Title:	Improved Diagnosis and Prognostication of Hypoxic Ischemic Injury in Neonates and Infants Using Contrast-Enhanced Ultrasound
Short Title	Contrast-Enhanced Ultrasound Evaluation of Hypoxic Ischemic Injury

Drug Name(s):	Sulfur hexafluoride lipid-type A microspheres (Lumason™)
FDA IND	137921
Regulatory Sponsor:	Misun Hwang, MD
eIRB Number	IRB 18-014912
Protocol Date:	January 10, 2018

Amendment 1 Date: 27 April 2018	Amendment 3 Date: 11 March 2019
Amendment 2 Date: 20 November 2018	Amendment 4 Date: 10 October 2019
Amendment 5 Date: 13 April 2020	Amendment 6 Date: 11 January 2021
Amendment 7 Date: 7 February 2022	Amendment 8 Date: 11 April 2022

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ABBREVIATIONS AND DEFINITIONS OF TERMS

AE	Adverse Event
Category B Drug	Animal reproduction studies have failed to demonstrate a risk to the fetus and there are no adequate and well-controlled studies in pregnant women
CEUS	Contrast-Enhanced Ultrasound
CFR	Code of Federal Regulations
CHOP	Children's Hospital of Philadelphia
CT	Computed Tomography
DSMP	Data Safety Monitoring Plan
DWI	Diffusion-weighted magnetic resonance imaging (MRI DWI) is the use of specific MRI sequences as well as software that generates images from the resulting data
Echo-PIV	Echocardiographic Particle Image Velocimetry
FDA	Food and Drug Administration
HII	Hypoxic Ischemic Injury
HIPAA	Health Insurance Portability and Accountability Act
ICU	Intensive Care Unit
IDS	CHOP Investigational Drug Service
IND	Investigational New Drug
IV	Intravenous
IRB	Institutional Review Board
Lumason™	FDA-approved for contrast-enhanced ultrasound; the investigational drug
LVT	Left Ventricular Thrombus
MRI	Magnetic Resonance Imaging
ORC	The CHOP Office of Research Compliance
PET	Positron Emission Tomography
PI	Principal Investigator
PIV	Particle Image Velocimetry
PHI	Protected Health Information
PTV	Particle Tracking Velocimetry
RMS	Root Mean Square
SAE	Serious Adverse Event
TCEUS	Transfontanellar Contrast Enhanced Ultrasound
US	Conventional Ultrasound Scan
PACS	Picture Archiving and Communication System

ABSTRACT

Context:

Neonates presenting with neurologic symptoms require rapid, non-invasive imaging with high spatial resolution and tissue contrast. The purpose of this study is to evaluate brain perfusion using contrast-enhanced ultrasound CEUS in bedside monitoring of neonates and infants with hypoxic ischemic injury.

Objectives: Two possible methods have been proposed to rapidly and accurately detect perfusion abnormalities:

- Primary: Identify regions of interest in gray versus white matter to detect ratio of perfusion changes, in cases of diffuse white matter ischemia.
- Secondary: Generate a microbubble velocity mapping technique to detect regions of ischemia.

Study Design:

Single site, open-label clinical study.

Setting/Participants:

Subjects in the neonatal or pediatric intensive care unit aged 1.5 years of age or younger with open fontanelles and suspected or diagnosed hypoxic ischemic injury.

The study will be performed at Children's Hospital of Philadelphia (CHOP). CHOP will be the only site of the study.

Study Interventions and Measures:

Contrast-enhanced ultrasound scan with a duration of approximately 20 minutes. Qualitative analysis with visual assessment and quantitative analysis of the acquired scans will be performed by the sponsor-investigator and reviewed by the second radiologist (co-investigator). The scans will be assessed for diagnostic quality of images, artifacts encountered, and the presence of additional contributory diagnostic information.

Protocol Synopsis

Study Title	Improved Diagnosis and Prognostication of Hypoxic Ischemic Injury in Neonates and Infants Using Contrast-Enhanced Ultrasound
Funder	Children's Hospital of Philadelphia
Clinical Phase	Phase II
Study Rationale	Neonates presenting with neurologic symptoms require rapid, non-invasive imaging with high special resolution and tissue contrast. Magnetic resonance imaging (MRI) is currently the most sensitive and specific neuroimaging modality for evaluation of neonatal neurological diseases. This modality does come with several challenges in the neonatal population, namely the need to transport a possibly critically sick neonate to the MRI suite and the necessity of the neonate to remain still for a significant length of time, occasionally requiring sedation. Cranial ultrasound has provided neuroradiologists and clinicians with an invaluable neuroimaging modality that allows a rapid, bedside point of care evaluation without ionizing radiation. The major drawback of cranial ultrasound is its lower sensitivity and specificity for subtle/early lesions. Contrast enhanced ultrasound (CEUS) has the potential to improve sensitivity and specificity for a variety of neonatal neurological diseases and expand the indications for cranial ultrasound. There is no current diagnostic tool with high soft tissue contrast that can assess brain perfusion of neonates and infants at the bedside. Critically ill neonates cannot be transported easily down to the magnetic resonance suite, and even when transported, ultrafast sequences are performed (which lack perfusion imaging) due to their clinical condition. In the case of neonatal hypoxic ischemic injury, our preliminary evidence has shown that contrast-enhanced ultrasound (CEUS) of the brain delineates areas of injury, as the perfusion to the injured regions of the brain become altered. Extensive literature exists in regard to the value of perfusion imaging in brain injury, especially in terms of prognostication. Since the CEUS of the brain can be performed at bedside, serially if needed over the course of injury evolution, the technique can be of significant clinical value in caring for neonates and infants with brain injury. It will be important to validate the initial evidence from early CEUS

	brain scanning studies, and establish standardized quantitative techniques to reduce interpretation variations and errors in the clinical research setting. There is a dire need to introduce better imaging tools such as contrast-enhanced ultrasound to the clinical setting that can detect HII at an early stage and prompt therapeutic implementation. In this regard, contrast-enhanced ultrasound (CEUS) enables safe, serial monitoring of dynamic quantification of brain perfusion at the bedside. Safety of intravenous use of Sulfur hexafluoride lipid-type A microspheres was based on evaluation of published literature involving use of Lumason™ in over 900 pediatric patients, as noted on the 2016 FDA product label. Non-fatal anaphylaxis was reported in one pediatric patient, but none in a neonate. Animal data of daily intravenous administration of Sulfur hexafluoride lipid-type A microspheres to rats (administered up to 10 times the recommended maximum human dose) and rabbits (administered up to 20 times the recommended maximum human dose) for 30 consecutive days and 14 consecutive days, respectively, resulted in no toxicity to the fetus in animal studies, as noted on the 2016 FDA product label. Specifically pertaining to the use of Sulfur hexafluoride lipid-type A microspheres in brain imaging in pediatric patients, we expect a similar risk of adverse events. For the proposed study, the same pediatric dosage, route of administration, safety monitoring guidelines, and low mechanical index used for contrast ultrasound settings will be used.
Study Objective(s)	**Primary** • The primary objective of this study is to identify regions of interest in gray versus white matter to detect the ratio of perfusion changes, in cases of diffuse white matter ischemia. **Secondary** • The secondary objective of this study is to generate a microbubble velocity mapping technique to detect regions of ischemia.
Test Article(s) *(If Applicable)*	Sulfur hexafluoride lipid-type A microspheres (Lumason™, Bracco Inc) is an FDA-approved ultrasound contrast agent which consists of active ingredients including Sulfur

	hexafluoride (strength 60.7 mg in 1 mg), Distearoylphosphatidylcholine, DL- (strength 0.19 mg in 1 mg), 1,2-Dipalmitoyl-Sn-Glycero-3-Phospho-(1'-Rac-Glycerol), Sodium Salt (0.19 mg in 1 mg). Inactive ingredients include Polyethylene Glycol 4000 (strength 24.56 mg in 1 mg) and Palmitic Acid (0.04 mg in 1 mg). The Sulfur hexafluoride lipid microspheres are composed of SF6 (molecular weight 145.9) gas in the core surrounded by an outer shell monolayer of phospholipids consisting of 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol, sodium salt (DPPG-Na) with palmitic acid as stabilizer. Sulfur hexafluoride lipid-type A microspheres fall under Category B, that is, animal reproduction studies have failed to demonstrate a risk to the fetus and there are no adequate and well-controlled studies in pregnant women.1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), with empirical formula C44H88NO8P, has a molecular weight of 790.6. 1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-glycerol sodium (DPPG-Na), with empirical formula C38H74 NaO10P, has a molecular weight of 745.
Study Design	Single site, open-label clinical study.
Subject Population key criteria for Inclusion and Exclusion:	**Inclusion Criteria** 1. Males and females aged 1.5 years or younger with open fontanelles and known or suspected hypoxic ischemic injury 2. Post menstrual age of 34 weeks or older 3. Patient in the CHOP NICU or PICU 4. Parental permission **Exclusion Criteria** 1. Medical history of Lumason hypersensitivity 2. Hemodynamic instability as defined by rapid escalation of cardiopulmonary support in the past 12-24 hours, as defined by the clinical care team including ≥1 intensive care physician not part of the study team

	3. Pulmonary insufficiency as defined by FiO2 requirements of >40% and/or subjects with pulmonary hypertension requiring nitric oxide
Number Of Subjects	200; CHOP will be the only site of the study.
Study Duration	The study duration per subject will be approximately 20 minutes including the time to prepare Lumason™ contrast agent and perform the pre-contrast imaging and the CEUS, as well as the 60 minute monitoring period after the first and second injection of Lumason™. CEUS will be performed at the time HII is first suspected or diagnosed. A second CEUS may be performed at short-term follow-up (approximately within 1-2 weeks from the first scan) for a total of two CEUS exams of 1 hour and 15-minute duration each. Study participation will be complete when the 60 minute monitoring period of the last CEUS performed is complete (after the first CEUS in patients who undergo one exam, or after the second CEUS is complete in patients who undergo two exams).
Study Treatment	CEUS has a total duration of 1 hour and 20 minutes: CEUS has a duration of approximately 20 minutes for the CEUS and 60 minutes for post-examination monitoring of potential adverse events.
Efficacy Evaluations	There are no efficacy evaluations for this diagnostic study. The endpoints of this study are: **Primary Endpoint** • To qualitatively evaluate perfusion abnormalities in correlation with clinical MRI results. **Secondary Endpoint** • To quantitatively evaluate perfusion abnormalities in correlation with clinical MRI results.

