. Limited focus on the underlying biology of outcomes
- III. Suboptimal ability to address clinical sequelae in patients, today
- IV. Treatments are palliative, not curative

In the following pages, we communicate the **four significant knowledge gaps** that are hindering progress within the SV field and provide a strategic roadmap that defines the broad and directed research avenues needed to solve these gaps. The comprehensive, multidisciplinary programmatic solutions are intended to align funder investments and highlight the infrastructure, timescale, and resources required to implement the program. We hope that such a roadmap will galvanize coordination of SV research and clinical care support globally.



Figure 2. Brief depiction of the workflow to generate the scientific and clinical roadmap for SV.

Overview of Critical Scientific Areas

Using a multidisciplinary lens and a systems-based approach, the key challenges were identified that hinder progress in improving and optimizing outcomes for patients with SV heart defects. As previously discussed, four areas emerged from this analysis, in which investment is required to move the needle:

I. POOR UNDERSTANDING OF DISEASE ETIOLOGY, RISK, AND PREVENTION

The genetic basis of SV heart disease is mostly unknown, with the molecular mechanisms underlying SV heart defects equally unexplored. Furthermore, there are no data on non-genetic factors impacting the etiology of SV heart defects, including the role of epigenetic, hemodynamic, and environmental (e.g. placental, maternal exercise) factors. Such a gap limits the development of predictive measures of disease, risk stratification tools, preventative non-therapeutic strategies, and therapeutic interventions to correct developmental abnormalities.

II. LIMITED FOCUS ON THE UNDERLYING BIOLOGY OF OUTCOMES

Due to major surgical advances, vast improvements in survival past the age of five have been observed. One caveat is that the majority of treated patients experience a number of complications and comorbidities that can severely impact quality and duration of life. The field is still early in understanding what drives the onset of each complication and comorbidity. Thus, a deeper exploration of the biological and environmental mechanisms of the correlates to outcomes are required to mitigate risk while also providing a personalized approach to each patient's journey.

III. SUBOPTIMAL ABILITY TO ADDRESS CLINICAL SEQUELAE

It is well appreciated that SV patients experience complications and comorbidities across a broad range of end-organ systems. Currently, no predictive measures (such as biomarkers) exist to map the trajectory of heart and other organ system function, nor are there preventative treatments to modify or mitigate outcomes. Furthermore, treatment options are limited, and novel interventions are underexplored. While efforts to understand and address the clinical sequelae are underway, they are limited in scope and scale, reducing the ability to draw relevant conclusions about potential treatment effectiveness and intervention.

IV. TREATMENTS ARE PALLIATIVE, NOT CURATIVE

The current standard of care of SV is palliative, not curative, as the passive flow caused by the Fontan procedure is thought to lead to long-term hemodynamic issues and palliation can fail over time. Additionally, other unknown issues may contribute to the high number of post-Fontan related deaths and heart failure cases leading to transplant. Unfortunately, the standard of care to treat a failing Fontan is often off-label, as clinicians primarily utilize adult-approved devices or a heart transplant; however, both techniques have challenges. As such, the field is shifting from a rescue focus to one of rebuilding the current paradigm, but this shift requires a concerted, multi-disciplinary effort guided by basic scientists, engineers, and clinicians, and supported by funders.

The challenges described above may seem insurmountable, with the science of today appearing deeply complex. Indeed, it is critical to recognize that no single scientist or lab can answer the multifaceted questions that exist; meaningful science and clinical care can no longer be siloed or performed in a vacuum. The overarching complexity is well demonstrated by the SV paradigm, a disease that requires not only a diverse set of expertise across the basic, translational, and engineering sciences, but also a breadth of knowledge across medical fields.

In the remaining sections, we highlight a series of recommendations that we believe can move the needle for SV understanding and care, which cross the boundaries of discipline and field, necessitating great minds across the scientific and funding communities to come together to tackle a common set of goals and coalesce as true partners to fund, manage, and support efforts moving forward.

Overview of Fieldwide Recommendations

From the working groups’ comprehensive targeted research programs and the subsequent analyses post-meeting, we developed the following scientific, clinical, and infrastructure recommendations for the entire SV field to promote aligned, collaborative investment.

Our recommendations are divided amongst the four specific areas of strategic investment (Figure 3), which are represented in each individual triangle. While lofty and difficult goals to achieve, we believe that collaborative, concerted investment in these areas will move us towards a future state that is rooted in a deep understanding of the etiology of disease, risk, and outcomes, results in the ability to address all complications and comorbidities, and realizes the introduction of true functional cures, not palliative options.

In the subsequent sections, we further define each area of strategic investment, and provide potential specific projects and programs, and with the scope and scale required to bring about marked progress. Where possible, we highlight avenues of investment that are currently “ready-to-go,” and those that may require the development of foundational tools or enabling technologies. Finally, we outline mechanisms of funding such endeavors, highlighting specific modalities. In providing these recommendations and the funding mechanisms, we aim to create a resource to the community that simplifies the path forward towards overcoming SV disease.

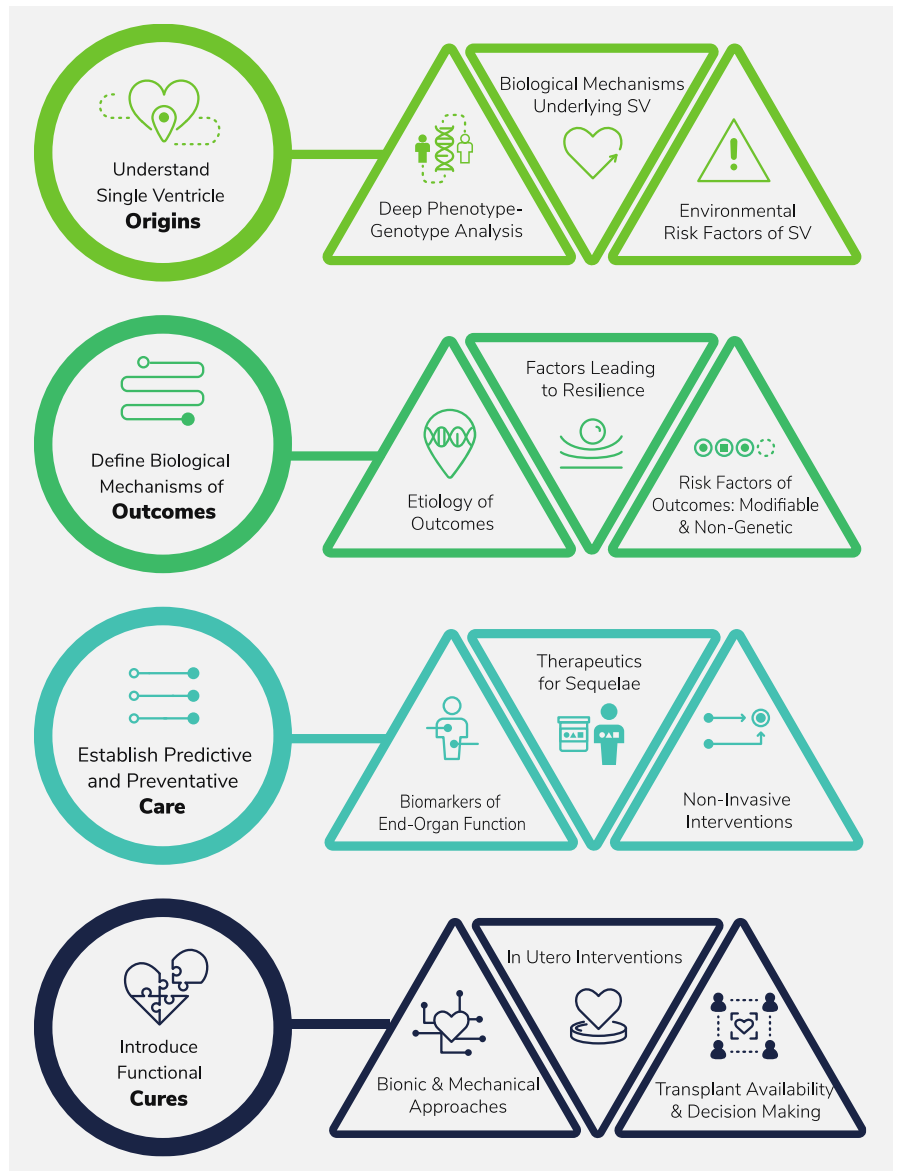
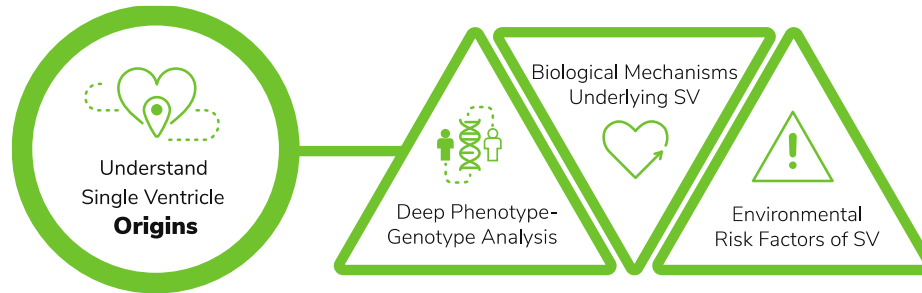


Figure 3. Through our work with the community, four areas emerged as important for SV disease research and clinical care. Each goal is listed in a circle and strategic investments are listed in the corresponding triangles.

