# Clinical Study Protocol

PROTOCOL NUMBER: PHN-Udenafil-04

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| **Title** | IMAGING AND BIOMARKER EVALUATION OF HEPATIC STIFFNESS IN CHILDREN ENROLLED IN THE FONTAN UDENAFIL EXERCISE LONGITUDINAL STUDY |
| **Phase** | Open Label Extension of parent study |
| **IND Number** | 121648 |
| **Investigational Study Products** | Udenafil |
| **Protocol Version** | Version 3.0 |
| **Final Date** | December 11, 2017 |
| **Sponsor:** | Mezzion Pharma Co. Ltd. in Partnership with Pediatric Heart Network (PHN) |
| **Sponsor Contact:** | Mezzion Pharma Co. Ltd. James L. Yeager, RPh, PhD, Tel: 847-283-9176<br>Pediatric Heart Network (PHN) Gail D. Pearson, MD, ScD, Tel: 301-435-0524 |
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***CONFIDENTIAL***

**PROTOCOL REVISION HISTORY**

| Version History | | |
|---|---|---|
| Version Number | Version Date | Comment(s) |
| Version 1.0 | November 1, 2017 | Original Protocol |
| Version 2.0 | November 17, 2017 | Administrative clarification as it relates to consent/assent timing. |
| Version 3.0 | December 11, 2017 | Change of planned sample size from 100-150 subjects to 50-150 subjects.<br>Removal of Certificate of Confidentiality language. |

**SIGNATURE PAGE**

IMAGING AND BIOMARKER EVALUATION OF HEPATIC STIFFNESS IN CHILDREN ENROLLED IN THE FONTAN UDENAFIL EXERCISE LONGITUDINAL STUDY

Protocol Number: PHN-Udenafil-04

**Sponsor Approvals:**

Signature: James L. Yeager Digitally signed by James L. Yeager Date: 2017.12.18 15:00:52 -06'00' Date: _____

James L. Yeager, RPh, PhD

Signature: Gail D. Pearson -S Digitally signed by Gail D. Pearson -S Date: 2017.12.19 16:08:33 -05'00' Date: _____

Gail D. Pearson, MD, ScD

**INVESTIGATOR AGREEMENT PAGE**

I have read the protocol and agree to conduct the study as outlined herein.

I will use the informed consent form approved by the NHLBI and will fulfill all responsibilities for submitting pertinent information to the Institutional Review Board or Ethics Committee responsible for this study.

I further agree that the NHLBI and/or its designee has access to any source documents from which case report form information may have been generated.

I also agree to handle all clinical supplies (including drugs, biologics, and/or devices) provided by Mezzion® and collect and handle all clinical specimens in accordance with the protocol.

Signature: _____ Date: _____

Name:
(print):

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## 1.0 PROTOCOL SYNOPSIS

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|---|---|
| **TITLE OF STUDY** | AN IMAGING AND BIOMARKER EVALUATION OF HEPATIC STIFFNESS IN CHILDREN ENROLLED IN THE FONTAN UDENAFIL EXERCISE LONGITUDINAL STUDY |
| **PHASE** | Open Label Extension |
| **STUDY OBJECTIVE** | Determine scope of hepatic stiffness in Fontan patients by ultrasound and magnetic resonance imaging elastography and evaluate the efficacy of udenafil in reducing liver stiffness. |
| **SIGNIFICANCE** | Study will obtain necessary and broad information regarding hepatic stiffness in Fontan patients and will be the first to evaluate a potentially efficacious treatment to reduce liver stiffness. |
| **STUDY DESIGN** | Prospective cohort study integrated with the Fontan Udenafil Exercise Longitudinal (FUEL) clinical trial |
| **PLANNED SAMPLE SIZE** | 50-150 Subjects |
| **KEY SUBJECT SELECTION CRITERIA** | Inclusion Criteria:<br>1. Enrollment in FUEL Extension Trial<br>2. Informed assent from subject, informed consent from parent/legal guardian as appropriate<br><br>Exclusion Criteria:<br>1. Non-enrollment in the FUEL Extension Trial<br>2. Subjects with contra-indications for MRI (these subjects will be excluded from the MRI component of this study)<br>3. Other exclusionary criteria will match those used for the FUEL Extension Trial |
| **TREATMENT** | One year of udenafil treatment (87.5 mg twice daily) |
| **PRIMARY AIM** | Define the range of liver stiffness in those who have had the surgical creation of a total cavopulmonary connection. |

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| **SECONDARY AIMS:** | • Determine the impact of udenafil treatment on liver stiffness in a large cross-section of adolescents who have undergone total cavopulmonary connection.<br>• Determine the relationship between clinical outcomes and liver stiffness.<br>• Assess the association between biomarkers of both heart failure and liver fibrosis and liver stiffness. |
| **EFFICACY PARAMETERS** | Reduction in liver stiffness after initiating udenafil therapy adolescents with Fontan physiology. |
| **EFFICACY ASSESSMENTS** | Efficacy will be assessed at FUEL-OLE initiation and conclusion. |
| **EFFICACY ENDPOINTS** | Absolute liver stiffness as measured by ultrasound (and MR) and change in liver stiffness. |
| **SAFETY PARAMETERS** | As this study has no active intervention, there are no specific safety measures beyond FUEL-OLE protocols. |
| **SAFETY ASSESSMENTS** | As this study has no active intervention, there are no specific safety assessments beyond FUEL-OLE protocols. |
| **STASTICAL ANALYSES** | **Analysis Populations:** All individuals enrolling in FUEL-OLE will be recruited for enrollment in the FALD study. At completion of FUEL-OLE, once FUEL assignments are unblended (udenafil or placebo), the cohort will be stratified into udenafil naïve vs. udenafil continuation groups. Analysis will be done both on an intent-to-treat and a per-protocol basis.<br><br>**General Statistical Methods:** This is prospective observational study of liver stiffness in Fontan patients. Absolute liver stiffness and association of degree of stiffness liver biomarkers will be assessed for the entire cohort. Change in liver stiffness over the 12-month FUEL-OLE duration will be assessed both for the overall cohort and after stratification into udenafil naïve vs. udenafil continuation groups. |
| **FINAL DATE** | December 11, 2017 |