Pharmacokinetic Evaluations	There are no pharmacokinetic evaluations.
Safety Evaluations	All subjects entered into the study and receiving at least one injection of investigational drug will be included in the safety analysis. The frequencies of AEs by type, severity, and temporal relationship to the CEUS scan will be summarized. SAEs (if any) will be described in detail.
Statistical And Analytic Plan	Baseline and demographic characteristics will be summarized by standard descriptive summaries. All subjects entered into the study and receiving at least one injection of investigational drug will be included in the safety analysis. The frequencies of AEs by type, severity, and temporal relationship to the CEUS scan will be summarized. SAEs (if any) will be described in detail. Details of sample size and power calculations for this study are described in Section 6 of the protocol.
DATA AND SAFETY MONITORING PLAN	The safety monitoring for this study is the primary responsibility of the sponsor-investigator. Monitoring the safety outcomes following IV administration of the investigational drug will be conducted primarily by the Principal Investigator and/or specifically designated study personnel. An independent safety monitor will be designated to oversee the safety reports, and help adjudicate attribution of serious adverse events, should they occur. Regular meetings to discuss the outcomes of the study, and of the safety events, will be conducted by the study team. The occurrence of adverse events, serious adverse events and unanticipated events will be reported by the study team in accordance with federal and institutional guidelines, as outlined in Section 8 of this clinical study. Prior to study initiation, the Office of Research Compliance (ORC) will conduct a pre-trial monitoring visit, to assess trial readiness of the study staff. Once the pre-trial monitoring visit has been successfully completed, the ORC will also monitor the IND study on at least an annual basis.

1 Background Information and Rationale

1.1 Introduction

Contrast-enhanced ultrasonography (CEUS) is a novel imaging technique in which gas-filled microbubbles, smaller than red blood cells, generate increased signal due to the acoustic impedance mismatch. Injection of ultrasound contrast agents into blood increase the echogenicity, allowing enhanced visualization of a blood vessel. Ultrasound contrast agents have been approved for use in Europe for almost two decades. In the case of SonoVue (now called Lumason™), a second-generation lipid/sulfur hexafluoride US contrast agent (Bracco, Milan, Italy), European Union approved its intravenous use in adults in 2001. The FDA in the United States just recently approved the use of Lumason™ for evaluation of focal hepatic lesions in pediatric population in 2016, and very recently (Jan 2017) approved its use in children for the evaluation of the urinary tract in pediatric patients with known or suspected vesicoureteral reflux. For the remainder of clinical applications, the ultrasound contrast agents are being used off-label in both Europe and the United States. All patients in this study will already be scheduled to have a CEUS study for clinical indications. This protocol focuses on imaging of hypoxic ischemic injury in neonates and infants.

Unlike CT or MRI contrast agents, ultrasound contrast agents have no associated renal toxicity, and there is no need for accompanying ionizing radiation or sedation. The risk of adverse events is the lowest of all contrast agents available, with CT contrast being the highest (0.6%), followed by MRI contrast (0.0088%) and ultrasound contrast (0.0086%) [1]. Several studies detail the safety profile of ultrasound contrast agents in children and have shown minor adverse events including altered taste, tinnitus, light-headedness, nausea [2-4]. One rare severe reaction in a child documented symptoms of generalized pruritus, nausea, hypotension with tachycardia initially then bradycardia [5]. Management in this instance consisted of oxygen, intravenous epinephrine, and fluids (0.9% normal saline) with resolution of symptoms in two hours. Treatment of both minor, mild, and severe adverse reactions post Lumason™ administration are the same as that of CT or MRI contrast agents. In comparison to CT or MRI contrast agents, however, ultrasound contrast agents have proven to be much safer in children over decades of its use to date (contrasting to approximately 15-20 adverse events per 2000 children if CT contrast agent were to be used).

Neonates presenting with neurologic symptoms require rapid, non-invasive imaging with high spatial resolution and tissue contrast. Magnetic resonance imaging (MRI) is currently the most sensitive and specific imaging modality for evaluation of neurological pathology. This modality does come with several challenges in the neonatal population, namely the need to transport a possibly critically sick neonate to the MRI suite and the necessity of the neonate to remain still for a significant length of time, occasionally requiring sedation. Cranial ultrasound has provided radiologists and clinicians with an invaluable imaging modality that allows rapid, bedside point of care evaluation without ionizing radiation. The major drawback of cranial ultrasound is its lower sensitivity and specificity for subtle/early lesions. Contrast enhanced ultrasound (CEUS) has the

potential to improve sensitivity and specificity for a variety of neuropathologies and expand the indications for cranial ultrasound.

CEUS has several advantages over traditional ultrasonographic grey scale and color-coded Doppler images. CEUS utilizes ultrasonographic contrast agents to allow for precise visualization of vasculature, determination of relative blood flow, and enhanced visualization of solid organs and lesions [6, 7]. Ultrasound contrast agents are gas-filled microbubbles that are injected into the systemic vasculature that appear echogenic on grey scale ultrasound [8, 9]. With respect to neuroimaging, CEUS not only provides a more accurate depiction of the cerebral macrovascular circulation but also has the ability to define regional cerebral blood flow, a surrogate marker of cerebral microcirculation. Early animal models of neonatal hypoxia in piglets have demonstrated that transcranial CEUS can be used to qualitatively track regional cerebral perfusion changes. Furthermore, CEUS measures of cerebral perfusion correlated with MRI [10]. There is, however, a paucity of published data on the subject of brain imaging using CEUS. This is due to a combination of lack of FDA approval and lack of familiarity with the technique amongst neonatal intensive care providers. Despite the favorable safety profile of ultrasound contrast agents, the institutional and governmental oversight as well as cumbersome consent procedures required for the off-label application of CEUS in the evaluation of neonatal brain conditions has limited its usage in this particular clinical setting throughout the United States.

Few studies have examined imaging findings in transfontanellar contrast enhanced ultrasound (TCEUS). Kastler and colleagues performed one of the first studies of TCEUS in 2013 [11]. Kastler examined several neonates with suspected neurological conditions using TCEUS. Specifically, the authors looked for abnormal parenchymal enhancement patterns on arterial, venous, and delayed phases to define regions of abnormal perfusion and masses/lesions. TCEUS findings were then correlated with brain MRI [11]. Twelve neonates underwent TCEUS and MRI. Diagnostic accuracy between TCEUS and MRI was graded based on the following scale: No correlation for discordant findings; Good correlation for accurate diagnosis with an underestimate of the extent of the lesion; Excellent correlation for accurate diagnosis and satisfactory estimation of the extent of the lesion. Ten out of 12 neonates exhibited enhancement abnormalities on TCEUS. TCEUS successfully defined brain perfusion abnormalities (9/10 neonates) and several hypovascular (4/10 neonates) and avascular lesions (5/10 neonates) which were felt to represent ischemic pathology [11].

When compared with MRI findings, TCEUS findings suggesting regional ischemia were accurate in the majority of cases, with either good or excellent correlation (10/12 neonates). In cases that were deemed to be discordant, TCEUS underestimated the extent of ischemic or hemorrhagic injury [11]. In this paper, calculated sensitivity and specificity for detecting pathological brain lesions in neonates was 88.9% and 66.6% respectively. For comparison, in larger samples of adults presenting after acute stroke, transcranial ultrasound has demonstrated a sensitivity and specificity for localizing areas of infarction ranging from 86-100% and 96-100% respectively [12, 13]. These figures are particularly impressive given the fact that adult examinations are limited by

significant acoustic impedance from the skull [11-13]. Thus, it is reasonable to postulate that sensitivity and specificity for detecting ischemic regions in the neonate may end up being more favorable than previously reported given the lack of acoustic impedance through the fontanelle. Further studies with larger samples sizes will of course be required to accurately determine sensitivity and specificity for a variety of neurological pathologies. No immediate or late adverse events were reported after TCEUS [11].

An area of deep interest is the application of cranial CEUS to investigate brain perfusion characteristics in neonatal hypoxic ischemic injury, specifically, elucidating quantitative parameters and qualitative perfusion patterns in neonates with a variety of hypoxic injuries. With contrast quantification software it is possible to determine contrast enhancement kinetics. When a region of interest is identified a time intensity curve is generated allowing for the calculation of the wash in (time from injection to peak intensity of contrast), peak intensity (maximum value of contrast on the time intensity curve), half washout (time between peak intensity and time when half the enhancement has disappeared), and washout (the time from peak enhancement to when enhancement has completely disappeared) can be calculated.

The area under the curve is calculated from the beginning of contrast enhancement to the end of washout which can be thought of as a surrogate for perfusion within that region of interest [14, 15]. Two quantitative methods are being developed by our group to rapidly and accurately detect perfusion abnormalities: 1) drawing regions of interest in gray versus the white matter to detect ratio of perfusion changes, in cases of diffuse white matter ischemia 2) generating a microbubble velocity mapping technique to detect regions of ischemia (unpublished data). With both qualitative and quantitative data, we believe that cranial CEUS can detect subtle ischemic injuries that cannot be detected with non-contrast cranial US that correlate well with gold standard MRI [14]. At Johns Hopkins, we initially started applying TCEUS for the evaluation of neonatal hypoxic ischemic injury. For extremely injured brain cases, nuclear scan (for diagnosis of brain death) instead of MRI may be performed as part of gold standard. Using both qualitative and quantitative parameters we have been able to characterize global perfusion abnormalities in neonates with different etiologies of hypoxic ischemic injury (see Preliminary Results section, below).