I. UNDERSTAND SINGLE VENTRICLE ORIGINS



The genetic basis of SV heart disease is mostly unknown, save for a couple of rare mutations, and the molecular mechanisms underlying SV heart defects is similarly opaque. Furthermore, there are no data on non-genetic factors impacting the development of SV heart defects, such as the role of epigenetic, hemodynamic, and environmental (e.g. placental, maternal exercise) factors. Excitingly, advancements in technology have dramatically enhanced our capacity to understand the contribution of variants, regulatory elements, and alterations in chromatin structure to the expression of genes.

- **Recommendation I:** Identify the genetic factors underlying SV in the context of deep phenotyping to develop a high-resolution map of SV-specific variants, including coding genes, regulatory elements, and chromatin structure, and evaluate the functional consequences of each variant.
- **Recommendation II:** Support efforts to define biological mechanisms underlying SV, including those involved in normal cardiac development, and perform a systematic analysis that yields a comprehensive, time-resolved developmental map of the molecular, cellular, and physiological alterations across the single ventricle heart.
- **Recommendation III:** Explore non-genetic factors, in the context of deep phenotyping, that contribute to the presentation and penetrance of SV heart defects, including modifiable risk factors, biomechanical forces, and environmental influences, through a combination of epidemiological and genetics approaches and assess the impact of intervention in the development of disease from a genetic, epigenetic, and anatomical perspective.

The understanding of the underlying causes of SV disease is in its infancy, and their relationship to clinical outcomes and treatment guidance predominantly unexplored. Thus, to fully uncover the factors needed to understand and effectively treat single ventricle disease, we recommend supporting the development of a large and comprehensive set of correlated genetics data, clinical records, and biospecimens that will be made available to the entire scientific and medical community.

Such a resource, inclusive of both advanced data infrastructure and the clinical expertise needed to capture the relevant pathophysiology, is a prerequisite to identifying variants and ultimately to studying variant-to-function.

II. DEFINE BIOLOGICAL MECHANISMS OF OUTCOMES

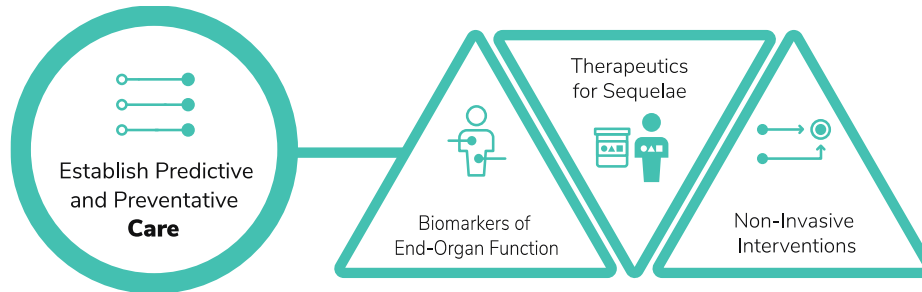


While survival has dramatically increased, the majority of treated SV patients experience significant complications and comorbidities which can severely impact quality and duration of life, yet the field does not yet understand what drives the onset. Thus, a deeper exploration of the biological and environmental mechanisms that correlate to outcomes is necessary to mitigate risk while also providing a personalized approach to each patient’s journey.

- **Recommendation I-A:** Isolate the genetic, gene regulatory, and epigenetic factors that drive the onset or impact the trajectory of common complications and organ-specific dysfunction in SV disease through a series of exploratory *-omics* and variant-to-function assays, as well as in the patient population. Additionally, examine the factors that appear to lead to resiliency.
- **Recommendation 1-B:** Develop appropriate model systems of the variants underlying clinical sequelae to create a comprehensive, time-resolved developmental map of the molecular, cellular, and physiological alterations across end-organs and the relationship to heart development and/or function.
- **Recommendation II:** Examine factors that lead to resiliency and systematically dissect those without SV disease-related clinical sequelae to risk stratify patients and move towards proactive versus reactive medicine.
- **Recommendation III:** Investigate how non-genetic factors, including alterations in flow, hypoxia, nutrition, and other post-natal exposures, interact with the underlying substrate to impact the development of complications and comorbidities that affect the heart and other end organs in the SV population.

The recommendations provided here should create the foundational framework to progress towards predictive, personalized medicine approaches. While the first two aims will require large-scale and concerted efforts that could take a full decade to accomplish, the resulting information will be critical to the development and implementation of predictive models defining risk, outcomes, and therapeutic guidance, while also providing actionable mechanisms to amplify factors promoting resilience in the SV population.

III. ESTABLISH PREDICTIVE AND PREVENTATIVE CARE

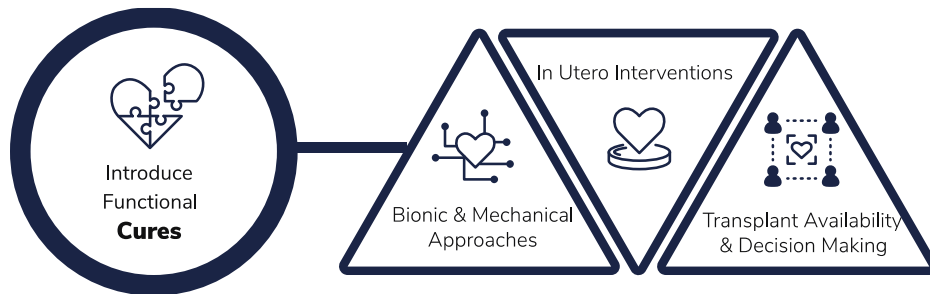


Efforts to predict, prevent, and treat SV-related clinical sequelae are limited in scope and scale, reducing the ability to draw relevant conclusions about treatment and/or intervention timing, effectiveness, and outcomes. Additionally, no measures exist to map the trajectory of heart and other organ system function, nor are there preventative treatments to modify or mitigate outcomes. Furthermore, treatment options are limited, and novel interventions are underexplored.

- **Recommendation I-A:** Map the trajectory of major complications and comorbidities of the brain, heart, and other end organs, and their impact on health, health-related quality of life, and well-being through unified, standardized protocols and long-term data collection on SV patients.
- **Recommendation I-B:** Generate functional assays or diagnostics that act as surrogate biomarkers for current heart and end-organ function, with the ultimate goal of adopting personalized medicine approaches to optimize outcomes and practice proactive medicine.
- **Recommendation II:** Develop etiology-based therapeutics and novel alternative interventions, such as diet, exercise, and lifestyle factors, and explore repurposing approved medications to address complications and comorbidities and prevent decline in functional and end-organ status.
- **Recommendation III:** Develop interventions to optimize expression of favorable outcomes, such as those observed in resilient populations, to promote enhanced health, health-related quality of life, and well-being.

Many of the recommendations provided throughout this document are aimed at bettering the lives of future cohorts of SV patients, but the aims of here more directly impact the thousands of patients who currently are living with SV disease and/or the Fontan circulation. While addressing each of the above recommendations will take time, application to the clinic is expected to be more near-term.

IV. INTRODUCE FUNCTIONAL CURES



The current treatment of SV is palliative, not curative, as Fontan procedure predisposes to early clinical deterioration, presumably because of its intrinsic hemodynamic consequences. While further improvements in the approach to Fontan palliation are possible, abnormal hemodynamics are an unavoidable consequence of this circulation and there is little reason to think that normalization of life expectancy and long-term quality of life can be achieved within the current Fontan paradigm. Further, in the absence of disease specific evidence-based therapies, clinicians primarily repurpose adult-approved devices or heart transplant to treat Fontan deterioration; at least partially successful, though both approaches have substantial limitations this is often. Recent advances in immunology, tissue engineering, and stem cell biology indicate the time is ripe for novel approaches to this decades-old problem.