## 2.0 LIST OF ABBREVIATIONS

| Abbreviation or Term | Definition/Explanation |
|---|---|
| ARFI | Acoustic Radiation Force Impulses |
| BNP | Brain natriuretic peptide |
| CAE | Common adverse event |
| CRF | Case report form |
| CHD | Congenital Heart Disease |
| DCC | Data coordinating center |
| DMS | Data Management System |
| EDC | Electronic data capture |
| ELF | Enhanced Liver Fibrosis |
| ELISA | Enzyme linked immunosorbent assay |
| FALD | Fontan-associated Liver Disease |
| FDA | Food and drug administration |
| FUEL | Fontan Udenafil Exercise Longitudinal Trial |
| HA | Hyaluronic Acid |
| ID | Identification |
| IRB | Institutional Review Board |
| MRI | Magnetic Resonance Imaging |
| NHLBI | National Heart, Lung, and Blood Institute |
| PAT | Pulse amplitude tonometry |
| PDE5i | Phosphodiesterase type 5 inhibitor |
| PHN | Pediatric Heart Network |
| PI | Principal investigator |
| PIIINP | Amino-terminal propeptide of type III collagen |
| RCT | Randomized control trial |
| ROC | Receiver operating characteristic |
| RT | Reverse Transcription |
| SAP | Statistical Analysis Plan |
| SWE | Shear Wave Elastography |
| SAE | Serious adverse event |

| | |
|---|---|
| TCPC | Total Cavo-pulmonary Connection |
| TIMP-1 | Tissue inhibitor of metalloproteinase-1 |
| UMPL | University of Michigan Pathology Laboratory |
| US | Ultrasound |

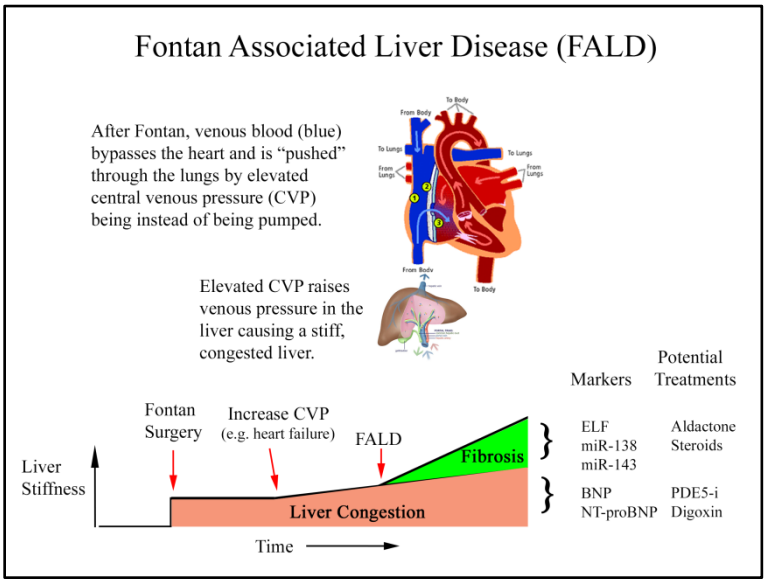
# 3.0 BACKGROUND INFORMATION AND RATIONALE

## 3.1 Discussion of Hepatic fibrosis and FUEL Trial Extension Study

### 3.1.1 FALD

Fontan-associated liver disease (FALD) is defined as abnormalities in liver structure and function resulting from the abnormal circulation created by the total cavopulmonary connection (TCPC) and not related to any other process (1). FALD is increasingly recognized as one of the most common complications of the TCPC (2, 3) and one which is universally present in patients with TCPC physiology (8-11). After the Fontan operation, blood from the inferior and superior vena cava are routed directly to the pulmonary arteries bypassing the heart (Figure 1). In this circulation, blood is not "pumped" through the lungs but is "pushed" through by chronic elevation of the central venous pressure. It is postulated that the Fontan's elevated, non-phasic systemic venous pressure results in obligate chronic hepatic venous hypertension and congestion that is likely an important driver of hepatic fibrosis. As part of the injury response, activated myofibroblasts deposit excess extracellular matrix in the perisinusoidal space, a process which, if it persists, can lead to increased hepatocellular injury, progressive fibrosis, organ dysfunction, and, ultimately, to cirrhosis and hepatic failure. The congestion and fibrosis result in a wide spectrum of liver disease, including synthetic dysfunction, ascites, portal hypertension, cirrhosis, and hepatocellular carcinoma, and may culminate in fulminant liver failure. The severity of liver fibrosis is known to increase with time after TCPC and occurs in the absence of any other identifiable etiology of chronic liver disease.

**Figure 1: Description of Fontan Associated Liver Disease**



### 3.1.2 Characterization of FALD

A roadblock to understanding and treating FALD is the lack of an established, noninvasive means of detecting it. Basic laboratory testing (platelet count, AST, ALT, bilirubin, GGT, INR) and standard imaging (conventional ultrasound (US), computed tomography (CT) or magnetic resonance imaging (MRI)) are insensitive to liver congestion and early liver fibrosis in these patients (8). Percutaneous core needle liver biopsy, while the current standard of care, is not an ideal surveillance tool as the core sample may falsely under- or over-estimate the degree of fibrosis given the significant heterogeneity that often exists. Liver biopsy is also invasive and carries a small but real risk of bleeding, particularly in patients who require anti-coagulation.

Ultrasound shear wave elastography (SWE), based on the measurement of tissue shear wave speed, provides a non-invasive measure of liver stiffness and is an extremely promising technology to monitor the congestion and early fibrosis that precedes overt FALD. The utility of ultrasound SWE in assessing liver fibrosis has been well-documented (9, 10) and image-guided ultrasound SWE has been used in TCPC patients to demonstrate that liver stiffness correlates with hepatic congestion (as determined by elevated central venous pressures) and hepatic fibrosis (as determined by liver biopsy) (11-13). MRI-elastography may be of even greater utility as it allows sampling over a much larger region of the liver (thus, minimizing sampling error) and may potentially allow differentiation of stiffness due to fibrosis versus congestion, a feature which may be particularly useful in the evaluation of Fontan patients and in determining the best approach to targeted therapy in this population (14).

### 3.1.3 FALD and FUEL Extension Study

The FUEL Extension trial is an NHLBI-sponsored study that will assess the efficacy of safety and efficacy of udenafil, a phosphodiesterase-5 inhibitor (PDE-5i) that causes relaxation and decongestion of the pulmonary and systemic venous vasculature, to improve Fontan cardiac function, peripheral endothelial function, and exercise performance in adolescents who have undergone TCPC. By partnering with the FUEL Extension trial, we will have the unique opportunity to 1) measure liver stiffness across a large cross-section of children and adolescents after TCPC; 2) assess how liver stiffness responds to vasodilator therapy determine; 3) determine if liver stiffness correlates with important functional outcomes; and 4) determine the relationship between biomarkers of heart failure and liver fibrosis and liver stiffness. Currently, PDE-5i therapy after the Fontan is reserved for those patients who have clinically symptomatic heart failure. However, a significant and sustained reduction in liver stiffness with PDE-5i therapy would suggest that earlier treatment might reduce the incidence, severity and rate of progression of FALD. This has important implications for a population in whom the rate of liver fibrosis is a critical determinant of overall long-term health.