The temporal evolution of perfusion following brain ischemia has been well studied in pediatric and adult patients [16-21]. A previous study on infants with hypoxic ischemic injury applied Doppler US to perform serial cerebral blood flow measurements and showed initial marked increase followed by decrease at 21 days in comparison to controls [18]. Another group evaluated infants with hypoxic ischemic injury with MR perfusion and also revealed similar results by noting lower cerebral blood flow in the basal ganglia and thalamus by the second week of life, likely reflective of decreased metabolic state of the central gray matter structures following irreversible brain injury [19].

Previous study with PET performed in infants with hypoxic ischemic encephalopathy showed decreased cerebral uptake at 2 weeks correlated with poor outcome at 2 years

[20]. Using PET, low cerebral blood flow has been shown during the first few days of life in two infants with basal ganglia injury [22]. The discrepant findings may be of clinical significance, as some infants with inadequate reperfusion in the acute post-ischemic phase may be correlated with increased neuronal cell death and poor long-term clinical outcome. Not only the hypoperfusion, but also continued or exaggerated post-ischemic hyperperfusion may cause brain injury: post-ischemic regional hyperperfusion to the basal ganglia and thalamus has been suggested to increase the vulnerability of metabolically active regions [22, 23]. An accurate delineation of perfusion changes following ischemia is important and may provide an additional therapeutic window of opportunity in infants with reduced or increased brain perfusion.

Prior CEUS study on post-ischemic reperfusion in a piglet showed hyperperfusion during hypoxia and early resuscitation state, and revealed the dynamic perfusion changes during the first hours of injury [10]. In the histologically injured brains, increased CEUS quantification parameters including time to peak and area under the curve was seen in both the basal ganglia and whole brain at the time of injury and resuscitation to a greater degree compared to 7 hours after injury. These findings emphasize the need for monitoring dynamic perfusion changes during the first few hours following ischemia, and show the convenience and practicality of CEUS in studying temporal perfusion changes of neonatal brain ischemia. This study also revealed compromised cerebral perfusion with 100% oxygen treatment, with a decrease of peak intensity and area under the curve during and shortly after resuscitation, and suggested a suspected pathomechanism of oxygen induced reduction of perivascular nitric oxide production and resultant vasoconstriction resulting in reduced cerebral blood flow [24].

In the near future, the investigative team anticipates that widespread use of contrast enhanced ultrasound will complement some of the conventional imaging modalities in specific patient populations. Contrast enhanced ultrasound will be an important imaging modality among the various tools in the arsenal of pediatric diagnostic imaging.

Preliminary Results: High Diagnostic Sensitivity of Contrast-Enhanced Ultrasound in Detection of Brain Injury

All patients were scanned at Johns Hopkins for clinically indicated reasons which in these cases was suspected brain injury. Contrast-enhanced ultrasound and elastography were performed as part of the standard of care brain ultrasound examinations. The studies were obtained due to the inconvenience or inability of transporting the patients to the MRI scanner at the time due to the nature of their illness, support staff or support devices limitations. Figure 1 demonstrates that from the qualitative evaluation of CEUS exams the injury patterns as validated on MRI can be inferred.

Figure 1

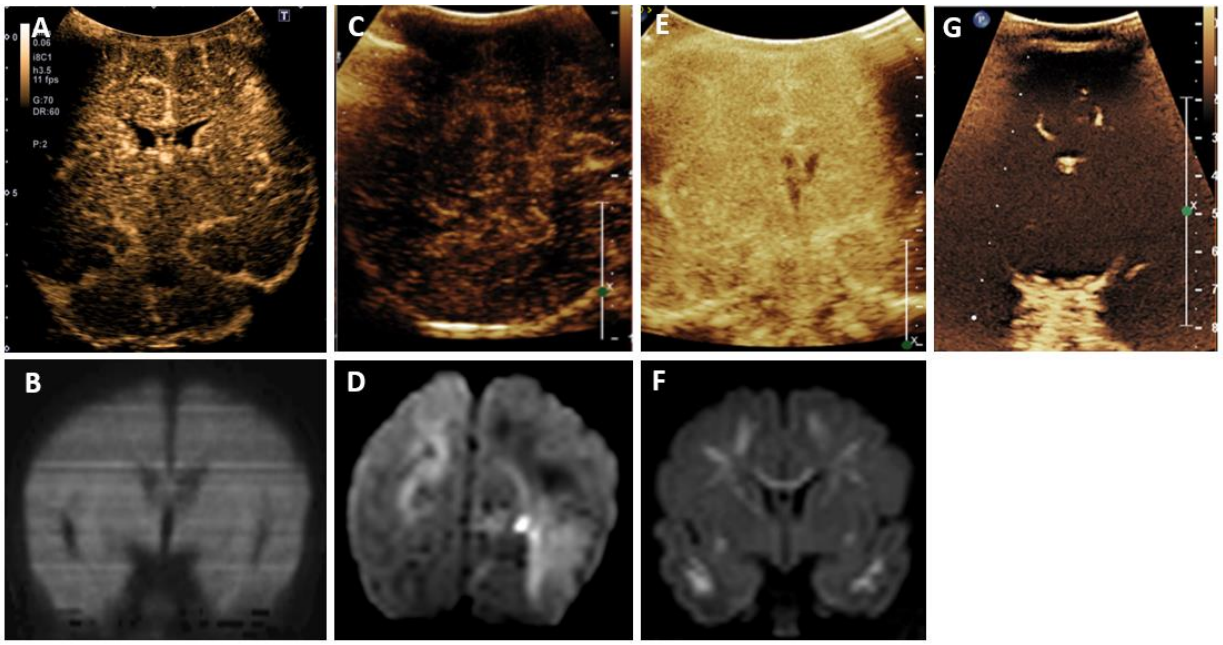
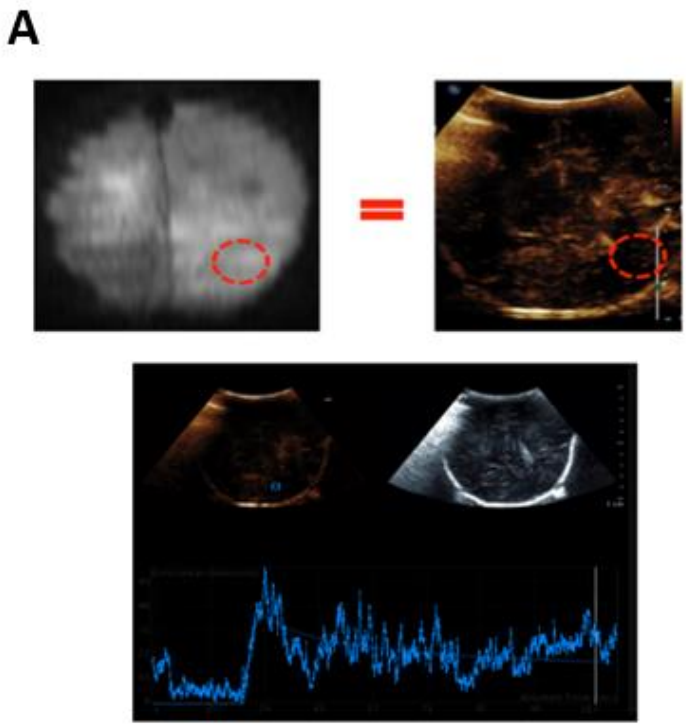
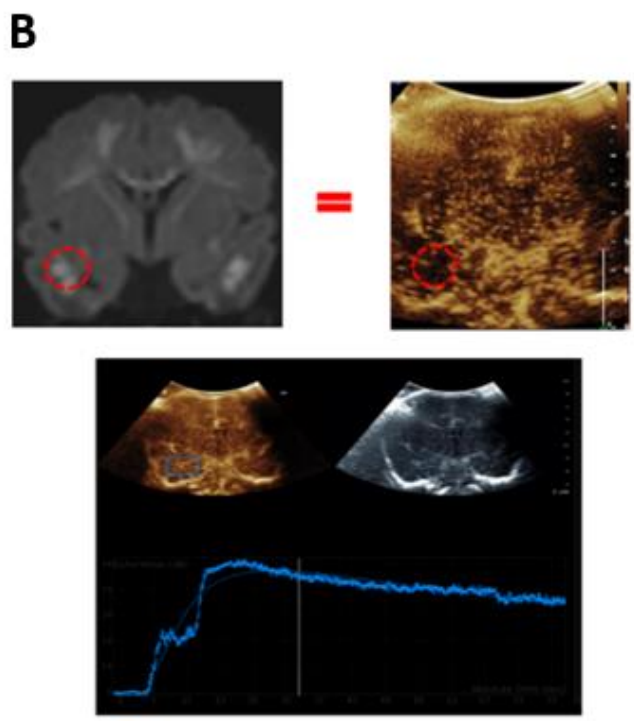


Fig 1. Top rows denote coronal brain CEUS exams of neonates with bottom rows representing corresponding coronal brain diffusion-weighted MRI (MRI DWI) images obtained hours after the CEUS exams. Figure 1A is of a 2-month-old male born at 35 weeks gestational age, with trisomy 21 and associated laryngomalacia and tracheomalacia status post supraglottoplasty scanned for respiratory distress and suspected brain injury. The CEUS scan depicts a coronal slice through the brain at the level of the frontotemporal lobes and basal ganglia showing homogeneous perfusion without focal or diffuse perfusion abnormality. Figure 1B shows the corresponding coronal MRI DWI slice obtained hours after the CEUS exam confirming the absence of brain injury. Figure 1C is a coronal brain CEUS of a 2-week-old male born at 40 weeks gestational age, delivered via caesarian section due to arrest of descent. The newborn presented with hypoglycemia and seizures with multifocal perfusion abnormalities in the parietooccipital regions bilaterally confirmed on the corresponding coronal MRI DWI of the brain (Figure 1D) to have multifocal injuries in the parietooccipital regions. Figure 1E is of a 3 day old male born at 35 weeks gestational age, with uncomplicated birth history who initially presented with seizures. Brain CEUS scan on Day 14, and brain MRI on Day 15, confirmed diffuse white matter injuries. Coronal CEUS brain images of the frontotemporal lobes showed generalized hyperperfusion. Comparison of coronal MRI DWI image in Figure 1F confirmed diffuse white matter injury. Figure 1G is a coronal CEUS scan of a 6-month-old male 3 hours after he suffered a prolonged cardiac arrest of unknown etiology. The scan shows no significant perfusion to the brain except for few intracranial vessels. Of note, wash-out of had not occurred 30 minutes after contrast administration in the post cardiac arrest patient signifying extremely poor cerebral circulation. Unfortunately, the 6-month-old died before any further imaging could be obtained or formal brain death evaluation was conducted.