- **Recommendation I-A:** Investigate novel “bionic” approaches to avoid or overcome the Fontan circulation challenges and restore adequate power to the circulatory system through 1) exploration into technologies to salvage or grow the hypoplastic ventricle or 2) the creation of an extracardiac conduit or implant to harness systemic circulatory power.
- **Recommendation I-B:** Create interdisciplinary physician, scientist, engineering, and modeling teams to create clinically informed, milestone-driven projects that enable the advancement of approaches to manufacture cardiac tissues, such as contractile patches, conduits, valves, ventricles, and whole hearts.
- **Recommendation II:** Explore for whether the regenerative capacity of the fetal environment can be leveraged to enable repair and/or reversal of single ventricle anomalies *in utero*.
- **Recommendation III:** Develop and refine strategies to improve heart transplantation, including improving identification of patients earlier in their course of failure and improving organ preservation of donor hearts to increase donor quality and availability and to extend the lifetime and outcomes of a transplanted heart to become a lasting cure for single ventricle.

If researchers are successful in developing such cures, SV could become a curable disease where patients can expect a normal duration and quality of life. Concerted efforts to solve these issues in an efficient and effective manner are critical and require alignment and collaboration from scientists, clinicians, engineers and funders.

Appendices: 1 - 4

Appendix 1. Understanding the Etiology of Single Ventricle Heart Defects

BACKGROUND

While it is appreciated that SV are multi-faceted with a number of distinct physiologies, the genetic, epigenetic, and environmental bases of disease remain unknown. This hinders efforts to both predict and prevent the disease and to define the molecular and cellular physiological differences that underpin risk and long-term outcomes.

- Around 4,000 infants are born with single ventricle anatomy each year in the United States, with an estimated prevalence of 35 per 100,000 (4,5).
- SV encompass a spectrum of diagnoses including, but not limited to: HLHS, Tricuspid Atresia, Double Inlet Left Ventricle (DILV), Double Outlet Right Ventricle (DORV), Pulmonary Atresia with Intact Ventricular Septum (PA/IVS), Single Left Ventricle, atrioventricular canal defect (CAVC) Unbalanced to the left, and Unbalanced to the right.
- One major caveat in the majority of studies examining etiology is that they are limited to HLHS and often do not account for the variability in the morphology, pathology, and presentation of SV patients (6,7).

GENETICS

Genetic Heterogeneity – The extent of the genetic contribution to SV is unknown, including the contribution of germline versus acquired (*de novo*) mutations. However, in some cases, heritability is reportedly high, suggesting a strong genetic component (8). The following discoveries have been made within the field regarding genetic heterogeneity:

- Germline mutations in several genes have been associated with SV and include GJA1, NKX2-5, NOTCH1 and MYH6 (Table 1). Other genes have been implicated in SV pathology but have not been confirmed and their impact is yet to be determined (Table 1).
- Currently, genes associated with SV overlap with genes implicated in other congenital heart defects (CHDs). Such data suggests that while there is a continuum of underlying causes, lessons learned from other CHDs may apply to SV, and that a candidate gene approach may be informative although robust genetics are lacking (9, 10).
- Human genetics and mouse models reinforce genetic complexity as hallmarks of SV. For example, a phenotype-driven mutagenesis screen identified 8 unique HLHS-like lines in mice (11). These lines included those with mutations in different single genes, as well as one line with mutations in two genes. This line, which had mutations in *Sap130* and *Pcdha9*, demonstrated their individual contributions to left ventricular hypoplasia and aortic valve abnormalities, respectively (1,11). Despite genetic heterogeneity, a subset of recovered genes converged into same pathway (Notch signaling).
- In another study, heterozygous mutations in three genes (Mkl2, Myh7 and Nkx2-5) segregated with left ventricular disease. Investigators used combinatorial mouse models that were sufficient to recapitulate the pathology, uncovering Nkx2-5 as a key genetic modifier in disease (12).

The current genetic understanding of SV is clearly lacking and is complicated by the complexity of the genetic landscape of SV, in which causes may be both Mendelian and non-Mendelian, multigenic, and multifactorial. Thus, more basic research and genetic analyses are needed.

In addition to looking into the genetics of disease, examining the genetics of normal heart development is critical. Such studies can shed light on the developmental processes that go awry in disease, such as morphogenesis, cardiac chamber specification and asymmetry, and conduction, and can provide valuable insights into the underlying factors responsible for SV. For example, HAND1 was shown to be expressed early in embryonic left ventricular myocardium and its loss of function affects left ventricular morphology. Mutations in Hand1 and its enhancer (which was shown to be necessary and sufficient to drive HAND1 expression in the left ventricle) thus may be clinically relevant (13,14). Furthermore, another study used single-cell analysis to identify cardiac lineage-specific transcription factors whose depletion from right ventricle is developmentally important, finding factors including Hand2 (15).

Table 1. Genes associated with SV

Candidate Gene Name	Gene Function	SV Type	References
Connexin43 (GJA1)	Gap-junction protein	HLHS	16
NKX2-5	Cardiac homeobox transcription factor	HLHS, DORV	17
NOTCH1	Membrane ligand-receptor	HLHS	18–21
MYH6	Gap-junction protein	HLHS	22
Other identified potential candidates			
RFX2, HAND1, FOX cluster (FOXF1, FOXC2, FOXL1), PTCH1, IRX4, TBX5, BMP2/BMPR2, ETS-1, JAG1, IRX4, MLL2/KMT2D, HUWE1			1,21,3

Chromosomal abnormalities – While ~18% of patients with CHD have chromosomal aberrations, their prevalence in SV is unclear (6,23). Although several studies have examined chromosomal abnormalities in SV, there is a limited pool of data on this topic.

- A retrospective study of infants with heart disease found that 17% of probands with single ventricle physiology had an abnormal karyotype (23).
- A range of chromosomal abnormalities and genetic syndromes are associated with SV (Table 2); their prevalence may differ between different SV types (24).
- The role of chromosomal abnormalities in the etiology or pathogenesis of SV is unknown and controversial, but their correlations with comorbidities and outcomes should be studied, especially those in candidate genes (25,26).

Table 2. Chromosome abnormalities associated with SV

Chromosome abnormality/syndrome	SV Type	References
Trisomy 13	HLHS	24,27–29
Trisomy 18	HLHS, MA/AA, RV, DORV, Unbalanced CAVC to Right	24,28,30
Trisomy 21 (Down)	HLHS	24,30,31
XO or 45X (Turner)	HLHS	11,24,27,28,30,32,33
11q terminal deletion (Jacobsen)	HLHS	27,28,34
Other observed chromosome abnormalities		
Trisomy 8, Trisomy 9, 16q deletion and others		35, 36

Genetics and Clinical Outcomes – Knowledge of disease-causing genetics can inform clinical practice and ultimately improve patient outcomes, by bringing precision approaches to care and treatment.

- Genetics can allow for risk and phenotype stratification:
 - In a recent analysis of 2,775 patients with Hypertrophic Cardiomyopathy, two subpopulations - sarcomere variant positive and negative - were identified. Mutation status correlated with phenotypic differences, such as more and less fibrosis, respectively (37).
- Genetic modifiers can significantly modify the presentation of disease:
 - For example, a homozygous mutation in the *Ets1* gene causes abnormal ventricular morphology in Jacobsen syndrome. However, when this mutation was modeled in two different mouse strains, one model had heart disease, while the other did not, indicating a likely role for a background genetic modifier (34).
 - Without the concurrence of a rare NKX2-5 variant, heterozygous mutations in *Myh7* and *Mkl2* did not cause severe heart disease phenotype (12).

To gain additional molecular insights, post-mortem genetic testing and molecular autopsy can be performed for patients with SV. Such data can provide a clinically-relevant source of susceptibility gene discovery and, as for example in Sudden Death Syndrome, the identified pathogenic variants could impact surviving families and provide closure (38).

NON-GENETIC

A vast majority of CHD cases are thought to be multifactorial and to arise from a combination of genetic and extra-genetic factors, such as environment, epigenetics, and mechanobiology (9,39). However, due to paucity of data linking specific extra-genetic factors to SV and the inconclusive nature of the existing studies, the extent of their contribution to SV is unknown.