## 3.2 Rationale for the Study and Study Outcomes

### 3.2.1 Study Rationale

While there have been many small studies that have used SWE to evaluate the degree of liver stiffness in patients who have undergone TCPC, there has not been a large-scale evaluation that defines "normal" in this population or seeks to correlate liver stiffness with functional outcome. Similarly, the impact of treatment with pulmonary vasodilators, medications that should lower systemic venous pressure, has not been evaluated. The FUEL Extension trial presents a unique opportunity to measure liver stiffness in those with TCPC, to evaluate the impact of udenafil on liver stiffness, to correlate liver stiffness with functional outcomes, and to gain valuable insight into the relationship between liver stiffness and biomarkers of heart failure and fibrosis.

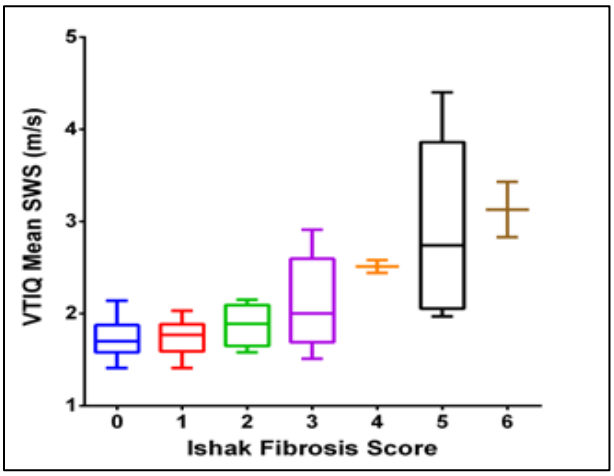
### 3.2.2 Rationale for Study Outcomes

#### 3.2.2.1 The use of ultrasound SWE to evaluate liver fibrosis

Ultrasound SWE uses one or more acoustic radiation force impulses (ARFI) or "push pulses" to generate shear waves in the tissue of interest. These shear waves can be tracked and their speed measured, with increasing tissue stiffness associated with increasing tissue shear wave speed. Shear wave speed can be mathematically converted to both shear and Young's moduli (in kPa), mechanical properties related to tissue rigidity and elasticity. There are two major forms of ultrasound SWE – "point" and "2D". Point ultrasound SWE provides a very focal estimate of liver stiffness using a small region-of-interest, while 2D ultrasound SWE provides a regional color parameteric map (elastogram) of image stiffness and allows measurement over a much larger area of the liver.

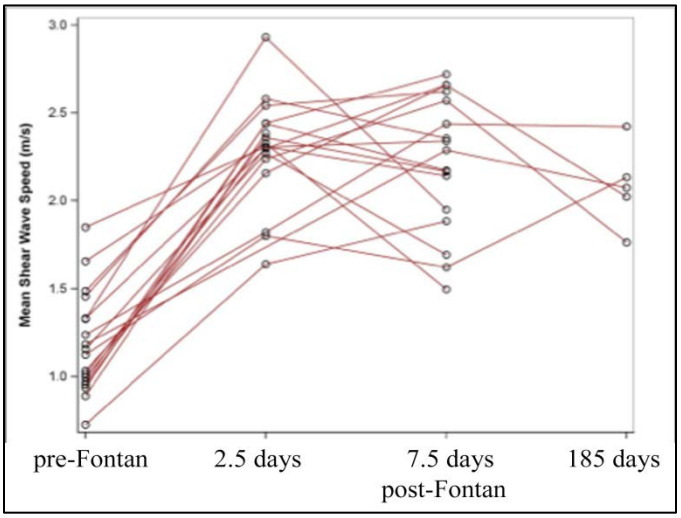
We have demonstrated excellent inter- and intra-observer reproducibility of ultrasound SWE measurements in soft and hard tissue-mimicking elasticity phantoms using two different US systems (Supersonic Imagine and Siemens Medical Solutions USA) and five different US transducers. Coefficients of variation between various combinations of US systems, transducers, phantoms, and phantom depths were low, ranging from 0.5-6.8%, and inter-operator agreement (four operators) was near-perfect (ICCs $\geq$ 0.99) (15). We also have explored the in vivo relationship between liver shear wave speed and histologic fibrosis and observed significant positive correlations between liver shear wave speed and histologic fibrosis using point and 2D ultrasound SWE (Siemens Acuson S3000) ($r = 0.68$ and $r = 0.73$; p-values $<0.0001$) (Figure 2).

**Figure 2: Box plot showing increasing liver shear wave speed measurements with increasing parenchymal fibrosis.**




Receiver operating characteristic (ROC) areas under the curve for discriminating Ishak fibrosis scores "0-2" (no/mild fibrosis) from "3-6" (moderate/severe fibrosis) were 0.84 and 0.86 for point and 2D ultrasound SWE, respectively. In a recent prospective longitudinal observation study, we have shown that children with single ventricle congenital heart disease experience marked liver stiffening immediately after the TCPC. Five subjects returned at a mean of 185 ± 28 days, and mean liver stiffness remained elevated above baseline but with a variable response between patients when comparing the change in stiffness from early (2.5 and 7.5 days post-op) to late (6 month post-op) time points, which may reflect individual differences in cardiovascular performance or functional reserve (Figure 3).

**Figure 3: Individual profile plots from subjects undergoing stage 3 Fontan palliation**



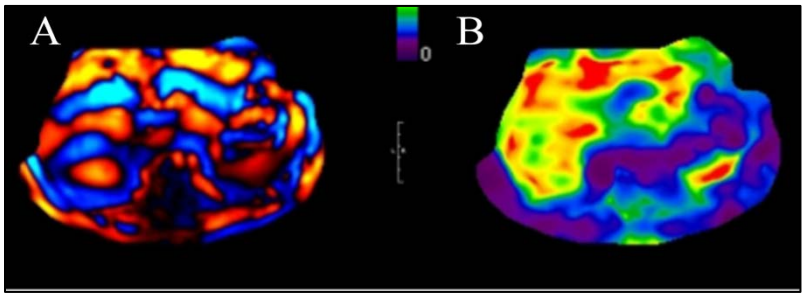

These plots show changes in liver shear wave speed (stiffness) over time (follow #1 = 2.5 days post-op, follow-up #2 = 7.5 days post-op, and follow-up #3 = 185 days post-Fontan).