Curve Type	Wash-in Slope (Intensity in dB/sec)	Time to Peak (Sec)	Peak Intensity (Intensity)	Area Under the Curve (Intensity x sec)	Mean Transit Time (Sec)	Time to Peak to ½ (Sec)
LDRW WIWO	10.59	27.84	24.44	1125.76	37.77	29.99



Curve Type	Wash-in Slope (Intensity in dB/sec)	Time to Peak (Sec)	Peak Intensity (Intensity)	Area Under the Curve (Intensity x sec)	Mean Transit Time (Sec)	Time to Peak to ½ (Sec)
LDRW WIWO	1.3	28.59	20.30	1230.23	54.97	14.02

Figure 2. Figure 2A shows a coronal DWI brain image (top left) and corresponding CEUS image (top right) with region of interest (dotted red circle) placed in the left occipital lobe based on which microbubble wash-in graph (second row) and graph (bottom row) are derived. Note the irregular, inhomogeneous perfusion to the region. Figure 2B shows a coronal DWI brain image (top left) and corresponding CEUS image (top right) with region of interest (dotted red circle) placed in the right temporal lobe based on which microbubble wash-in graph (second row) and graph (bottom row) are derived. Note the double peak wash-in phase with a more regular, homogeneous perfusion to the region without significant variability in intensity as on Figure 2A. Notable differences include significantly quicker wash-in and higher area under the curve representative of total microbubble or blood volume in Figure 2B as compared to Figure 2A [15].

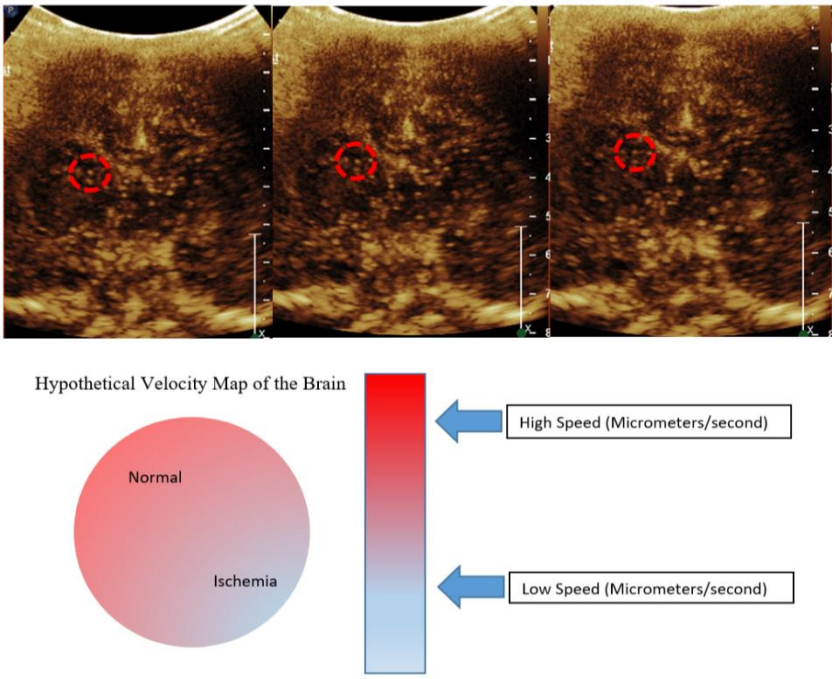


Figure 3. Hypothetical Microbubble Velocity Map of the Brain. Cine (movie) clips of contrast scans will be analyzed to track the movement and velocity of an individual microbubble across hundreds of frames. The data will be used to generate a super-resolution velocity maps of the brain. Above is an example of how an individual microbubble can be tracked across three successive frames; the same microbubble as denoted by a dotted red circle is traced (arrows) from the left to mid to right temporally sequential images, each obtained within milliseconds of each other. The acquired information across hundreds of successive frames will be compiled to generate an image detailing regional velocity information, with the high speed denoting normal velocity (red) and low speed denoting abnormal velocity as in ischemia (blue). [Hwang et al, J Neuroimaging 2017, in press]

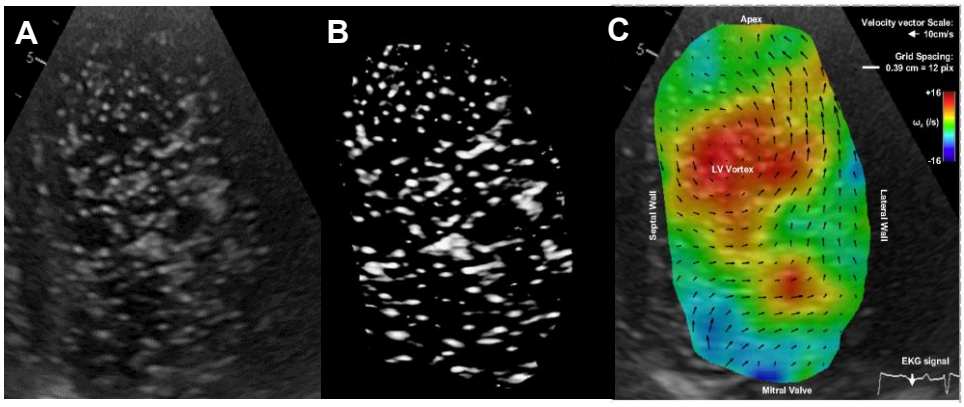


Figure 4: (a) Sample original clinical echocardiographic images containing ultrasound contrast agent; (b) Enhanced image of the bubble traces of the same image; and (c) Vectors sowing the corresponding velocity distribution and flow direction calculated using the new echo-PIV/PTV procedures. The color map shows the vorticity (angular velocity) distribution (Sampath et al., submitted for publication).

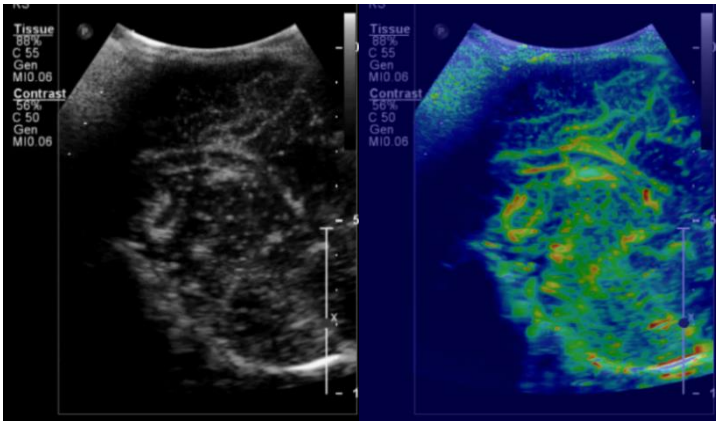


Figure 5: (a) A sample instantaneous brain CEUS showing clear bubble traces; and (b) Distribution of RMS values of contrast over time. Bright (red) regions represent regions with rapid motions and dark (blue) regions represent stagnant zones with weak seeding and/or circulation.

Microbubble Velocity Mapping Technique

As a further advancement to the existing CEUS quantification tools, we developed a novel microbubble velocity mapping technique for quantifying the instantaneous distributions of brain perfusion abnormalities that may serve as an alternative to diffusion-weighted sequence of MRI.

Echocardiographic particle image velocimetry (echo-PIV) has been introduced in recent years to obtain quantitative velocity distributions from CEUS data [25, 26]. However, images acquired from *routine clinical* CEUS are prone to noise as well as spatially and temporally non-uniform distribution of tracers, adversely affecting the quality of data obtained using standard PIV algorithms. Consequently, we have developed an optimized procedure that integrates the image enhancement, PIV, and particle tracking velocimetry (PTV), which tracks individual tracers in time, to process clinical ultrasound images and obtain reliable, time-resolved, two-dimensional velocity distributions from non-uniformly distributed bubbles [27]. So far, to demonstrate the clinical value of the optimized procedures, the new tools have been used for analyzing cardiac contrast images acquired from four patients with left ventricular thrombus (LVT). A sample original contrast echo image is presented in Figure 4a, the enhanced traces are shown in Figure 4b, and the corresponding velocity and vorticity distributions calculated using the new PIV/PTV procedure is presented in Figure 4c. Time series of such velocity maps enable us to follow the formation, decay and fragmentation of the left-ventricular vortex (large swirling structure in Figure 4c) as it migrates towards the LV apex. The flow induced by this vortex is believed to play a key role in apical washing, an important factor in determining susceptibility to LVT formation Furthermore, the velocity distribution enables direct quantification of the local washing around the LVT, which might be useful for assessing risk of LVT. A sample of a recently acquired brain CEUS is presented in Figure 5a. The bubble traces are clear and can be readily followed in time. In some areas, they move at high speed while in others, they are slow or even stagnant. As a preliminary indication of the advantages offered by mapping the velocity, Figure 5b shows the root mean square (RMS) of the contrast intensity over time. Bright areas indicate high speed flow regions, and dark regions indicate sites with weak or no flow. Our objective is to integrate the new procedures into routine CEUS imaging, providing bedside real time data on the velocity distribution, flow directions, and clearance rates of injured brains.