Environmental factors – While several labs are focused on understanding the genetics and cellular biology of SV, very few are exploring the relationship between environmental factors and disease prevalence, severity, or outcomes. In such a complex disease, environmental insults may have a profound impact depending on the context of certain genetic backgrounds, tipping the scales to cause a developmental abnormality. Finding these linkages could uncover risk factors that could provide useful therapeutic and/or interventional targets.

- A range of extrinsic and intrinsic factors have been implicated in cardiac heart disease to date (40). These include maternal diabetes, maternal use of alcohol, and exposure to teratogens like thalidomide (9,41,42).

Epigenetic factors – Epigenetic markers are molecular modifications of genes that can influence how much a certain gene is expressed by a cell; however, little is known about how epigenetics modifies risk in SV and contributes to disease. As epigenetic markers are a consequence of both maternal patterning and environment, identifying epigenetic linkages may also help to define the altered molecular signaling pathways.

It is well appreciated that epigenetic regulation is required for proper heart formation, which includes DNA methylation, histone modification, and non-coding RNAs. Defective epigenetic pathways are associated with CHD (39). Examples of a link between epigenetics and SV include:

- De novo mutations in genes encoding epigenetic regulators and chromatin modifiers are overrepresented in CHD; a subset of the patients in the study had HLHS, DILV, DORV, and other left ventricular obstructions (36,43).
- HLHS patient cardiac progenitor cell-derived iPSCs exhibit significant changes in H3K4me2, acH3 and H3K27me3 within the NKX2-5 promoter (44).
- The miRNA profiling of the right ventricle in a pediatric case of HLHS demonstrated a unique miRNA profile and differential regulation of miRNAs, as compared to a control heart (45).

Mechanobiology – Biomechanical forces are required for normal embryonic heart development; these forces are particularly necessary during cardiac chamber development, contractility, fluid dynamics, and valve formation, in which mechano-sensing proteins are critical (39,46). The biology of these processes and their contribution to disease etiology and progression are poorly understood, but new studies have demonstrated their relevance.

- In HLHS, the right ventricle is increasingly stretched due to a high-pressure load. Using an unbiased screen as a starting point, a recent study showed that miR-486 levels are increased in response to stretch in vitro, in patient samples, and in an animal model of ventricular dilation (47).
- Heart chamber formation is a blood flow-dependent process (39); Zebrafish mutants with disturbed blood flow and/or contractility exhibited defects in endocardial cell proliferation and cardiac chamber formation (48).
- Cardiac cilia are essential for proper myocardium development and cilia-related mutations play an important role in the etiology of CHDs (49,50). For example, reports have shown that primary cilia activates endocardial Notch signaling in response to flow in zebrafish, and that cilia-mediated flow sensing is critical for cardiac morphogenesis (51, 52). Cilia also makes a connection to other organs that require ciliary function. Thus, genetic mutations in motile cilia may help explain extracardiac anomalies, such as respiratory complications in heterotaxy patients (53).
- Emerging themes contributing to the landscape of cardiac disease etiology and pathology include metabolites and mitochondria dysfunction, whose role in pathological heart tissue remodeling is being investigated (54, 55).

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Appendix 2. Complications & Comorbidities in Single Ventricle Heart Disease

RELEVANCE

The survival rate post-Fontan surgery is rising, with excellent transplantation-free outcomes in recent surgical history (5-year survival of 95%, 10-year survival of 91% and an estimated 30-year survival of 85%) (1–3). Yet, these survival milestones are undermined by significant comorbidities and complications in individuals with the Fontan circulation, leading to premature morbidity and mortality, and a poor quality of life (1,3).

While efforts to understand the drivers of these complications are underway, they are limited in scope and scale, reducing the ability to draw relevant conclusions about the causes, treatments, and prevention. To some degree, many of the current knowledge gaps are rooted in an incomplete understanding of the disease etiology and epidemiology. Moreover, complications are further compounded by a lack of clinical tools, including a suboptimal detection timeline, limited diagnostics, and a lack of treatment options, and an insufficiently interdisciplinary research lens. These solvable challenges preclude the robust and timely identification of risk factors, hinder the potential to stratify this complex population, and limit progress on the development of improvements to disease management, treatment, and therapeutic development.

BACKGROUND

In SV patients, overworked ventricles are often layered on top of abnormalities in other organs that have an increased likelihood of failure, possibly attributed to the altered hemodynamics of the cardiovascular system. These issues worsen over time, reducing both the quality and duration of life – a condition sometimes referred to as “Fontan syndrome” – and can lead to Fontan Failure and ultimately, heart transplant or death.

Failing Fontan – The Fontan operation results in a single functional ventricle; however, the procedure is palliative, not curative, as it does not create a “normal” heart or circulatory system. As such, those with the Fontan anatomy, physiology, and abnormal circulation often experience challenges, which over time, can result in premature morbidity and mortality through multiple mechanisms, a condition represented by the term “Fontan Failure” (1,4).

- Fontan failure is not clearly defined, but is broadly characterized by a series of overlapping features including systolic or diastolic ventricular dysfunction, Fontan pathway/circuit failure, lymphatic failure, and extra-cardiac organ failure (4).
- In a Failing Fontan, ventricular failure (~50%) and Protein Losing Enteropathy (PLE) (~40%) account for the most common reasons for transplant referral (1,4).

Outcomes Post-Fontan – While it is well appreciated that the Fontan operation itself has excellent post-operative survival statistics, post-surgery complications are not yet eliminated, leading to variable outcomes.

- Although the primary cause of post-Fontan death is largely cardiac, other major causes include respiratory failure, sudden death, PLE, and liver disease (1,5).
- Outcome disparities are evident from the follow-up studies (1,5). This variation is most readily explained by the operative year, institution, surgical technique, and type of Fontan operation

(1,3,5). However, some of the variation is due to the heterogeneity of pre- and post-operative complications and comorbidities, as well as socioeconomic and geographic status.

- Currently, it is not possible to predict which complications will occur and when they will occur, nor is it possible to identify which Fontan patients are at risk of developing each complication (1). A combination of factors, including a lack of risk-stratification strategies, a paucity of biomarkers, and insufficient longitudinal and incremental tracking of patient health, limit progress in this area.

FONTAN-ASSOCIATED COMPLICATIONS & COMORBIDITIES (“FONTAN CIRCULATORY SYNDROME”)

The altered circulatory state of Fontan patients, and likely other underlying abnormalities, lend themselves to complications and comorbidities. Indeed, the list of potential issues in Fontan patients is extensive and includes both cardiac and extra-cardiac anomalies. However, the underlying basis for such clinical issues is not fully understood and the causes are likely multifactorial, and may include (1,3,6):

- | | |
|-----------------------------------|-------------------------------|
| • Pre-Fontan Biology & Physiology | • Surgical load & technique |
| • Genetic predisposition | • Medical complications |
| • SV etiology | • Socioeconomic status |
| • Fontan circulation | • Yet-to-be-discovered causes |

Cardiovascular Complications – As expected, patients often experience cardiac complications post-Fontan, likely due to the single ventricle physiology and post-operative geometry and/or hemodynamic pressure (Figure 1, Table 1 for most common forms and treatments).

- The most frequent cardiac complications in Fontan patients include heart failure (up to ~35% of Fontans) and arrhythmias (up to ~50% of Fontans) (1,7). Such complications can increase the risk of other events; for example, patients with atrial tachycardia are at increased risk of heart failure and other complications that require hospitalization (1).
- Early and late post-Fontan complications are roughly defined as <30 days (early) versus >30 days (late), but are not clearly documented or demarcated in patient records. Early complications, however, appear largely cardiovascular, although not exclusively. Cardiac deficits, such as decreased ejection fraction or thromboembolic events, present throughout lifetime (1,8).
- Cardiac defects in Fontan patients may differ from the general population. For example, causes and phenotypes of Fontan-associated heart failure are more heterogeneous and complex, as well as may worsen over time (1,9).

Extracardiac Complications – In addition to the cardiac complications and comorbidities, Fontan patients experience a host of other issues that severely impact quality and duration of life (Figure 1, Table 1).

- The associated extracardiac complications are multi-organ and can involve the liver, lung, kidney, brain, lymphatic system, and other organs (1). The most common complications are hepatic fibrosis (in 100% of Fontan adolescents), renal (in 20-50% of Fontan patients), and PLE (in 5-12% of Fontan patients) (1,9).