### 3.2.2.2 The use of Magnetic Resonance Elastrography to evaluate liver fibrosis

MR elastography uses a vibrating passive driver or "paddle" placed on the abdominal wall over the liver in order to generate in vivo shear waves. These shear waves can be tracked using an MRI modified phase contrast pulse sequence. Shear wave data is then used to generate color parameteric maps (elastograms) of liver stiffness that allow quantitative assessment (Figure 4). We have demonstrated excellent agreement when using intra-class correlation coefficients to assess reproducibility of MR elastography measurements across MRI scanners (Philips and GE Healthcare), field strengths (1.5T and 3T), and pulse sequences in 24 adult healthy volunteers. Pairwise ICCs ranged from 0.67-0.82 when agreement was assessed between individual sequences across manufacturers (GE vs. Philips); the greatest agreement while fixing the pulse sequence was at 1.5T using 2D GRE sequences (ICC=0.82, r=0.85) (16).

A study by Wallihan et al (11) showed that MRI-derived liver stiffness was universally elevated in a cohort of children and young adults that had undergone the Fontan procedure. They also found a statistically significant inverse correlation between liver stiffness and cardiac index (p=0.02) and ejection fraction (p=0.002). Patients with long Fontan duration (>20 years post-op) had greater liver stiffness compared with those having a shorter duration (p=0.02). The study provided evidence that elevated liver stiffness in Fontan patients is likely due to both fibrosis and congestion.

**Figure 4: 13 year-old patient status post Fontan palliation due to Single Ventricle Congenital Heart Disease.**




**A**, MR elastography color wave image shows mechanically-induced shear waves in the liver. **B**, Color parametric map (elastogram) shows the distribution of stiffness within the upper abdomen, including the liver. The liver is very stiff and heterogeneous.

### 3.2.2.3 Biomarkers and liver stiffness

As noted previously, increased liver stiffness can be secondary to hepatic congestion and/or hepatic fibrosis. Circulating biomarkers previously demonstrated to be associated with congestive heart failure or tissue fibrosis may help to determine the primary driver of liver stiffness in a particular patient and help to monitor the response to targeted therapy. For instance, we would anticipate that patients with increased liver stiffness due to hepatic congestion would have increased biomarkers of heart failure (e.g., BNP, NT-proBNP). Similarly, patients in whom fibrosis significantly contributes to increased liver stiffness may have elevation in biomarkers associated with liver fibrosis (e.g., ELF panel, miR-138, miR-143, or galectin-3). The relative contribution of congestion or fibrosis (as determined by biomarker profiles) to the observed level of liver stiffness may correlate with which therapeutic strategies are likely to be successful in a particular patient. Therefore, in this aim, we will determine which circulating biomarkers correlate with measures of liver stiffness and which correlate with a change in liver stiffness with udenafil treatment. We will use both a directed approach, with an analysis of candidate biomarkers validated for other disease processes, and a non-directed approach, which will identify novel biomarkers potentially associated with FALD.

# 4.0 CLINCIAL TRIAL OBJECTIVES, AIMS, AND HYPOTHESES

## 4.1 Clinical Trial Objective

Determine scope of hepatic stiffness in Fontan patients by ultrasound and magnetic resonance imaging elastography and evaluate the efficacy of udenafil in reducing liver stiffness.

## 4.2 Primary Aim and Hypothesis

Primary Aim: Define the range of liver stiffness in those who have had the surgical creation of a total cavopulmonary connection.

Hypothesis: Following total cavopulmonary connection, children with single ventricle congenital heart disease will have elevated measures of liver stiffness.

## 4.3 Secondary Aims and Hypotheses

Secondary Aim 1: Determine the impact of udenafil treatment on liver stiffness in a large cross-section of adolescents who have undergone total cavopulmonary connection.

Hypothesis: Udenafil treatment will reduce liver stiffness as measured by non-invasive elastography by decreasing venous congestion.

Secondary Aim 2: Determine the relationship between clinical outcomes and liver stiffness.

Hypothesis: Liver stiffness will be associated with measures of ventricular diastolic function, exercise performance, and endothelial function.

Secondary Aim 3: Assess the association between biomarkers of both heart failure and liver fibrosis and liver stiffness.

Hypothesis: Biomarkers of heart failure and liver fibrosis will directly correlate with liver stiffness as measured by ultrasound and magnetic resonance elastography.

## 5.0 STUDY DESIGN

This is an open-label extension study that integrates with the FUEL Extension Trial. FUEL-enrolled patients will have been randomized and double-blinded to either placebo or udenafil, and all patients entering the FUEL Extension trial will be placed on udenafil therapy. Therefore, approximately 50% of patients eligible to be enrolled in our study will be udenafil-naïve (U-) and 50% will have already completed 6-months of therapy (U+). As a part of FUEL-OLE, each participant will receive one year of udenafil treatment (87.5 mg twice daily). Both the U- cohort and the U+ cohort will have liver stiffness measured by ultrasound and MR elastography (where available), as described above. Thus, the U- subjects will have elastography measurements pre-udenafil and after 1 year of treatment. This will determine the impact of udenafil therapy on liver stiffness. Additionally, the U+ cohort from FUEL will allow us to determine if potential benefits of udenafil therapy are sustained over time by comparing measurements after 6 (baseline entry into our study) and 18 months of therapy (after 1-year of our study). The study visits are as follows:

1. Baseline visit: during the baseline visit, subjects will undergo ultrasound SWE and, if available at their center, magnetic resonance elastography. These will be coordinated with FUEL extension testing to ensure that the primary study is not interrupted. The additional blood work for this protocol will be obtained at the same time as the blood work for the FUEL extension study.
2. Follow-up visit: during the follow-up visit, subjects will undergo ultrasound SWE and, if available at their center, magnetic resonance elastography. These will be coordinated with FUEL extension testing to ensure that the primary study is not interrupted. The additional blood work for this protocol will be obtained at the same time as the blood work for the FUEL extension study.

### 5.1 Study Duration

The planned clinical trial duration from the start of subject screening and enrollment to last subject out is anticipated to be from 12 to 24 months concurrent with FUEL-OLE. The final evaluation within the current study will be concurrent with the 12-month FUEL-OLE completion visit. Thus, each individual subject will be enrolled in the current study for only 12 months.

# 6.0 SELECTION AND DISPOSITION OF SUBJECTS

## 6.1 Number of Subjects

Subjects for this protocol will be recruited from the pool of patients in the FUEL Extension trial, from all participating centers that have the capacity to perform either ultrasound SWE or magnetic resonance elastography. We anticipate that we will have access to 100-200 subjects, although this will vary based on the start date for this study. If we achieve between a 50-75% consent rate, it would provide a sample size of 50-150 subjects.