The goal of the study is to perform contrast enhanced ultrasound (CEUS) for diagnosis and monitoring of brain injury in neonates and infants. The CEUS technique offers several advantages over MRI in that the technique can be performed at bedside, at lower cost, and without the need for sedation, and thus may serve as a valuable alternative to MRI in critically ill neonates and infants. Our preliminary evidence has shown that CEUS can delineate regions of brain injury due to associated altered perfusion. Extensive literature demonstrates the value of monitoring injury associated perfusion response in prognostication and therapy. This study will enroll neonates and infants in the neonatal intensive care unit or pediatric intensive care unit at Children’s Hospital of Philadelphia who are already scheduled to receive a standard of care grayscale brain ultrasound scan.

1.2 Name and Description of Investigational Product or Intervention

Sulfur hexafluoride lipid-type A microspheres (Lumason™, Bracco Inc) is FDA-approved ultrasound contrast agent which consist of active ingredients including Sulfur hexafluoride (strength 60.7 mg in 1 mg), Distearoylphosphatidylcholine, DL- (strength 0.19 mg in 1 mg), 1,2-Dipalmitoyl-Sn-Glycero-3-Phospho-(1'-Rac-Glycerol), Sodium Salt (0.19 mg in 1 mg). Inactive ingredients include Polyethylene Glycol 4000 (strength 24.56 mg in 1 mg) and Palmitic Acid (0.04 mg in 1 mg). The sulfur hexafluoride lipid microspheres are composed of SF6 (molecular weight 145.9) gas in the core surrounded by an outer shell monolayer of phospholipids consisting of 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol, sodium salt (DPPG-Na) with palmitic acid as stabilizer. Sulfur hexafluoride lipid-type A microspheres fall under Category B, that is, animal reproduction studies have failed to demonstrate a risk to the fetus and there are no adequate and well-controlled studies in pregnant women.1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), with empirical formula C44H88NO8P, has a molecular weight of 790.6. 1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-glycerol sodium (DPPG-Na), with empirical formula C38H74 NaO10P, has a molecular weight of 745. In pediatric patients, after reconstitution 0.03 mL per kg is administered intravenously. The weight-based dose of 0.03 mL per kg will be repeated twice during a single examination. Following each injection, an intravenous flush of 0.9% Sodium Chloride is injected.

1.3 Compliance Statement

This study will be conducted in full accordance of all applicable Children's Hospital of Philadelphia Research Policies and Procedures and all applicable federal and state laws and regulations including 45 CFR 46. All episodes of noncompliance will be documented. Research will also be conducted in full accordance with Food and Drug Administration (FDA) regulations 21 CFR 50 (Protection of Human Subjects), 21 CFR 56 (Institutional Review Boards) and 21 CFR 312 (Investigational New Drug).

The investigators will perform the study in accordance with this protocol, will obtain consent, and will report unanticipated problems involving risks to subjects or others in accordance with The Children's Hospital of Philadelphia IRB Policies and Procedures and all federal requirements. Collection, recording, and reporting of data will be accurate and will ensure the privacy, health, and welfare of research subjects during and after the study.

2 STUDY OBJECTIVES

The purpose of this study is to evaluate brain perfusion using contrast enhanced ultrasound CEUS in neonates and infants with hypoxic ischemic injury.

2.1 Primary Objective (or Aim)

The primary objective is to identify regions of interest in gray versus white matter to detect the ratio of perfusion changes, in cases of diffuse white matter ischemia.

2.2 Secondary Objective (or Aim)

The secondary objective is to generate a microbubble velocity mapping technique to detect regions of ischemia.

3 INVESTIGATIONAL PLAN

3.1 General Schema of Study Design

Patients scheduled for routine cranial ultrasound as part of clinical care for screening or monitoring of hypoxic ischemic injury will be recruited for the study. Following parental consent, the subject will undergo an investigational CEUS exam, which will be performed separately from any clinically indicated conventional ultrasound. The CEUS exam includes a pre-contrast ultrasound evaluation with FDA-approved technologies (e.g. gray-scale ultrasound, Doppler ultrasound, microvascular imaging). Pre-contrast ultrasound and CEUS duration is of approximately 20 minutes, followed by 60 minutes of monitoring.

3.1.1 Screening Phase

Potential subjects will be identified by referral from neonatologists of patients during the neonatal or pediatric intensive care unit stay for suspected, at risk of or diagnosed hypoxic ischemic injury and review of orders for cooling blankets, brain imaging and/or EEG exams in neonates.

Before discussing participation in the study, we will confirm eligibility by reviewing the subjects' medical records. Participation will be discussed between the PI and the referring neonatologist. Participation will be discussed with the parents/guardian by the neonatologist and/or radiologist after identification and confirmation of eligibility. Consent of the parents/guardian will be obtained prior to the exam by the PI, co-investigator, or neonatologist co-investigator in a private setting. Questions will be answered by the PI, co-investigator, or referring neonatologist co-investigator.

3.1.2 Study Treatment Phase

Investigational CEUS scan will be performed separately from clinically indicated conventional US, in the ICU. CEUS will be performed at the time HII is first suspected or diagnosed. A second CEUS may be performed at short-term follow-up (approximately within 1-2 weeks from the first scan) for a total of two CEUS exams of 1 hour and 20-minute duration each.

Injection of Lumason™ contrast agent will be performed via the existing peripheral intravenous line or central line using the FDA-recommended dose of up to 0.03 mg/kg. Contrast-agent injection will be performed twice per CEUS scan to ensure image quality and test reproducibility. In the case of more stable patients without an IV line, a peripheral IV line will be started to conduct the investigational CEUS. Two bolus

injections will performed to evaluate for dynamic brain perfusion and several 2-minute cine clips as well as static images will be acquired during the exam.

3.2 Allocation to Treatment Groups and Blinding

Not applicable. The CEUS scan will be interpreted by the sponsor-investigator only.

3.3 Duration of Study Participation

The study duration per subject will be approximately 20 minutes including the time to prepare Lumason™ contrast agent, perform the pre-contrast imaging, and perform the CEUS, as well as the 60 minute monitoring period after the first and second injection of Lumason™.

CEUS will be performed at the time HII is first suspected or diagnosed. A second CEUS may be performed at short-term follow-up (approximately within 1-2 weeks from the first scan) for a total of two CEUS exams of 1 hour and 20-minute duration each.

Study participation will be complete when the 60 minute monitoring period of the last CEUS to be performed (after the first CEUS in patients who undergo one exam, or after the second CEUS is complete in patients who undergo two exams).

3.4 Total Number of Study Sites/Total Number of Subjects Projected

The study will be conducted at one site, The Children's Hospital of Philadelphia. It is expected 200 subjects will be enrolled to produce 100 evaluable subjects.

3.5 Study Population

3.5.1 Inclusion Criteria

1. Males and females aged 1.5 years or younger with open fontanelles and known or suspected hypoxic ischemic injury
2. Post menstrual age of 34 weeks or older
3. Patient in the CHOP NICU or PICU
4. Parental permission

3.5.2 Exclusion Criteria

1. Medical history of Lumason hypersensitivity

2. Hemodynamic instability as defined by rapid escalation of cardiopulmonary support in the past 12-24 hours, as defined by the clinical care team including ≥1 intensive care physician not part of the study team
3. Pulmonary insufficiency as defined by FiO2 requirements of >40% and/or subjects with pulmonary hypertension requiring nitric oxide

In this regard, there is a published report of infusion of the investigational drug in twelve neonates, ranging from 26.9 to 41 weeks gestational age, including four premature infants from 26.9 to 29.5 weeks gestational age [11] as well as 3 additional premature subjects from unpublished data of the sponsor. The infusion of the investigational drug in these neonates and premature infants was safe, and imaging results of good quality.

Brain CEUS has been performed successfully in intubated subjects without alteration of image quality or microbubble pharmacokinetics, as illustrated by the extensive evidence of intrasurgical brain CEUS after bone flap removal and/or transtemporal and transforaminal bone windows [28-33]. The specific ventilator settings and nitric oxide administration for pulmonary hypertension were chosen as exclusion criteria because they are indicative of unstable pulmonary status. These settings were determined according to clinical practice as communicated by Dr. John Flibotte (Co-medical Director of the Neonatal Neurocritical Care Program at CHOP), attending neonatologist with extensive clinical experience of HII patient care and supervision of clinical trials in this population.

Subjects who do not meet all of the enrollment criteria may not be enrolled. Subjects will be excluded from the study, if in the judgement of the primary clinical team, they are too unstable to tolerate the procedure.

Any violations of these criteria must be reported in accordance with IRB policies and procedures.

4 STUDY PROCEDURES

4.1 Screening

- Identify patients with suspected or diagnosed hypoxic ischemic injury
- Review of medical records (for inclusion and exclusion criteria)
- Discussion of the case with neonatologists and study team to determine eligibility
- If subject is considered eligible, written consent can be obtained at this stage by PI, co-investigator, or neonatologist co-investigator
- Coordinate cranial ultrasound schedule

4.2 Study Treatment Phase

Subjects enrolled to this clinical trial are anticipated to receive the study drug and have CEUS imaging conducted in the ICU setting. In this case, the study drug and US imaging unit will be brought to the ICU for study drug administration and imaging. Any clinically indicated non-contrast cranial ultrasounds be performed prior to contrast-enhanced cranial ultrasound so as not to delay clinical care.

Pre-injection documentation

Prior to injection, Vital signs and baseline assessment will be recorded and documented from the medical record and clinically in-place monitoring. Baseline assessment will consist on neurological status as described in the clinical evaluations recorded in the medical chart prior to intervention.

Pre-injection evaluation

The exam will include a pre-contrast injection evaluation with FDA-approved technologies (e.g. gray-scale ultrasound, Doppler ultrasound, microvascular imaging) to assess anatomical structures and guide contrast evaluation. This will last approximately 5 minutes.

CEUS scan

CEUS duration is of approximately 15 minutes.

CEUS will be performed at the time HII is first suspected or diagnosed. A second CEUS may be performed at short-term follow-up (approximately within 1-2 weeks from the first scan) for a total of two CEUS exams of 1 hour and 20-minute duration each.

Study participation will be complete when the 60-minute monitoring period of the last CEUS to be performed (after the first CEUS in patients who undergo one exam, or after the second CEUS is complete in patients who undergo two exams).