- In addition, there are significant complications related to neurocognitive deficits and mental health challenges (discussed below) (1,9).
- End organ disease in Fontan patients can differ from similar diseases in non-Fontan patients. For example, Fontan-associated liver disease (FALD), despite its overlapping features with non-Fontan liver disease, does not correlate well with standard liver tests, making the diagnosis more challenging for clinicians not well versed in Fontan physiology (6).

Extracardiac complications often may appear late. However, “late” complications range from 30 days to 40+ years after Fontan surgery, and are in part determined by the follow-up time. More systematic and multi-variable data collection is needed to provide insight into both the onset and progression of these comorbidities, and to allow for subpopulation enrichment.

Mental Health & Neurodevelopmental Defects – Fontan patients often suffer from neurocognitive challenges, thought to be due to both developmental defects and surgical burden, and/or mental health decline, as a result of their precarious health situation.

Manifestations – Mental health decline is a major concern in all Fontan survivors. Fontan individuals face outcomes that are associated with chronic illness, including worse physical health and self-esteem, worse education and job prospects, and difficulties with social interactions. These, in addition to concerns about mortality, lead to anxiety, depression, and other mental health problems.

- Currently, mental health status is severely under-recognized by health professionals. Addressing these issues is paramount to improving the health and quality of life in all Fontan patients (1,10,11).
- Neurocognitive outcomes of children with SVD include difficulties with memory and attention, learning disabilities, and behavioral abnormalities, although their prevalence is unclear. Importantly, some studies indicate that post-Fontan patients operate within the normal range of neurocognitive testing, suggesting there is a wide range of outcomes. (11).
- Neurological impairments in Fontan patients may include seizures, stroke, white matter damage, cortical atrophy, neurological and neurocognitive deficits, and other forms of brain damage (1,3,12).
- A prenatal HLHS diagnosis was associated with improved early neurologic outcomes, despite no association with survival (13). However, the association with other forms of SVD is not yet known.

Systematic studies are required to identify the full spectrum and frequency of neurocognitive outcomes in post-Fontan children and adults, as well as a concerted effort for treatment.

Etiology – The etiology of neurodevelopmental and neurocognitive deficits is poorly documented and not well understood, but is likely multifactorial, deriving from underlying genetics, surgical load, and other complications.

- Operative procedures are likely a critical factor for the development of neurocognitive problems. For example, Fontan children and adolescents had reduced cortical volume and thickness, as compared to normal children, and this reduction correlated with operative history (12).

- Furthermore, in this study, although not fully deconstructed, the HLHS patients had worse outcomes than other SVD patients. For example, they had smaller regional brain volume. The differences could be due to additional surgery only HLHS patients underwent (Norwood), although it may also have something to do with causes inherent to HLHS (e.g. reduced cerebral perfusion). Notably, operative causes are potentially modifiable and are good candidates for interventions.
- Post-Fontan patients are at increased risk of cerebrovascular events, accidents, and stroke (14).
- Pre-operative abnormalities and biological reasons also may be contributors. For example, genetic mutations may provide a link between heart and brain development and the observed abnormalities, as evidence by the following findings:
 - Both cardiac and neural crest cells originate from neural tube (15)
 - Neural crest-cell specific Dicer mouse mutants (with mutation in Dicer, a key endonuclease of miRNAs), show severe defects in both, cardiovascular system and craniofacial structures, with cardiac phenotypes including DORV (16).

Exercise Intolerance – Fontan physiology and circulation are thought to limit exercise capability. This exercise intolerance is related to complications including lack of subpulmonary pump, cyanosis, and lung restriction (1). Indeed the decline in peak oxygen uptake (VO_2), a key endpoint used to evaluate exercise performance, is a predictor of adverse cardiovascular prognosis, death and transplantation (17). As a result, in the past, Fontan patients were advised against intensive exercise. However, new evidence suggests that exercise training is beneficial to Fontan patients for both, physiological and physical health and quality of life (1,18). Below are some examples of mounting evidence in support of lifting restrictions on exercise in Fontan patients and promoting exercise paradigms in this population.

- High-intensity whole body resistance training increased cardiac output and exercise performance in a small cohort of adult patients with Fontan physiology, demonstrating increased muscle strength and muscle mass, increased peak VO_2 , and cardiac output (19).
- Across different ages (8 years or older with diverse SVDs), Fontan patient’s sports practice (leisure, competitive, exercise training) was strongly associated with improved functional capacity as measured by changes in VO_2 and other cardiopulmonary exercise test (CPET) aspects (18).

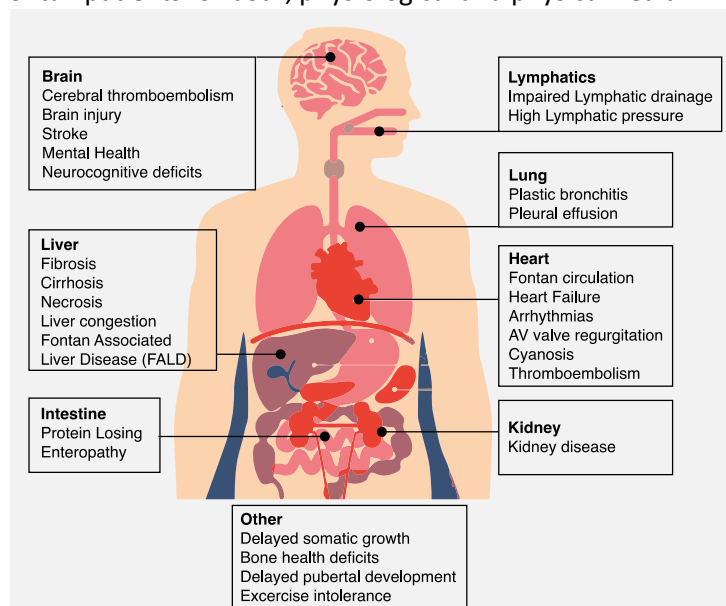


Figure 1. Known complications in a Fontan patient. In the above diagram, known complications and comorbidities are outlined. While this is not an exhaustive list, it demonstrates systemic and multi-organ risks and morbidities and emphasizes the need for systems approach to managing and treating the Fontan population.

- ‘Super-Fontans’, or Fontan individuals with normal or supranormal exercise capacity, are all individuals who practice intense sporting activities and whose characterization may provide therapeutic insights (20).
- The recent multi-center Fontan Udenafil Exercise Longitudinal (FUEL) Phase III clinical trial of 400 participants found that adolescents receiving udenafil (a PDE-5 inhibitor) showed some improvements in exercise performance at the ventilatory anaerobic threshold (21).

Major limitations to exercise studies include bias towards inclusion of individuals in good enough shape to actually perform the exercise tests; deficiency of tests of pulmonary function and hemodynamics and of long term safety evaluation or end-organ damage analysis; unknown impact of medications and devices; and unknown contribution of baseline characteristics including weight and obesity. Differences in exercise capacity can potentially be used to assess and stratify patients and offer recommendations, but the prognostic capacity has not been tested.

Table 1. Common comorbidities in Fontan patients

Complication	Risk Factors	Current Treatment
Arrhythmias	Genetics, elevated pre-operative pulmonary artery pressures, older age at Fontan, type of Fontan, heterotaxy, AV regurgitation, sinus node dysfunction	Antiarrhythmic drugs, beta-blockers, pacing, cardioversion, pacemaker, Fontan conversion, arrhythmia surgery (1,3)
Heart Failure	Ventricular dysfunction, atrial tachycardia, valvar regurgitation, volume-loading shunts	Cardiac transplantation
Protein Losing Enteropathy & Plastic Bronchitis	‘Failing Fontan’ physiology and circulation, chronic venous pressure, venous congestion; lymphatic congestion	Diuretics, afterload reduction, albumin replacement, heparin or steroid therapy, anti-inflammatory agents, dietary changes; fenestration, atrial pacing; transplantation (1,22,26); dietary modifications e.g. high-protein diet (1); lymphatic drainage in consideration (23)
Liver disease	Fontan hemodynamics (high venous pressure), lymphatic overload, older age, hepatitis B or C, alcohol use, hepatotoxic drug use, obesity	Preventing clinical heart failure, optimizing Fontan circulation; liver transplantation, combined heart-liver transplantation (6,24)
Atrioventricular valve regurgitation	Structural valvar abnormalities (most commonly tricuspid valve), annular dilatation, reduced ventricular contractility	Diuretic therapy, afterload-reducing medications, AAV repair; transplantation

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Appendix 3. Finding Functional Cures for Single Ventricle Heart Defects

RELEVANCE

The three-staged palliation is viewed as a modern miracle of medicine, routing a new pathway to survival beyond age five. While survival has indeed increased significantly, SV patients are not functionally cured. Further, the duration and quality of life in these patients is far from ideal, as the passive flow caused by the Fontan procedure is thought to lead to long-term hemodynamic issues, and palliation often fails over time.