## 6.2 Clinical Trial Population Characteristics

In order to be eligible for the clinical trial, subjects must fulfill the following criteria. Male and female subjects 12-19 years of age who meet all the Inclusion criteria and none of the Exclusion criteria will be enrolled.

## 6.3 Subject Inclusion Criteria

1. Enrollment in FUEL Extension Trial
2. Informed assent from subject, informed consent from parent/legal guardian as appropriate

## 6.4 Subject Exclusion Criteria

1. Non-enrollment in the FUEL Extension Trial
2. Subjects with contra-indications for MRI including presence of any indwelling, significant, ferromagnetic objects (including pacemakers and implantable cardiac defibrillators, or those unable to lie in the scanner without sedation (these subjects will be excluded from only the MRI components of this study)
3. Other exclusionary criteria will match those used for the FUEL Extension Trial

## 6.5 Subject Withdrawal Criteria

Subjects may voluntarily withdraw from the study at any time. In addition, the site principal investigators are free to withdraw a subject at any time for reasons of medical prudence. Any subject withdrawn from the FUEL Extension study will also be withdrawn from this ancillary study.

# 7.0 TREATMENT DESCRIPTION

## 7.1 Description of Treatment

All subjects participating in the present protocol will be participants in the FUEL-OLE protocol. Subjects in FUEL-OLE will be treated with udenafil for one year's time (87.5 mg twice daily). No additional treatment will occur in the present study.

## 7.2 Subject Identification Number

Each subject is assigned a subject identification number (SID). All interview and clinical research data are stripped of identifiers and labeled with the study number. The enrollment log with participant identifiers will be maintained at each site in a secured, locked location available only to the study staff. The informed consent form states that study data will be made available to the Data Coordinating Center (DCC) and NIH/NHLBI to ensure study safety and quality control. The subject's name and any other identifying information will not appear in any presentation or publication resulting from this study.

## 7.3 Method of Treatment Assignment and Blinding

Not applicable. This study is open label.

## 7.4 Sample Management

Sample collection and brain natriuretic peptide (BNP) testing will be performed during the collection of samples for the FUEL-OLE study. A plasma sample for BNP level will be obtained at the time of entry into the study and at the end of the 1-year open label extension trial. The samples will be collected and processed on-site and the plasma will be shipped on dry ice to the FUEL-OLE Central Laboratory located at the University of Michigan. The Central Laboratory will aliquot a portion of the sample for BNP testing in the University of Michigan Pathology Laboratory (UMPL) which performs this test for all patients seen in the University of Michigan Health System. In addition to this sample, FALD specific labs will be obtained at the same venipuncture. The samples that will be collected for those patients enrolled in the FUEL-OLE and the ancillary study are as follows:

*Blood Samples*

- 3 mL in a purple top (K2EDTA) PPT tube for BNP measurement (FUEL-OLE sample)
    - Invert the tube gently five times. Further inversion may cause alterations in sample integrity.
    - Centrifuge 10 minutes at 1600 x g using a refrigerated centrifuge (4°C)

    - For each sample, the separated plasma will be transferred into two labeled polypropylene tubes (approximately 750 µL each), which will be stored at approximately -70°C within 2 hours of collection.
- 3.5 mL in a yellow top (SST) tube for serum isolation (Liver study sample)
    - Invert the tube gently five times. Further inversion may cause alterations in sample integrity.
    - Allow to clot for 30 min at room temperature
    - Centrifuge 15 minutes at 1600 x g using a refrigerated centrifuge (4°C)
    - For each sample, the separated serum will be transferred into two labeled polypropylene tubes (approximately 750 µL each), which will be stored at approximately -70°C within 2 hours of collection.

*Biomarker Testing*

- BNP testing will be performed by the UMPL
- NT-proBNP levels measurements will be performed on the residual plasma sample using a commercially available enzyme linked immunosorbent assay (ELISA) that will be run in the FUEL-OLE Central Laboratory.
- MicroRNA measures will be performed on the residual plasma sample in the FUEL-OLE Central Laboratory on total RNA isolated using miRNeasy total RNA preps (QIAGEN, Valencia, CA). miRNA qRT-PCR analysis will be performed using Taqman miRNA assays (Applied Biosystems, Foster City, CA). 5 ng total RNA will be used as an input into each reverse transcription reaction (RT) for each miRNA. Four replicates will be done for each miRNA, consisting of two replicate PCR reactions from each of the two replicate RT reactions, and the results were averaged. PCR reactions will be run on a 7500 Real Time PCR machine (Applied Biosystems) and analyzed using 7500 System SDS software (v1.4). For the candidate biomarkers, an equivalent number of anonymous, age-matched, healthy controls will be used for comparison. The control cohort will be derived from healthy individuals who present to the University of Michigan for elective surgical procedures.
- Enhanced Liver Fibrosis (ELF) score which is based on the serum levels of hyaluronic acid (HA), amino-terminal propeptide of type III collagen (PIIINP), and tissue inhibitor of metalloproteinase-1 (TIMP-1). Testing will be performed by the FUEL-OLE Central Laboratory using the serum sample. In accordance with prior studies, we will assign those patients with scores of <7.7 to the no or mild fibrosis group, 7-7-9.8 to the moderate fibrosis group, and >9.8 to the severe fibrosis group.

BNP testing will be performed by the UMPL, as described above. NT-proBNP levels measurements will be performed using a commercially available enzyme linked immunosorbent assay (ELISA) that will be run in the Russell laboratory. MicroRNA measures will be performed

in the Russell laboratory on total RNA isolated from plasma using miRNeasy total RNA preps (QIAGEN, Valencia, CA). miRNA qRT-PCR analysis will be performed using Taqman miRNA assays (Applied Biosystems, Foster City, CA). 5 ng total RNA will be used as an input into each reverse transcription reaction (RT) for each miRNA. Four replicates will be done for each miRNA, consisting of two replicate PCR reactions from each of the two replicate RT reactions, and the results were averaged. PCR reactions will be run on a 7500 Real Time PCR machine (Applied Biosystems) and analyzed using 7500 System SDS software (v1.4). For the candidate biomarkers, an equivalent number of anonymous, age-matched, healthy controls will be used for comparison. The control cohort will be derived from healthy individuals who present to the University of Michigan for elective surgical procedures.