For those neonates/infants not undergoing hypothermia therapy, the first CEUS scan will be performed at the time HII is first suspected or diagnosed and the second CEUS scan will be performed within 24 hours of clinically indicated MRI. It is part of standard of care to obtain a clinically indicated MRI as follow-up of diagnosed or suspected hypoxic ischemic injury.

60-minute monitoring period

The study team, who are composed of personnel trained in recognizing signs of infusion reaction, will conduct the 60 minute monitoring period and record any untoward reaction that may be related to the infusion of the contrast drug. The 60 minute monitoring post-CEUS will be conducted by study team members.

Vital signs will be recorded and documented from the medical record and clinically in-place monitoring at 1) 30 minutes post-scanning, and 2) 60 minutes post-scanning. During monitoring, subjects will be assessed for rash, allergic reactions, anaphylaxis, and abrupt deviations from the subject's baseline hemodynamic parameters trend not related to medical intervention.

Adverse event assessment and documentation
Adverse events will be recorded at 1) 60 minutes post-scanning when the monitoring period is completed, and 2) through 48 hours post-scanning, with documentation at 48 hours post-scanning if no adverse event presents until this point. During the 48-hour AE assessment period, the following AEs of special interest will also be assessed:
a. worsening cardiopulmonary status,
b. worsening pulmonary hypertension, which may be suggested by new requirement of nitric oxide use, elevated pulmonary artery pressures on echocardiography, or differential limb pulse oximetry measurements,
c. worsening neurological status, and
d. serious gastrointestinal complications

4.2.1 CEUS #1

- Obtain consent prior to scheduled CEUS exam (if not previously obtained by, PI, co-investigator, or neonatologist co-investigator)
- Record vital signs and baseline assessment prior to injection of contrast
- Perform pre-contrast injection evaluation.
- CEUS scan performed at the time HII is first suspected or diagnosed
- Monitor subject for 60 minutes following the CEUS scan for documentation and treatment of potential adverse events
- Documentation of any adverse events through 48 hours post-scanning

4.2.2 CEUS #2

A second CEUS may be performed at short-term follow-up (approximately within 1-2 weeks from the first scan) for a total of two CEUS exams of 1 hour and 20-minute duration each.

- Record vital signs and baseline assessment prior to injection of contrast
- Perform pre-contrast injection evaluation.
- CEUS scan performed on the same day of MRI exam
- Monitor patient for 60 minutes following the scan for documentation and treatment of potential adverse events

Documentation of any adverse events through 48 hours post-scanning
Study participation will be complete when the 60-minute monitoring period of the last CEUS to be performed (after the first CEUS in patients who undergo one exam, or after the second CEUS is complete in patients who undergo two exams).

The study team will not use sedation or general anesthesia to conduct the research CEUS scans. The lowest mechanical index (MI) possible will be implemented in both CEUS, with a maximum MI value <0.2.

The results of the CEUS imaging will be collected for research purposes only. The results of the CEUS will not be used to direct clinical care decisions, without

confirmation of diagnosis by another medically established diagnostic product or procedure

4.3 Concomitant Medication

No concomitant medications will be recorded, with the exception of rescue medications, as noted below.

4.4 Rescue Medication Administration

All the rapid response equipment and resuscitation staff are readily available 24/7 in the intensive care unit setting at Children's Hospital of Philadelphia. In the rare event that a significant allergic reaction occurs, or anaphylaxis results following injection of Lumason™, standard clinical medication to treat the subject will be administered. For presumed allergic reactions, medications may include intravenous diphenhydramine and bolus corticosteroids (prednisolone), based on clinical care. For anaphylaxis, medications may include epinephrine as well as fluid and oxygen administration for emergency treatment, based on clinical care decision making and on the severity of symptoms. Any severe allergic or anaphylactic reaction will be reported to both the IRB and the FDA.

4.5 Subject Completion/Withdrawal

Subjects' families may withdraw their child from the study at any time without prejudice to their child's care. A study investigator may withdraw a subject to protect the subject for reasons of safety or for administrative reasons. It will be documented whether or not each subject completes the clinical study. If the Investigator becomes aware of any serious, related adverse events after the subject completes or withdraws from the study, the adverse events will be recorded and reported.

4.5.1 Early Termination Study Visit

A subject may be withdrawn by a parent prior to or during a CEUS scan, provided the 60 minute monitoring is completed after injection of the investigational drug. The CEUS examination will be terminated if there is a deterioration in the subject's clinical status during imaging.

4.5.2 Review of medical records from EPIC and/or other sources

- Date of birth
- Weight
- Clinical diagnosis
- Treatment history (medications, chemotherapy, antibiotics, steroids)
- Surgical history
- Pathology report

4.5.3 Review of diagnostic images from the PACS (iSite Radiology or iSite Enterprise)

- Review of cranial ultrasound and CEUS images (iSite)
- Review of available MRI images

5 STUDY EVALUATIONS AND MEASUREMENTS

The results of the pre-contrast injection and CEUS imaging will be collected for research purposes only. The results of the CEUS will not be used to direct clinical care decisions, without confirmation of diagnosis by another medically established diagnostic product or procedure.

Qualitative analysis
Visual rating by 2 teams consisting of primary investigator and second radiologist (co-investigator). Each scan will be rated for diagnostic quality and qualitative rating of cranial perfusion. The visual rating scale used will be: 0 (absent flow), 1 (decreased flow), 2 (normal flow), 3 (increased flow).

Quantitative analysis
Qontrast (Bracco Diag Inc., 510K regulatory status granted in 2004) contrast quantification software or similar software will be used to analyze the obtained CEUS scans. For each scan, wash-in and wash-out curves will be generated to quantify the rate of wash-in, time to peak intensity, peak intensity, and area under the curve.

The CEUS scans will be interpreted by the sponsor-investigator, and a second interpretation by a “second reader” in Radiology, who is part of a group of radiologists with sufficient training and expertise to read CEUS scans.

Monitoring After Investigational Drug Administration:

The monitoring post-administration will encompass 60 minutes, during which time the subject will be observed for the occurrence of infusion reactions. Any concern by the investigative team or attending staff for severe allergic reaction or anaphylaxis will be managed as all similar reactions are managed as part of clinical care, with close observation and treatment as clinically indicated, which may include diphenhydramine, corticosteroids, fluids, oxygen and epinephrine. Post-infusion reactions will be recorded and reported following established IRB and FDA reporting guidelines.

6 STATISTICAL CONSIDERATIONS

Up to 100 evaluable patients should be adequate for assessment of clinical feasibility and optimization of protocol. Qualitative analysis will be used as detailed above. Quantitative analysis will be performed by drawing a region of interest in brain segments and compared to control subjects (those with suspected or at risk of HII but turns out to be normal on imaging and clinical evaluation) using non-parametric Wilcoxon testing.

The study will optimize current techniques in the application of contrast ultrasound. For instance in brain imaging, the optimal plane in which to obtain the contrast perfusion kinetics curve is suggested but not established. The region of interest placement for perfusion kinetics quantification is also suggested by few publications, but not established as the standard practice. All this is part of the optimization process that is pivotal to improving the current contrast ultrasound techniques.

Specific analysis plan consists of both qualitative and quantitative assessment in which description of focal perfusion deficit or lesion, for instance, is reported in correlation with clinical information. Perfusion abnormalities will be qualitatively evaluated (absent – 0, mild hypoperfusion – 1, normal – 2, hyperperfusion – 3) and quantitatively (region of interest placed on brain regions for acquisition of wash-in curves using QLab or Qontrast or other similar software). These abnormalities will be correlated to MRI findings as gold standard, non-contrast ultrasound, and clinical information (neurologic exam, hemodynamics, behavioral outcomes) between serial exams. For extremely injured brain cases, nuclear scan (for diagnosis of brain death) instead of MRI may be performed as part of gold standard

The sample size will be based on the available cases, and based on the current number of patients presenting to Children's Hospital of Philadelphia neonatal or pediatric intensive care unit with clinical suspicion for hypoxic ischemic injury. It is estimated that the study will achieve a sample size of up to 50 neonates and infants per year for up to two years.

Baseline and demographic characteristics will be summarized using descriptive measures. Statistical analysis will consist of comparing diagnostic tests, in this case conventional grayscale ultrasound and contrast ultrasound, on each patient using the McNemar's test. Differences between perfusion before and after resuscitation (in the setting of hypoxic ischemic injury) will be evaluated with the Wilcoxon signal rank test for related samples.

6.1 Primary Endpoint

Qualitative analysis of perfusion abnormalities in correlation with clinical MRI results.

6.2 Secondary Endpoints

Quantitative analysis of perfusion abnormalities in correlation with clinical MRI results. This will be achieved by quantitative analysis, identifying regions of interest and generating time intensity curves.

6.3 Statistical Methods

6.3.1 Baseline Data

Baseline and demographic characteristics will be summarized by standard descriptive summaries.

6.3.2 Safety Analysis

All subjects entered into the study and receiving at least one injection of investigational drug will be included in the safety analysis. The frequencies of AEs by type, severity, and temporal relationship to the CEUS scan will be summarized. SAEs (if any) will be described in detail.

Adverse events will be recorded at 1) 60 minutes post-scanning when the monitoring period is completed, and 2) through 48 hours post-scanning, with documentation at 48 hours post-scanning if no adverse event presents until this point.

6.4 Sample Size and Power

Power analyses indicate that the study is sufficiently powered to detect group perfusion differences. For example, given a basal ganglia PE mean value in the control group of 1.37 (SD = 0.23) and in the patient group of 0.96 (0.09), with an alpha p = 0.05, power to detect group differences with a sample of 25 per group approaches 1.00. As another example, given a peak cortical perfusion mean value in the control group of 2.46 (SD = 1.96) and in the patient group of 0.69 (0.20), with an alpha p = 0.05, power to detect group differences with a sample of 50 per group approaches 0.84.

Given a sample of about 100 imaged patients, pilot data suggest that approximately one-half will show abnormal perfusion, thus providing a final sample of approximately 50 patients with abnormal perfusion and 50 patients with normal perfusion. The above sample sizes were used to compute the power to detect group differences in the ratio of basal ganglia to cortex perfusion, using means and standard deviations obtained from the pilot sample (see Preliminary Data).