Currently, heart transplantation is the closest option to a functional cure; however, this procedure faces tremendous challenges that compromise the overall quality of life (1). Indeed, a combination of extensive organ waiting list time, limited donor supply, immune complications, organ rejection, and mounting complications and comorbidities limit the feasibility of heart transplant as a curative approach. For example, the overall availability of donor hearts is limited, causing the process of donor-recipient matching to be highly selective. This selectivity is further exacerbated by the fact that many Fontan patients are referred too late and are thus ineligible for a transplant. Moreover, although a recent post heart transplantation analysis in 33 Fontan children demonstrates a 5-year survival of 70%, 18.5% of survivors required re-transplantation and multiple patients were hospitalized and sustained on ventilator support (2). Therefore, in order to move closer to a functional cure for SV patients, the field should examine alternative therapeutic approaches, and transition from the concept of rescue to that of heal, replace, and/or rebuild.

BACKGROUND

Currently, researchers are investigating regenerative medicine approaches to overcome the major hurdles associated with SV and Fontan physiology, potentially creating new approaches that will functionally cure patients prior to palliation. Specifically, researchers are aiming to:

- Identify mechanisms to heal/repair a failing heart through tissue engineering and/or regenerative medicine.
- Develop biological devices to serve as a conduit for SV flow, both *in utero* and in those with Fontan circulation.
- Create a *de novo* tissue, ventricle, or entire organ.

If researchers are successful, SV could become a curable disease where patients can expect a normal duration and quality of life. However, there are not concerted efforts to solve these issues in an efficient and effective manner. Exploration into tissue engineered grafts, devices, or hearts would help to overcome many of the challenges associated with the palliative approach. However, additional understanding of the cell types, immunological components, and hemodynamics would be beneficial. The highlighted topic areas below represent some of the most cutting-edge research and development currently in ideation or underway.

REGENERATIVE MEDICINE

The complexity of developing a *de novo* heart requires both incredible feats of engineering, while also recapitulating the functional, structural, and niche complexity of the heart and associated vasculature.

While the development of a fully functional *de novo* heart is likely decades in the future, there is promise that the engineering of parts and components of the heart could be accomplished in the near term, such as cardiac cells, blood vessels, cardiac patches, and valves. Focusing on these smaller scale advances may be the key to unlocking breakthroughs and offer a true path to functional cures. At the frontier of cardiac regeneration are stem cells, cardiac tissue engineering, and gene therapy, which are detailed below.

Stem Cells – Stem cells are undifferentiated cells whose capacity to both self-renew and differentiate into another (uni- or multi-potent) or any other (pluripotent) cell type, is sought after for cardiac healing, repair, and regeneration. A major goal of stem cell-based, regenerative cardiac therapy is to treat heart failure by replacing the damaged myocardium. Such a therapy requires generation of cardiomyocytes (CMs) from stem cells and then transplantation of these cells into the damaged heart (3,4). However, the potential of stem cell-based therapies in treating SV-associated heart failure, arrhythmias, and other cardiac complications is largely unknown and unexplored. Currently, the successes with cardiac stem cell therapy are few, and there are many challenges. There are many different kinds of stem cells, including: adult stem cells (ASCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs); their therapeutic potential is discussed in more detail below. To date, all FDA-approved regenerative cell therapies use adult stem cells (4).

Adult Stem Cells (ASCs) – Cells obtained from adult tissues and organs exhibit stem cell-like properties, but they do not have unlimited lineage potential (so are multipotent but not pluripotent). The different ASC populations attempted for cardiac repair (5) include:

- Mononuclear cells (MNCs) from bone marrow or umbilical cord blood
- Hematopoietic stem cells (HSCs)
- Mesenchymal stem cells (MSCs)
- Peripheral blood progenitor cells
- Skeletal myoblasts
- Endothelial progenitor cells (EPCs)
- Cardiac stem cells

Perhaps surprisingly, the use of cardiac stem cells derived from heart niches like the atrial myocardium is challenging, in part because the stem cell pool is limited and also because these cells experience senescence. However, several pre-clinical and clinical studies using ASCs have been conducted:

- Direct implantation of bone marrow-derived cells, MNCs, MSCs and EPCs into the heart resulted in improved ventricular function, regeneration of contracting cardiomyocytes, and vascular bends or decreased fibrosis. However, these findings were mixed (5,6).
- The first reported case of intramyocardial injection of autologous UCB-derived MNCs into the right ventricle of HLHS neonate during stage II surgical palliation was published in 2015 (1). In this study, improvement in right ventricular ejection fraction was noted during a 3-month follow-up, although whether this improvement was driven by cell therapy, surgery, or both was unclear.
- Most recently, a Phase 1 clinical trial using UCB-MNC therapy in 10 infants with HLHS demonstrated safety and feasibility of direct injection of these cells into the right ventricular myocardium of infants at time of stage 2 palliation. However, up to 6-month follow-up showed no change in right ventricle function. Follow-up studies will determine if there is any therapeutic benefit or risk; a multi-center phase 2b study is anticipated (2).

The cause of any of the demonstrated benefits from ASCs in patients and in animal models is controversial, requiring substantial research to untangle the effects. For example, the following are outstanding questions that must be addressed:

- The capacity of ACSs to differentiate into cardiac cell types upon implantation is questionable.
- Engraftment may be minimal or even none suggesting stem cells may not directly contribute, but rather, contribute through an indirect method.
- Data overwhelmingly suggests that paracrine signaling is responsible for the ability of EPCs to contribute to angiogenesis, and other outcomes. Thus, understanding underlying paracrine mechanisms is essential for defining the therapeutic potential of stem cell therapy in heart regeneration.

While regenerative therapy for SV is highly sought after, experience with these approaches in children is very limited and different SV may also have different cardiac repair needs. As such, the development of a variety of therapy approaches and strategies is likely required. For example, Hypoplastic Left Heart Syndrome (HLHS) poses unique demands on the right ventricle, which is not equipped for systemic flow, and often fails over time; thus, this creates a need for a therapeutic intervention specifically targeted at strengthening the right ventricle.

Pluripotent Stem Cells – Unlike ASCs, the embryonic stem cells (ESCs) and inducible pluripotent stem cells (iPSCs) are considered truly pluripotent, indicating that there is great promise for their use in cardiac regeneration and bioengineering efforts for cardiovascular therapy. However, while efforts are currently underway, they face many challenges, requiring more basic research to understand their clinical relevance and applicability to SV (7,8).

Embryonic Stem Cells – ESCs are derived from an embryo, specifically from the inner cell mass of the preimplantation blastocyst. Their theoretic regenerative capacity is unlimited, and they can be differentiated into any somatic cell.

- Injecting human ESC-derived CMs has been shown to successfully ‘re-muscularize’ infarcted monkey heart, making CM cell therapy attractive for use in humans. However, serious concerns include arrhythmic complications (9).
- To date, no controlled clinical trial of ESC-derived cells in cardiovascular disease has been conducted or even initiated. This may be due in part to the low clinical tolerance for potential malignancies as a result of treatment, the lack of evidence showing clinical doses are relevant, and the unclear mechanism of action driving repair (7).

Inducible pluripotent stem cells – iPSCs are derived from differentiated somatic cells, which are then induced to become pluripotent stem cells using a cocktail of transcription factors, in a process called ‘cell reprogramming’. The most common tissue sources for iPSCs are peripheral blood cells, cord blood cells, and skin fibroblasts, but it is unclear which have the best potential to differentiate into cardiac cells. Perhaps unsurprisingly, lines of iPSCs are unique and depend on the host cells used for iPSC reprogramming. As a therapy, iPSCs are thought to be extremely promising, in part because they can be derived from patients, thereby eliminating rejection concerns.

- A major barrier is generating fully mature CMs from iPSCs (e.g. with proper contractility and energetics). Current protocols often yield a variety of iPSC-CM populations, which may represent different stages of CM maturation. To overcome this issue, one recent study suggested that heart tissues should be formed from more plastic early-stage iPSC-CMs and use physical conditioning to mimic mechanical loading (10).