Proteomic and transcriptomic analysis will take place using a multiplex Tandem Mass Tag (TMT) system allowing concurrent analysis of multiple samples. Plasma samples will be depleted of 14 high-abundant proteins using MARS14 (Agilent) or Seppro IgY14 (Sigma) columns. Depleted plasma samples (~50ug) will be alkylated and digested overnight. Samples will be labeled with the TMT Reagents per manufacturer's recommended protocol and mixed before sample fractionation (12 fractions). Each fraction will be analyzed by high-resolution, nano-LC-MS/MS using Orbitrap Fusion Tribrid mass spectrometer (ThermoFisher, Waltham, MA) to identify peptides/proteins and quantify reporter ion relative abundance. Data will be collected using the recently developed multinotch-MS3 method which improves the quantitation accuracy by minimizing the reporter ion ratio distortion resulting from fragmentation of co-isolated peptides (17). The data analysis will be performed using both commercial (Proteome Discoverer v 2.1) and publicly available software (e.g., MaxQuant). The performance and analysis of the proteomic profiling will be done in the Proteomics Resource Facility at the Department of Pathology, University of Michigan.

## 8.0 STUDY ASSESSMENTS/MEASUREMENTS AND PROCEDURES

### 8.1 Primary Assessments

- Range of liver stiffness in subjects with a surgically created total cavopulmonary connection

### 8.2 Primary Assessment Measurement

- Liver stiffness as determined by ultrasound shear wave elastography
- Liver stiffness as determined by magnetic resonance elastrography

### 8.3 Additional Measurements

- Evaluate serum biomarkers of heart failure, brain type natriuretic peptide (BNP) and n-terminal BNP (NT-BNP)
- Evaluate biomarkers of liver fibrosis
- Proteomic and transcriptomic analyses on subcohorts of patients with the following:
  - Low levels of liver stiffness
  - Very high levels of liver stiffness
  - Decrease in liver stiffness with udenafil treatment

## 9.0 PLANNED STATISTICAL ANALYSES AND STATISTICAL CONSIDERATIONS

### 9.1 Statistical Analysis Plan

A Statistical Analysis Plan (SAP) will be developed and finalized prior to database lock. The SAP will contain detailed descriptions of data conventions and statistical procedures. Any deviation(s) from the SAP will be described and justified in the clinical study report.

### 9.2 Sample Size Determination

Given the primary outcome is descriptive, sample size estimate is provided for secondary outcome 1 – evaluating the change in liver stiffness before and after udenafil treatment compared to a patient receiving on-going udenafil treatment. Elastography indices measured by US or MRI elastography from baseline to 12 months will be examined in both udenafil treatment groups (U+ and U-), using paired t-test or Wilcoxon signed- rank test, as appropriate. DiPaola et al. (17) demonstrated that liver stiffness measured by US-SWE significantly increased from baseline to 6-month post-TCPC (mean 1.18 ± SD 0.29 m/s to 2.08 ± 0.24 m/s, $p<.0001$). Assuming a mean liver sheer wave speed (SWS) of 2.08 with known SD 0.24 as our baseline liver stiffness, a total sample size of 24 subjects (12 per group) is required to achieve 85% power with alpha=0.05 to detect 10% reduction of liver stiffness (deemed clinically meaningful in an individual) at 12 months from baseline in the U- group. Assuming a maximal enrollment of 100 subjects, (approximately 50% of whom will be from the FUEL U- cohort), we would be powered to detect a difference of 3.75% reduction in liver stiffness.

### 9.3 Analysis Populations

All individuals enrolling in FUEL-OLE will be recruited for enrollment in the FALD study. At completion of FUEL-OLE, once FUEL assignments are unblinded (udenafil or placebo), the

cohort will be stratified into udenafil naïve vs. udenafil continuation groups. Analysis will be done both on an intent-to-treat and a per-protocol basis.

## 9.4 Statistical Analysis

### 9.4.1 General Methods

This is a prospective, observational, cohort study.

### 9.4.2 Primary Outcome

The absolute stiffness by SWE and by MR elastography will be reported using descriptive statistics as appropriate.

### 9.4.3 Secondary Outcomes

*Secondary outcome 1*

Baseline liver stiffness measured by ultrasound or MRI elastography will be compared between the two groups (U+ vs. U-) using appropriate statistics. Next we will assess change in liver stiffness from pre- to post-1 year udenafil treatment in the U-group as well as from 6 to 18 months of therapy in the U+ group.

*Secondary outcome 2*

Changes in liver stiffness, diastolic cardiac function (including E/E' and MPI), PAT measurement, and exercise measurements (including peak VO2 and submaximal exercise measures) from baseline to 12 months will be quantified. Correlation between change in liver stiffness and changes in diastolic cardiac function and exercise measurements in the entire cohort and in the U- group and U+ group separately.

*Secondary outcome 3*

Bivariate analyses will examine 1) the correlation between each biomarker and liver stiffness measured by ultrasound and MRI elastography and 2) the correlation between change in each biomarker and change in liver stiffness from baseline to 12 months in both the U+ and U- cohorts.

The proteomic and transcriptomic data will be presented primarily as a descriptive report. Pairwise differences between groups may be sought using appropriate 2-group statistics, but we expect the limited sample size in this exploratory outcome to limit analysis to descriptive statistics.

# 10.0 ETHICS AND GENERAL CLINCIAL TRIAL CONDUCT CONSIDERATIONS

## 10.1 Institutional Review Board or Independent Ethics Committee

## 10.2 Ethical Conduct of the Clinical Trial, Confidentiality, and Potential Risks

The clinical studies in this proposal meet the criteria in the NIH Supplemental Instructions for Preparing the Human Subjects Section of the Research Plan for Scenario B: Non-exempt human subject research, non-clinical trial. Although this will be a study ancillary to a clinical trial, this study is not a clinical trial; it collects additional data and measurements on all patients regardless of their status within FUEL-OLE.

## 10.3 Potential Risks

*Human Subjects Involvement, Characteristics, and Design*

Young adults and pediatric patients with single ventricle congenital heart disease will be managed as per the parent FUEL-OLE trial and otherwise per the standard of care; laboratory, radiologic, and echocardiographic testing will be performed within the framework of the FUEL-OLE trial and at interim time points per the standard of care. The research team will not be involved in clinical decisions. Patient demographics and available clinical and laboratory data will be recorded for the purposes of research.