Power analyses indicate that the study is sufficiently powered to detect group perfusion differences. For example, given a basal ganglia PE mean value in the control group of 1.37 (SD = 0.23) and in the patient group of 0.96 (0.09), with an alpha p = 0.05, power to detect group differences with a sample of 50 per group approaches 1.00. As another example, given a peak cortical perfusion mean value in the control group of 2.46 (SD = 1.96) and in the patient group of 0.69 (0.20), with an alpha p = 0.05, power to detect group differences with a sample of 50 per group approaches 0.85.

An interim statistical analysis may be considered to review image quality and comparisons between the results of each subject’s CEUS scans and available clinical US exams, to better inform the number of subjects required for statistical significance.

7 STUDY DRUG

7.1 Description

Lumason™ is currently FDA approved for use in the pediatric population for echocardiography and evaluation of focal hepatic lesions, and very recently (Jan 2017) approved for use in children for the evaluation of the urinary tract in pediatric patients with known or suspected vesicoureteral reflux. Previously, the presence of cardiac shunts was a contraindication for its use, but this was recently cancelled by the FDA as of December 2016. However, for this clinical study, the FDA has recommended that subjects with significant PDA and right-to-left cardiac shunts be excluded from study participation. Ultrasound contrast agents have been approved for use in Europe for almost two decades. In the case of SonoVue (now called Lumason™), a second-generation lipid/sulfur hexafluoride US contrast agent (Bracco, Milan, Italy), the European Union approved its intravenous use in adults in 2001. The FDA in the United States just recently approved the use of Lumason™ for evaluation of focal hepatic lesions in the pediatric population in 2016. Through decades of clinical utilization of ultrasound contrast agents, there are established recommended doses for the intravenous route of administration. Recommended intravenous dose for Lumason™ is weight-based, 0.03mL/kg as an intravenous injection, up to a maximum of 2.4mL per injection. As an example, for the intended study population it may be roughly estimated that the maximum weight of an infant 1 year of age may be up to approximately 10 kg, for a maximum dose of 0.30 mL. Two injections per exam will be performed.

7.2 Dosing

Through decades of clinical utilization of ultrasound contrast agents, there are established recommended doses for the intravenous route of administration. Recommended intravenous dose for Lumason™ is weight-based, 0.03mL/kg as an intravenous injection, up to a maximum of 2.4mL per injection. As an example, for the intended study population it may be roughly estimated that the maximum weight of an infant 1 year of age may be up to approximately 10 kg, for a maximum dose of 0.30 mL. Two injections per exam will be performed. Since investigational findings are subject to pre-analytic variability, performing two contrast-agent injections facilitates validation of findings if reproducibility is found. No increased risk was found at initial experience at Johns Hopkins Hospital, where 5 of 10 patients required double injection of contrast-agent. Clinical cases at Johns Hopkins Hospital and The Children's Hospital of Philadelphia have required double injection due to variable factors such as microbubble trapping within intravenous tubing. Adverse effects are not dose-dependent, thus risk is not increased by modifying the timing of interventions or increasing the number of contrast-agent injections.

Even though the initial dosing of investigational drug for this study is 0.03 mL/kg, it is possible that the optimal dose for CEUS imaging of hypoxic ischemic injury may be less than 0.03 mL/kg. Since there would be no apparent safety concern regarding the administration of a lower dose of the investigational drug, the study team would proceed with a lower dose administration, if initial imaging studies suggest that a dose less than 0.03 mL/kg may provide more optimal imaging results. Therefore, the study team proposes that a dose range of 0.01-0.03 mL/kg be considered for dose optimization of

the initial subjects, as indicated. Based on the published report of safe infusion of the investigational drug in four premature infants from 26.9 to 29.5 weeks gestational age [11] as well as 3 additional premature subjects from unpublished data of the sponsor, this dose range seems appropriate. For each subject, the dose per injection, as well as the total dose delivered, will be recorded in the study file for each administration of study drug. The expectation is that the intravenous injection of this ultrasound contrast agent will permit noninvasive, non-ionizing delineation of physiology and pathophysiology with higher resolution and accuracy than conventional ultrasound techniques. Since preliminary studies at Hopkins showed that half of neonates required two injections of contrast agent to achieve evaluable CEUS images, two injections will be performed per CEUS scan in all subjects to ensure adequate image quality and reproducibility.

7.3 Investigational Drug Handling and Accountability

Bulk supplies of drug will be directly shipped to IDS, by drug supplier. On an as-needed basis, only 1 box of either 5 or 20 vials, will be ordered from IDS by the principal investigator. The 1 box supply will be located in a storage unit, inside the Ultrasound Suite, with access limited to study personnel and dedicated Ultrasound personnel.

The investigational imaging drug, Lumason™, will be maintained as a separate supply from the Radiology Departments and central supply's Lumason™ that is used for clinical care purposes. Specifically, the investigational drug Lumason™ for this IND will be physically segregated from the clinical use Lumason™, and stored in a location that identifies the study drug as "Investigational Drug Lumason™, For IND Research Only" so that all Radiology personnel know to limit the use of the investigational drug Lumason™ for study in this particular IND research exclusively.

The investigational drug Lumason™ will be labeled according to FDA regulations, and identified as for IND research use only. The study team will use the investigational drug exclusively for the purposes of this IND study, and will not distribute or administer the investigational drug to persons not participating in the clinical study.

The study team will ensure that each Lumason™ vial used in the IND clinical trial is entered into an investigational drug log that contains at least the following details of each vial: Lot #number and expiration date. For each subject, the Lumason™ lot #number, expiration date, # of vials used, disposition of unused Lumason™, and discard procedure, will be recorded as part of the study record. Expired lots of Lumason™ will not be used in the clinical trial, and will be discarded.

8 SAFETY MANAGEMENT

8.1 Clinical Adverse Events

Clinical adverse events (AEs) will be monitored throughout the study. Subjects enrolled to this clinical trial are anticipated to receive the study drug and have CEUS imaging conducted in the ICU setting. In this case, the study drug and US imaging unit will be

brought to the ICU for study drug administration and imaging. CEUS has a duration of approximately 20 minutes. The 60 minute monitoring post-CEUS will be conducted by the study team members. The study team, who are composed of personnel trained in recognizing signs of infusion reaction (rash, allergic reactions, anaphylaxis) will conduct the 60 minute monitoring period and record any untoward reaction that may be related to the infusion of the contrast drug. Adverse events will be recorded at 1) 60 minutes post-scanning when the monitoring period is completed, and 2) through 48 hours post-scanning, with documentation at 48 hours post-scanning if no adverse event presents until this point. All adverse events suspected of being related to study drug infusion will be reported to the regulatory authorities.

In addition to assessing each patient after dosing, after 10 subjects, a complete safety analysis to determine the safety of continuing the study will be performed.

8.2 Adverse Event Reporting

Unanticipated problems related to the research involving risks to subjects or others that occur during the course of this study (including SAEs) will be reported to the IRB in accordance with CHOP IRB SOP 408: Unanticipated Problems Involving Risks to Subjects. AEs that are not serious but that are notable and could involve risks to subjects will be summarized in narrative or other format and submitted to the IRB at the time of continuing review.

AEs will be recorded and graded per the International Neonatal Consortium (INC) Neonatal AE Terminology.

8.3 Definition of an Adverse Event

An adverse event is any untoward medical occurrence in a subject who has received an intervention (drug, biologic, or other intervention). The occurrence does not necessarily have to have a causal relationship with the treatment. An AE can therefore be any unfavorable or unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product.

All AEs (including serious AEs) will be noted in the study records and on the case report form with a full description including the nature, date and time of onset, determination of non-serious versus serious, intensity (mild, moderate, severe), duration, causality, and outcome of the event. Adverse events will be recorded at 1) 60 minutes post-scanning when the monitoring period is completed, and 2) through 48 hours post-scanning, with documentation at 48 hours post-scanning if no adverse event presents until this point.

8.4 Definition of a Serious Adverse Event (SAE)

An SAE is any adverse drug experience occurring at any dose that results in any of the following outcomes: death, a life-threatening event (at risk of death at the time of the event), requires inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect in the offspring of a subject.

Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse drug event when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. A distinction should be drawn between serious and severe AEs. A severe AE is a major event of its type. A severe AE does not necessarily need to be considered serious. For example, nausea which persists for several hours may be considered severe nausea, but would not be an SAE. On the other hand, a stroke that results in only a limited degree of disability may be considered a mild stroke, but would be an SAE.

8.4.1 Relationship of SAE to study drug or other intervention

The relationship of each SAE to the study intervention should be characterized using one of the following terms in accordance with CHOP IRB Guidelines: definitely, probably, possibly, unlikely or unrelated.

8.5 IRB/IEC Notification of SAEs and Other Unanticipated Problems

The Investigator will promptly notify the IRB of all on-site unanticipated, serious Adverse Events that are related to the research activity. Other unanticipated problems related to the research involving risk to subjects or others will also be reported promptly. Written reports will be filed using the eIRB system and in accordance with the timeline below. External SAEs that are both unexpected and related to the study intervention will be reported promptly after the investigator receives the report.

Type of Unanticipated Problem	Initial Notification (Phone, Email, Fax)	Written Report
Internal (on-site) SAEs Death or Life Threatening	24 hours	Within 2 calendar days
Internal (on-site) SAEs All other SAEs	7 days	Within 7 business days
Unanticipated Problems Related to Research	7 days	Within 7 business days
All other AEs	N/A	Brief Summary of important AEs may be reported at time of continuing review

8.5.1 Follow-up report

If an SAE has not resolved at the time of the initial report and new information arises that changes the investigator's assessment of the event, a follow-up report including all relevant new or reassessed information (e.g., concomitant medication, medical history) should be submitted to the IRB. The investigator is responsible for ensuring that all SAE are followed until either resolved or stable.