Table 1. A comparison between three stem cell types (adult, embryonic, and inducible pluripotent stem cells) for use in regenerative medicine.

TYPE	PROS	CONS
ASC	<ul style="list-style-type: none"> • Differentiate into CMs in culture • Well-characterized • Some, like MSCs, are less immunogenic and don't require immune suppression • Can be directly implanted • Can be easily procured and manipulated 	<ul style="list-style-type: none"> • Limited cardiac potential (different ASCs have different potential); differentiation into CMs may not occur upon implantation • Heterogeneity and potentially mixed fate • Limited fraction in residing tissue • Clinical translation challenges (dose, concentration, volume) • Regeneration benefit upon transplantation unproven • Conflicting data on engraftment potential • Direct benefit unproven; improvements may be paracrine in nature
ESC	<ul style="list-style-type: none"> • Truly pluripotent • Excellent cardiac potential 	<ul style="list-style-type: none"> • Ethical and regulatory issues (embryonic) • Tumorigenic and can form teratomas • Pro-arrhythmic • No robust evidence supporting long-term engraftment • Prone to genetic instability when expanded in culture • Typically differentiated in culture as embryoid bodies • Immunogenic • Argued to have no advantage over iPSCs
iPSC	<ul style="list-style-type: none"> • Can theoretically be reprogrammed into any cell type • Can be generated from patient cells, avoiding ethical issues and rejection of transplanted cells • Excellent source of autologous cells 	<ul style="list-style-type: none"> • Low iPSC reprogramming efficiency • Lack of clarity as to which somatic tissue sources are best for cardiac potential • Human iPSC-derived CM identity – similar but equal? • Human iPSC-derived CM heterogeneity

Stem Cell Applications:

Basic Research – Efforts in stem cell biology research are unlocking key components of this cell population, which can be applied to disease models. Some examples of recent advances include:

- *Solving cardiac cell heterogeneity.* While human iPSC- or ESC- derived CMs recapitulate many aspects of cardiac development and disease, their underlying biology and environment is still unclear. For example, there is still incomplete matching of stem-cell derived CMs with native tissue (e.g. in terms of electrophysiology and biology) (7,8). New research is underway to uncover the observed CM heterogeneity, using single cell sequencing methods to deconstruct hPSC-CM populations into distinct, heterogeneous clusters (11).
- *Understanding cardiac cell context.* A healthy myocardium includes cardiac myocytes that are embedded in an extracellular matrix (ECM), which is crucial for structure, signaling, and communication. Many cardiac diseases are associated with pathogenic myocardium remodeling or abnormalities, including HLHS (12).
- *Building organoids before organs.* Many congenital heart diseases can be chamber or ventricle specific or impact the left or right side of the heart differentially. This anatomical complexity comes from specification by first and second heart fields. However, the mechanisms that induce the specification of first and second heart fields themselves are unclear, and this information may be crucial for engineering the many heart parts.

- In recent studies, 3D pre-cardiac spheroids were generated from iPSCs, allowing for a unique opportunity to interrogate the molecular identity and mechanisms of heart field specification (13). Such data supports the importance of 3D spheroids with heart-like properties (heart organoids) as both a research tool and for building and engineering a fully *de novo* heart.

Cardiac Tissue Engineering – Applications in bioengineering for iPSCs are extremely promising. Currently, they are under exploration for several key areas, including tissue-engineered patches, tissue-engineered biological devices (i.e. tube pumps), and creation of *de novo* organs or organ parts. These concepts are explored below.

Tissue Engineering – Engineering of an entire heart, and more realistically at present, of blood vessels, cardiac tissues, pumps, and valves is a current direction in the field and a true alternative to heart transplantation. The complexity of developing a *de novo* heart or heart components requires extensive knowledge of a vast array of scientific disciplines; such an accomplishment is still in the distant future, due to the several of the following challenges:

- **Perfusion and vascularization.** The Fontan operation requires the implantation of vascular conduits or grafts, which face the significant challenge of thrombosis. Growing advances in Tissue Engineered Vascular Grafts (TEVGs) promise to overcome these current limitations; however, engineering patient-specific pediatric vasculature still remains a challenge (14).
 - While several labs have demonstrated the ability to manufacture large scale vascular networks, no research group has yet been able to produce the microvasculature needed to fully perfuse thick heart tissue.
 - Current synthetic vascular grafts do not have sufficient flexibility to adapt to the growth of children, often requiring additional surgeries, and also can lead to further complications (14).
 - There have been significant advancements in TEVGs, which include improvements in cell sources, development of new natural and synthetic materials, improved elasticity, improved mechanical properties, long term potency, nonthrombogenicity, and new methods of 3D bioprinting (14).
 - For example, it was demonstrated that implantation of cell-free, small-diameter vascular grafts containing heparin and VEGF promote endothelialization (important for preventing thrombosis and occlusion) and regeneration in mice, recapitulating the structure of native artery (15).
 - An innovative pulsatile vascular conduit known as Tissue Engineered Pulsatile Conduit (TEPC) was designed for use in Fontan circulation to assist the single ventricle. Recent optimization of this approach features a modular design, optimized biomaterial scaffolds, use of decellularized porcine ventricle heart tissue, and hiPSC-CMs, which resulted in enhanced contractile function and overall enhanced composition (16).
- **Stem cells.** Labs are increasingly more proficient at maintaining large quantities of iPSCs and differentiating these to CMs. Despite these advances, challenges still remain:
 - New cell types within the heart are emerging, indicating that there is more complexity than originally anticipated.

- iPSCs are immature, suggesting that matrices used to develop volumetric tissues must also house developmental cues.
- The cell volume needed for such complex structures is incredibly high – and incredibly expensive.
- **Alignment.** While 3D printing apparatuses can produce a longitudinal axis of stress to allow for alignment of cells to form structures like septa, this model is too simplistic to develop the complex structures of the entire heart. A biologically relevant pattern of stress is required to build a heart to beat in the correct fashion and develop the appropriate compartments. Currently, the engineering field has only mastered 1D alignment.
- **Conduction.** Heart tissue requires extremely quick conduction, a process that relies on a cell type called Purkinje cells. Without such cells, the tissue operates on slow wave conduction, which causes arrhythmias. To develop a *de novo* heart, the field must develop robust means of differentiating these cell types and incorporating them appropriately within the heart structure.
- **Pressure and Valves.** Hearts rely on pressure gradients to function properly and they do so through the use of valves. However, valves are incredibly complex structures that require extensive engineering, and current usage has limitations, including 1) *mechanical* valves are prone to failure and deterioration 10-15 years post-implantation, and 2) *biological/tissue* valves have a propensity for thromboembolism, requiring a lifetime of anticoagulation treatments. Premature structural valve deterioration is also a problem that is pronounced in pediatric patients (17,18).
 - Implantation of Tissue Engineered Heart Valves (TEHVs) may overcome current valve limitations and replace traditional (mechanical and biological) heart valves. However, clinically successful TEHV paradigms need to feature properties such as repair, remodeling, and growth, while at the same time, allow for endogenous cell infiltration and neotissue formation (18).
 - Many current methods use de-cellularization (a process used to isolate the scaffold of a tissue from its inhabiting cells) of pig valves to reconstruct human valves, to avoid immunological rejection and/or calcification. However, this method is still flawed and requires more development (17).

While creating a *de novo* heart requires mastery of the above components, engineering smaller components, such as a cardiac patch or a tubular pump, is a much simpler task that could likely be accomplished in the near term. Focusing on these smaller stepwise advances in the individual components of a *de novo* heart may be the key to unlocking this breakthrough while also having collateral benefit in the process.

3D Bioprinting – Important advances in bioprinting tissues have been made that have indicated the promise of this technology, but significant challenges remain, as some have been noted above. In addition to these, the development of soft and elastic structures and/or matrices, printing intact structures, and supporting living cells in a sterile environment are still challenging. Some key advances in the field include:

- Recent technical advances in 3D bioprinting have allowed researchers to transition away from synthetic materials towards using biocompatible materials, such as collagen and gelatin.