*Biospecimen Collection*

No additional venipuncture beyond that already being obtained in FUEL-OLE will occur. Minor temporary discomfort may be associated with the removal of blood by venipuncture. There is a risk of bruising and a very small amount of bleeding associated with the blood drawing. There is also a very small risk of infection at the site. Whenever possible, blood samples will be gathered when the participant is scheduled for routine blood testing or procedures. Serum collection will occur via venipuncture simultaneously with FUEL-OLE collection. The total amount of serum obtained for both studies will be 6mL (3mL for FUEL-OLE and 3mL for this study), which is within safe range for adolescent patients within FUEL-OLE's height and weight criteria. No DNA samples specific to FALD beyond what is already collected in FUEL-OLE will be obtained.

*MRI*

Risks related to MRI include subject distress/claustrophobia, projectile injury, and scanning with ferromagnetic implanted material (e.g., pacemaker or aneurysm clip) in the subject. Subjects experiencing distress/claustrophobia during MRI will be allowed to opt out of the MRI portion of the study; their US results will still be included in our results. Additionally, individuals with any

significant ferromagnetic objects, specifically implantable cardiac defibrillators or pacemakers, should not undergo MRI and will have US results included in the study. MRI scanners will be operated within U.S. Food and Drug Administration guidance to minimize energy imparted into the subject and tissue heating.

*Ultrasound*

There are no known risks to ultrasound SWE. FDA-approved US systems will be employed that operate within U.S. Food and Drug Administration guidance with respect to energy imparted into the patient (thermal index and mechanical index).

## 10.4 Protection against Risks

Investigators will take all reasonable measures to protect the confidentiality of subjects and their families, including the following:

*Reporting of Test Findings*

The results of future tests on biological specimens will not be released to the subject/family. There is a reasonable possibility that no findings will result from this research effort. If findings are detected, it may be years before any utility of these findings is realized. Further, if samples are "anonymized" prior to release to other investigators for research, it may not be possible to trace the results back to the subject.

If an incidental finding is found on a study clinical test such, the PI or other qualified member of the research team will take full responsibility for disclosing the findings to the patients/parents, communicating with their primary cardiologist with permission, or making appropriate cardiology referrals as indicated. The subject may choose to seek a second opinion and/or appropriate clinical care. This might change the subject's insurability and employability as it relates to the clinical finding only. The presumption is that detection of a potentially clinically significant finding will prove to be beneficial.

## 10.5 Potential Benefits

*Study Findings*

At the end of the study study results will be provided to treating/referring physicians (cardiologist, etc). If udenafil is shown to reduce liver stiffness, it is possible that this can modify progression of FALD and could directly benefit the enrollee. This may directly benefit an individual by attenuating the disease. It is possible that the liver scans obtained for the study will demonstrate significant abnormalities in liver stiffness, and they may also find areas of significant fibrosis or cirrhosis. Although the clinical significance of such findings remains

unclear to the field, appreciation of these individual findings is likely to be beneficial for an individual's care.

*Biospecimens*

Identification of optimal serum biomarkers of liver stiffness may directly benefit the individual by allowing serial monitoring of FALD. In addition, we hope that the proteomic and transciptomic portion will help investigators to learn more about the relationship between genetic factors and pathophysiology of FALD. This information may help physicians provide better answers to families' questions regarding causes and risk. It may also provide clues to future interventions and/or treatments.

*Indirect Benefit*

There might be an indirect benefit from the awareness that study results may help to improve the care of children with similar problems in the future. Families may derive a sense of altruism, accomplishment, and contribution to furthering understanding of the problem through their participation.

### 10.6 Risk/Benefit Ratio and Importance of Information to be Obtained

The risk/benefit ratio is favorable for this study and adverse events are not anticipated. The baseline risk is minimal because there are no therapeutic interventions specifically for the FALD study. There are direct potential benefits as described above. In addition, although an individual subject may not benefit from participation, the results of this study will make important contributions to the improvement of knowledge of the causes of congenital heart disease (CHD), in the development of new diagnostic tests, and ultimately in the improvement of treatment and prognosis.

### 10.7 Study Limitations

While integrating with FUEL-OLE is a clear advantage and makes this FALD study feasible, the study will not have a placebo control arm. However, an active control (U+) should still allow assessment of how udenafil affects liver stiffness in a naïve patient. Each center may have access to only US SWE, MR elastography, or both. We expect most, if not all, centers to have access to US SWE, as it is now widely available, commonly employed in practice, and straightforward to use. Our (CCHMC) radiology core (including MRI physicist Suraj Serai, PhD, and imaging post-processing laboratory) will be able to facilitate MR elastography capabilities at participating centers, ensuring the use of a standardized protocol across scanners. Experience in processing and analyzing/interpretation of this relatively new measurement technique should be mitigated by use of a radiology core. MR elastography is more difficult to add for currently non-capable centers, thus, MR elastography measurements will be limited to centers that have capabilities.

# 11.0 REGULATORY/ADMINISTRATIVE PROCEDURES AND DOCUMENTATION

## 11.1 Data Collection

Data for each SWE and magnetic resonance elastography will be generated from each site and recorded on a case report form. This form will serve as the source document for each test and will be used to enter data into an electronic data capture (EDC) system (eCOS). US SWE data will be recorded from each site. MREL data will need to be sent anonymized to CCHMC for central processing/analysis.

The biomarker core lab will generate results for each blood specimen and will transmit that data electronically into the EDC system (eCOS). Subject identification will follow the same system as for the FUEL Extension trial. Each imaging study will be associated with the FUEL Extension identification number and each blood sample will be labeled with that same number.

FUEL-OLE enrollment takes place over a 2 day period. For clarity, often the initial FUEL-OLE day (called hereafter OLE-D1) and the final day of FUEL are the same visit. This visit consists of informed consent and assent, blood sample collection, EndoPAT testing, an echocardiogram, and exercise testing. Based on experience with the Udenafil study, this day takes 5-6 hours in total. Subjects will then return the next day (OLE-D2) for drug administration and 6-minute walk. Subjects will return at 12 months of FUEL-OLE for a final day repeating the above tests. FALD will ideally be integrated into this schedule with keen attention to preserving the integrity of the FUEL-OLE parent trial and preventing study fatigue as detailed below for each step. This plan was vetted and approved by the FUEL study subcommittee.

Subject recruitment: FALD will only be recruited by either the site PI or a study coordinator after a commitment is given to participate in FUEL-OLE. Recruiting should be done at the same time point as OLE to prevent unnecessarily repetitive patient contact.

Consent/Assent: Consent and assent for FALD will be obtained immediately following but only after consent/assent for FUEL-OLE has been obtained.

Serum testing: Serum for the ancillary study will be obtained during the same venipuncture as used in FUEL-OLE. The serum sample will be processed, stored and shipped in parallel with the plasma sample that is being collected for the FUEL-OLE Trial. The processed serum (and the processed plasma for the FUEL-OLE protocol) will be shipped to the FALD core lab (University of Michigan) for testing. The ancillary study will require an additional 3 ml sample of blood (for a total of 6 ml).