8.6 Notifications of SAEs/IND Safety Reports to the FDA

Unexpected fatal or life-threatening adverse events that are related to the study drug, will be reported to FDA as soon as possible but no later than 7 calendar days following the sponsor's initial receipt of the information.

Unexpected serious adverse events that are related to the study drug but not fatal or life-threatening, will be reported to FDA as soon as possible but no later than within 15 calendar days following the sponsor's initial receipt of the information.

Follow-up reporting: Any relevant additional information obtained by the sponsor that pertains to a previously submitted IND safety report will be submitted as a Follow-up IND Safety Report. Such report will be submitted as soon as the information is available, but no later than 15 calendar days after the sponsor receives the information.

All other adverse events, will be reported to the FDA at or by the time of the Annual Report.

8.7 Medical Emergencies

Any medical emergencies that develop following injection of the investigational drug will be managed according to clinical care. See protocol Section 4.4 for a more complete description of clinical care management for serious adverse events following infusion of the investigational drug.

8.8 Study Stopping Rules

The study will be stopped for image futility, if non-diagnostic imaging is obtained in the first 3 subjects.

Affected neonates and infants with hypoxic ischemic injury have substantially increased mortality and long-term neurological sequelae. There are some circumstances which potentially may arise, requiring temporary study stop. The FDA requires that the study be stopped for all patients (to allow for review of the protocol and procedures based on study related events) after one episode of anaphylaxis or death or other serious adverse event, regardless of relation to study drug. Therefore, anaphylaxis or death or another SAE will prompt a temporary stop to formally discuss the event with the FDA. As such an event (if deemed unrelated) may not meet the prompt reporting criteria for the IRB, the IRB will be notified as applicable (in accordance with CHOP IRB SOP 408). The

study will proceed only with documented concurrence of the FDA (and the IRB, as applicable).

The occurrence of two non-fatal SAEs directly related to use of the study drug or one death directly attributed to use of the study drug will stop the study.

9 STUDY ADMINISTRATION

9.1 Data Collection and Management

Confidentiality: All subjects will be assigned a number unrelated to their medical record number and this will be kept in a master list. All of the data collected will be recorded using the respective “research number” to maintain anonymity. Only the master list will contain patient identifiers and a link to the research-specific code, and the data collection sheet will not contain patient identifiers.

Security: All files (master list and data collection sheet) will be password protected and will be stored on a password protected computer at CHOP on the secure storage network on the secure Hospital server. A password protected excel spreadsheets will be used for data collection. The paper scoresheets will be identifiable only by the coded # of each subject and will be stored in a binder in a locked cabinet located in the study coordinator's office. The only way we will use to transfer information between co-investigators will be [send secure] emails using the study members @email.chop.edu account. These e-mails will be only accessed from CHOP network computers and will be erased after the download of the password-protected excel sheet. All computers will meet CHOP IT Policy A-3-6: Acceptable Use of Technology Resources.

Anonymization, de-identification, or destruction: After the study is finalized (results published in a scientific journal), the master list containing the reference to PHI will be archived in accordance with FDA and CHOP requirements. De-identified scoresheets and data gathered from the study will be archived in a password-protected folder on the primary computer of the P.I. These data will be destroyed only after a period of time compliant with federal and institutional guidelines.

9.2 Confidentiality

All data and records generated during this study will be kept confidential in accordance with Institutional policies and HIPAA on subject privacy and that the Investigator and other site personnel will not use such data and records for any purpose other than conducting the study. Patient names will be removed from images for use in the educational setting.

No identifiable data will be used for future study without first obtaining IRB approval. The investigator will obtain a data use agreement between the provider (the PI) of the data and any recipient researchers (including others at CHOP) before sharing a limited dataset (PHI limited to dates and zip codes).

9.3 Data and Safety Monitoring Plan

The safety monitoring for this study is the primary responsibility of the sponsor-investigator. Monitoring the safety outcomes following IV administration of the investigational drug will be conducted primarily by the Principal Investigator. An independent safety monitor will be designated to oversee the safety reports, and help adjudicate attribution of adverse events, should they occur. Regular meetings to discuss the outcomes of the study, and of the safety events, will be conducted by the study team. The occurrence of adverse events, serious adverse events and unanticipated events will be reported by the study team in accordance with federal and institutional guidelines, as outlined in Section 8 of this clinical study.

Prior to study initiation, the Office of Research Compliance (ORC) will conduct a pre-trial monitoring visit, to assess trial readiness of the study staff. Once the pre-trial monitoring visit has been successfully completed, the ORC will also monitor the IND study on an annual basis.

9.4 Regulatory and Ethical Considerations

9.4.1 Risk Assessment

The pre-contrast scan is non-invasive and poses risk no greater than minimal. The potential risks associated with contrast-enhanced ultrasonography have been extensively studied, and the risk associated with the technique is less than that of CT or MRI contrast agents. The risk of adverse events is the lowest of all contrast agents available, with CT contrast being the highest (0.6%), followed by MR contrast (0.0088%) and ultrasound contrast (0.0086%) [1]. Further studies detail the safety profile of ultrasound contrast agents in children and have shown minor adverse events including nausea, tinnitus, lightheadedness, altered taste sensation [3-5]. Serious cardiopulmonary reactions, including fatalities, have occurred uncommonly in adults that had complex comorbidities. Risk for these reactions may be increased among patients with unstable cardiopulmonary conditions.

One documented severe reaction in a child documented symptoms of generalized pruritus, nausea, hypotension with tachycardia initially then bradycardia [6]. Management in this instance consisted of oxygen, intravenous epinephrine, and fluids (0.9% normal saline) with resolution of symptoms in two hours. Treatment of both minor, mild, and severe adverse reactions post Lumason™ administration are the same as that of CT or MRI contrast agents. In comparison to CT or MRI contrast agents, however, ultrasound contrast agents have proven to be much safer in children with only one serious adverse event over decades of its use to date (contrasting to approximately 15-20 adverse events per 2000 children if CT contrast agent were to be used). No serious adverse event has been reported in a neonate since its clinical use in this population. Animal studies on its toxicity profile also validate no fetal toxicity and the ultrasound contrast agent belongs to category B.

In the case of SonoVue (now called Lumason™), a second-generation lipid/sulfur hexafluoride US contrast agent (Bracco, Milan, Italy), the European Union approved its intravenous use in adults in 2001. The FDA in the United States just recently approved the use of Lumason™ for evaluation of focal hepatic lesions and vesicoureteral reflux in pediatric population in 2016. For the remainder of clinical applications, the ultrasound contrast agents are being used off-label in both Europe and the United States.

Risks of the administration of the study drug are considered a minor increase above minimal risk, without the prospect of direct benefit. Adverse effects are not dose-dependent, thus risk is not increased by modifying the timing of interventions or increasing the number of contrast-agent injections.

Another risk of the study includes the insertion of a peripheral IV line. This is a no greater than minimal risk procedure, with the main risks of discomfort, bruising, and infection which are generally self-limited. There is a no greater than minimal risk of breach of confidentiality, which is minimized by having all of study personnel undergo HIPAA training.

Interference of CEUS with MRI is not expected since CEUS contrast-agent Lumason clears within minutes after injection. Elimination of Lumason (Sulfur Hexafluoride Lipid-Type A Microspheres) occurs via the lungs in the first minutes following contrast-agent injection (please see Package Insert's section 12.3 Pharmacokinetics for more detail, attached in Application's section 12.02 (3.0)). Additionally, Misun Hwang, the sponsor-investigator, has also previously performed brain CEUS before MRI in neonatal patients without adverse events.

Steps Taken to Minimize Risks

Parents and/or legal guardians of participants will be asked about contraindications to contrast enhanced ultrasonography examinations, as listed in the exclusion criteria. In order to appropriately treat potential rare adverse events, patients will be monitored by the study team for 60 minutes following contrast administration.

Vital signs will be recorded from the medical chart and in-place monitoring and documented at 1) 30 minutes post-scanning, and 2) 60 minutes post-scanning. During monitoring, subjects will be assessed for rash, allergic reactions, anaphylaxis, and abrupt deviations from the subject's baseline hemodynamic parameters trend not related to medical intervention.

Adverse events will be recorded at 1) 60 minutes post-scanning when the monitoring period is completed, and 2) through 48 hours post-scanning, with documentation at 48 hours post-scanning if no adverse event presents until this point.

9.4.2 Potential Benefits of Trial Participation

The patient will not receive a direct benefit as a result of participating in the study. Indirect benefits may include improvement in current diagnostic algorithm for detection and monitoring of HII and downstream reduction of high mortality and morbidity associated with HII.

9.4.3 Risk-Benefit Assessment

The benefit to society outweighs the risks of this study.

9.5 Recruitment Strategy

Potential subjects will be identified by reviewing the subjects' medical chart and referral from neonatologists of patients during the neonatal or pediatric intensive care unit stay for suspected, at risk of or diagnosed hypoxic ischemic injury and review of orders for cooling blankets, brain imaging and/or EEG exams in neonates. Potential participation will be discussed by the PI with the referring neonatologist (co-investigator). Parents/legal guardians may be approached over phone/e-mail by a member of the study team to determine if they are interested in receiving more information about the study. Participation will be discussed with the parents/guardian by the neonatologist and/or radiologist. Parental/guardian permission (informed consent) will be obtained.

Additionally, the CHOP Research Discovery Finder, e-mails, and a tearpad flyer will be used in the recruitment strategy

9.6 Informed Consent and HIPAA Authorization

Approved members of the study team will obtain parental/guardian consent prior to the proposed study in a private setting. The investigators will assure that parents/guardian comprehend the nature of the study, the study procedures and the risks and benefits of participation, steps that will be taken to avoid coercion and documentation of consent. A combined HIPAA consent-authorization document will be used.

9.7 Payment to Subjects/Families

Families will be offered a gift card of 50 USD value for participation in the study.

10 PUBLICATION

The investigative team plans to publish the data collected in a scientific journal. Data may also be presented as abstract, podium presentation, or poster presentations at scientific meetings and conventions. No patient identifying information will be used in publications.

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