- Advances in 3D bioprinting techniques have allowed researchers to manufacture more complex 3D objects. For example:
 - The 3D bioprinting technique FRESH (freeform reversible embedding of suspended hydrogels) has enabled the printing of complex 3D biological structures by embedding said structures in biocompatible hydrogels during printing (4).
 - Multimaterial Multinozzle 3D (MM3D) printing allows switching between up to eight different materials within a single nozzle (19).
- The ability to 3D-bioprint collagen, a critical component of extracellular matrix (ECM), with control, resolution and precision is necessary to engineer tissue components of human heart in a patient-specific manner. A further refinement of the FRESH method using human ESC-derived CMs enabled researchers to develop a model of the left ventricle, which was printed and subsequently cultured. This small ventricle showed contractility and other cardiac features throughout the entire printed structure (5).

Tissue-Supporting 3D Scaffolds – Endogenous scaffolds, like ECM, are required to provide physical support for creating tissues and/or organs, and to provide the necessary microenvironment for cell signaling, cell communication, biochemical reactions, and cellular function. Such scaffolds are essential for complex organs like the heart; thus, scaffolds must be engineered in parallel with the tissues themselves.

- Available synthetic materials or materials that elicit immune reaction (e.g. decellularized or interspecies materials) jeopardize the success of engraftment of engineered tissues, creating a great need for a biomaterial scaffold that is compatible with any recipient.
- Recent advances include a method to process patient-specific ECM into a hydrogel to support efficient iPSC reprogramming and differentiation, and to ensure engraftment without rejection (6). This method was used to efficiently differentiate iPSCs to CMs used to create patient-specific cardiac patches.
- A combination of advanced 3D printing techniques and ECM-derived hydrogel support matrix enabled the recent bioengineering of vascularized and perfusable cardiac patches that offer a path to bioengineered heart (7).

Gene Therapy – Gene therapy is a controversial mechanism for treating SV, in part because heart form so early in development. Currently, no gene therapies are available or in clinical trials for SV, but interest in cardiovascular gene therapy is mounting (20). Current challenges to gene therapy in SV include:

- *Insufficient knowledge of genetic and epigenetic SV etiology and progression.* Therapeutic target selection requires knowledge of both the biology and mechanisms that drive SV and/or its comorbidities.
 - For example, labs have shown altered atrial action potential duration as a mechanism of arrhythmias. Indeed, a selective potassium channel blockade with KCNH2-G628S (a dominant negative mutant) using epicardial atrial gene transfer in porcine AF model prevented atrial fibrillation (21). Subsequently, preclinical testing in pigs with post-operative AF confirmed this result (22).

- *Suboptimal targeted delivery to disease sites.* While there are ongoing trials using adenoviral, retroviral, and plasmid-based vectors, these are limited and have been mostly negative.
 - Although considered generally safe, major problems include low gene transfer efficiency, short expression, potential immunological reactions and placebo or off-target effects (20). Promising recent directions from the lab bench include: Coated Cationic Lipoparticles (CCLs) -based delivery of anti miRNA-712 oligo (against pro-atherogenic, mechanosensitive miR-712 upregulated in atherosclerosis) to inflamed mouse endothelial cells in vivo (23).

MEDICAL DEVICES

Creating implantable biological devices to improve blood flow in SV patients could prove to be a functional cure that eliminates the long-term complications and comorbidities associated with the Fontan circulation. Below, ventricular assist devices, the Rodefeld pump, and biopumps are explored.

Ventricular Assist Devices (VADs) – Clinicians utilize VADs in certain patients to help with heart function and/or to save a failing Fontan, if needed. In fact, VADs could serve as a long-term solution, providing mechanical circulatory support to fix the Fontan circulation and alleviating end organ dysfunction, as well as use for a bridge to transplant. However, while currently available VADs can improve ventricular function and blood flow, these are not built specifically for the SV or Fontan physiology.

Two additional major issues limit mechanical device functionality and application in very small children: 1) the devices currently on the market are too large and 2) the device does not grow with the child, even if miniaturized, and would eventually require replacement (24,25).

- A recent study in 13 children with advanced heart failure in Hong Kong using 5 different types of VADs showed overall promising results; however, use was associated with two deaths and severe morbidities. Of these patients, one was an SV patient with a failing Fontan circulation; this patient had generally positive outcomes (25).
- Jarvik 2015 is the first miniature continuous-flow VAD specifically designed for small children and is currently in the NHLBI PumpKIN Clinical Trial for testing in kids, infants, and neonates, including those with SV (26).
- The ACTION network is currently focusing on improving outcomes for VADs in pediatric patients.

Rodefeld Fontan Blood Pump – Fontan patients display a wide range of abnormal heart anatomies, and as such, anatomically-specific, Fontan circulation support devices are in great demand. Such devices ultimately hope to alleviate the need for heart transplantation or at least to effectively bridge Fontan patients to transplant. One such device-in-progress is a blood pump designed for implantation into the cavopulmonary connection proposed by Dr. Rodefeld (27). Designed to specifically assist the univentricular Fontan circulation, this implantable rotary propeller is a blood pump meant to achieve proper hemodynamics in Fontan patients and prevent Fontan failure. More research is required to develop the functional prototype of Rodefeld’s small implantable pump, to demonstrate its feasibility, safety, and durability and to translate it to clinical practice.

Tissue Engineered Device – Tissue-engineered biological devices that can grow and function in a child and accommodate multiple physiologies would be transformative. The impact of such devices could be two-fold:

- *Fix the Fontan circulation.* A biological pump could be used in Fontan patients to create pulsatility and functionally cure the altered hemodynamics caused by surgical intervention, thus alleviating downstream complications. Such a pump could be inserted at the time of or post-Fontan procedure in those currently living with the Fontan circulation. It may even be possible for the biopump to replace the Fontan procedure entirely.
- *In utero intervention.* Performing tissue engineering approaches in combination with *in utero* procedures could be curative for some SV. In patients where a small imperfection in the underlying cellular physiology lead to blood flow changes which cause poor ventricle or valve formation, surgical interventions could occur *in utero* where a tissue-engineered conduit is inserted to restore flow, allowing the ventricle to form normally.

PERSONALIZED MEDICINE APPROACHES

Computational Modeling and Simulations – In the adult heart disease field, computational modeling and simulation tools have enjoyed great success in the diagnosis of disease, surgical decision making, and surgical guidance. Modeling methods are becoming crucial tools for clinical interventions, as these hold the promise of providing individualized, patient-specific approaches to treatment. As such, modeling approaches are emerging in clinical trials. For example, the application of computational fluid dynamics to coronary computed tomography images provided an accurate and non-invasive way to diagnose coronary artery disease (28,29). Additionally, in the context of arrhythmias (specifically atrial fibrillation) personalized computational modeling of fibrotic atria offered optimal ablation targets for guiding surgical elimination of AF (30).

Similar advances are still scarce in the pediatric and congenital heart disease clinical spaces (29).

Excitingly, there is an increase in computational modeling and simulation in congenital heart diseases, including SV. However, the scope and impact of computational modeling in SV remains largely limited to the surgical realm, rather than in other aspects of SV including diagnosis, management, and treatment paradigms.

- Single ventricle physiology and Fontan circulation, primarily in the context of HLHS, have been extensively computationally modelled (31,32). Such modeling, for example, has been useful for exploring the impact and advantages and disadvantages of various surgical innovations and their effect on hemodynamics.
- Computational 3D modeling has become an important tool in the SV field for comparing the effects of various surgical techniques on the hemodynamics in the resulting single ventricle physiology (29). For example:
 - Modeling of various shunts (Sano, Blalock and Taussig) and Hybrid Norwood in HLHS patients suggested that a hybrid approach is inferior to shunts in terms of systemic and cerebral oxygen delivery (33).
 - Modeling of Hemi-Fontan or bidirectional Glenn in order to achieve superior cavapulmonary circulation suggests no difference between the two in terms of hemodynamic or physiological outcomes, including cardiac output (34).
 - Postoperative complications remain a significant concern for Fontan patients and it is plausible that further modeling, accounting for new information on long-term complexity of palliative ‘side effects’, can inform surgical procedures.

- Similarly, computational modeling and simulation are helpful in understanding how a mechanical support device alters hemodynamics in a Fontan patient. For example, computer simulation experiments were used to examine the effect of Rodefeld's cavopulmonary assist device on hemodynamics, hydraulics and other responses (35).
- In a recent study, a group conducted a retrospective simulation of the response to exercise in Fontan patients. The work attempted to understand the relevant physiologic responses and enable prediction of patient-specific exercise capacity for Fontan physiology and highlights the gap in clinical data collection (36,37).

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