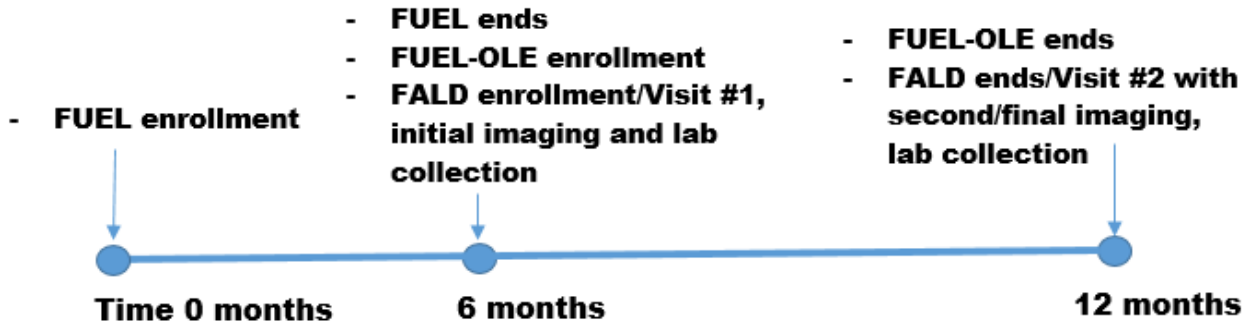
Imaging: We have identified the morning of OLE-D2 as the optimal time for hepatic imaging.

- US SWE: A complete US SWE exam lasts 5-10 minutes only. It is a portable test, and thus may be performed in either a radiology suite or any other clinical cardiology location used by the study. Additional limited anatomic imaging will also be obtained that takes approximately 1 minute.
- MRE: A complete MRE exam lasts approximately 15-20 minutes. Additional limited anatomic imaging will also be obtained that takes approximately 1 minute. Including patient travel to and from the MR scanner we anticipate an additional 20-40 minutes, making the total duration of MR testing 35-60 minutes.

All liver imaging should be able to be completed in 60-90 minutes.

- For initial FALD imaging, by starting OLE-D2 immediately with hepatic imaging at 7:30 or 8:00 AM, we would anticipate FUEL-OLE can begin its formal day's schedule with study drug administration by 9-9:30 AM. In total, we would expect OLE-D2 including hepatic imaging to still conclude by midday as anticipated by the FUEL-OLE protocol.
- For the OLE and FALD 12-month follow-up visit, we would again anticipate beginning the day for with hepatic imaging. To make this feasible, we can pay for hotel costs for those individuals enrolled in FALD at the 12-month visit so that the testing day may start early. Alternatively, imaging may be obtained at the end of the day following FUEL-OLE testing, although this is less ideal because exercise testing may influence FALD measures. Integration of same day testing should be feasible based on projected length of the testing day.

Imaging identifiers for these research studies will include only the site and subject study-specific ID. Following image obtainment at both time points, images from both US SWE and MRE will be sent to the imaging core. Images transferred will contain the site and subject identifiers and no protected health information.




## 11.2 Quality Assurance

The DCC has primary responsibility for QC/QA activities of the phenotypic data. The DCC also requires that the sites complete certain QC activities, most of which are monitored by the DCC.

The key QC/QA activities include the following:

- Development of a Study Manual;
- Clearly formatted and carefully constructed Data Forms with clear, up-to-date manuals of instruction;
- Sign-Off Procedures for all CRFs;
- Central protocol training and certification of all site data collection staff with the use of standardized checklists;
- Data management training and certification of site personnel completing data entry and/or data management;
- Verification of patient eligibility;
- On-going monitoring of all protocols/data collection activities;
- Completion of reliability and/or pilot studies for key measurements as appropriate;
- Inclusion of repeat measurements, as feasible, in the course of the study; and
- Monitoring visits to sites as required with pre-specified goals and/or remote monitoring activities.

The DCC may conduct site visits to the Core Laboratories and/or Biorepository to review QA and QC procedures and data transfer to the DCC. Review of central laboratory-related reports will be conducted at least monthly to identify overall or site-specific problems in data or specimen acquisition and reporting of results.

### 11.3 Data Management

An EDC system will be used for the study that is designed to support reliable and secure data entry for clinical research purposes. The system also provides seamless integration of eCRFs and paper-based CRFs within a single protocol if desired; implementation of protocol amendments; and SAS and XML study data exports.

#### 11.3.1 Data Entry

Data can be entered directly from multiple study sites via a fully validated and 21 CFR Part 11 compliant, secure Web application and stored centrally. A configurable sample-based double data entry system is available. Data are entered by subject study identification number; names will not be linked with subject data in the database. Study sites will maintain records in secure areas linking the subject name with the identification number assigned for the study. Study sites will have full access to their own data and be able to view these data remotely. Study staff will not be able to view subject data associated with other sites.

### 11.3.2 Data Validation and Monitoring

Integrated into the data entry system are real time validations, including both inter- and intra-instrument data checks. Inconsistent or questionable values are flagged during entry, and an edit report is automatically generated to the data entry client. These edit reports provide the information necessary to investigate any data entry errors or resolved questions regarding out-of-range or questionable values. Second level query tracking allows monitors and data managers real time access to unresolved queries as well as the date and time of query generation and resolution.

### 11.3.3 Data Security and Integrity

All data changes are written to an audit trail. The audit trail identifies the data item by table, column and key field. The entry includes the user, date and time, as well as the old value and new value. Both patient related data as well as trial configuration data are written to the audit trail. Data are saved at regular intervals during data entry to prevent loss of information in the event of a disruption of the Internet connection. In the unlikely event of a major disruption, a backup connection allows full access to the DMS.

Several levels of security are employed to ensure privacy and integrity of the study data, including the following: Study access requires use of assigned user names and passwords. Individual roles and access levels are assigned by the study data manager. Passwords are changed regularly. Web-based entry uses secure socket layer data encryption. Data will not be stored on laptop computers.

### 11.3.4 Biospecimen Tracking

Specimen tracking is started from the time of receipt at the site, through shipment to the central biorepository. Each specimen will be labeled with a bar-coded label identified by a unique specimen number that is different from the subject's unique study ID number. The master list linking the barcode numbers to the subject study ID numbers will be maintained under password protection in the data management system at the DCC. This blinding code system will maintain the confidentiality of the specimens yet allowing linkage of the specimens with clinical study data for analyses.

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