Clinical Study of the BreathID® MCS System to train the algorithm for the ^{13}C-Methacetin Breath Test (MBT) in assessment of Portal Hypertension in Patients with Compensated Liver Cirrhosis

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	Name	**Position**
Author / Owner	Avraham Hershkowitz	Clinical Trial Manager
Supervisor / Revised by	Gil Guggenheim	Clinical Application Development Specialist and Database Manager
Approved by	Melina Arazy	Director of Clinical Affairs
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Exalenz Bioscience Ltd. 4 Ha'Maayan St. Modiin, Israel 7177872

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2 ABBREVIATIONS

AASLD - American Association for the Study of Liver Diseases

AE - Adverse Event

ALF - Acute Liver Failure

ALT - Alanine aminotransferase

APAP - n-acetyl-p-aminophenol (acetaminophen)

aPTT - activated Partial Thromboplastin Time

AST - Aspartate aminotransferase

AUC - Area Under the Curve

BUN - Blood Urea Nitrogen

CFR - Code of Federal Regulation

CO_2 - Carbon dioxide

CPDR - Cumulative Percentage Dose Recovery (expressed as %)

CRA - Clinical Research Associate

CRF - Case Report Form

CRO - Clinical Research Organization

CSPH - Clinically Significant Portal Hypertension

CT - Computed Tomography

CTCAE - Common Terminology Criteria for Adverse Events

CVP - Central Venous Pressure

DOB - Delta over baseline

EASL - European Association for the Study of the Liver

EC - Ethics Committee

eCRF - Electronic Case Report Form

EDC - Electronic Data Capture

FDA - Food and Drug Administration

FHVP - Free Hepatic Venous Pressure

GCP - Good Clinical Practice

GI - Gastrointestinal

GTTP - Gamma-Glutamyl Transpeptidase

HCC - Hepatocellular Carcinoma

HCO_3 - Bicarbonate

HE - Hepatic Encephalopathy

HVPG - Hepatic Venous Pressure Gradient

ICH - International Conference of Harmonization

IDE - Investigational Device Exemption

INR - International Normalized Ratio (for prothrombin time)

IRB - Institutional Review Board

KOL - Key Opinion Leaders

K+ - Potassium

LF - Liver Function

MBT - ^{13}C-Methacetin Breath Test

MCS - Molecular Correlation Spectrometry

MELD - Model for End-stage Liver Disease

Na+ - Sodium

NIH - National Institute of Health

NPV - Negative Predictive Value

NSBB - Non-Selective Beta Blocker

OUS - Out of the United States

O_2 - Oxygen

PAP - Pulmonary Artery Pressure

PEEP - Positive End Expiratory Pressure

PHT - Portal Hypertension

PPV	-	Positive Predictive Value
PDR	-	Percent Dose Recovered (expressed as % per hour)
ROC	-	Receiver Operating Characteristic
SAE	-	Serious Adverse Event
SUSAR	-	Suspected Unexpected Serious Adverse Reactions
TIPS	-	Transjugular Intrahepatic Portosystemic Shunt
US	-	United States (of America)
USA	-	United States of America
WBC	-	White Blood Cells
WHVP	-	Wedged Hepatic Venous Pressure

3 PROTOCOL SYNOPSIS

Protocol Title:	Clinical Study of the *BreathID® MCS* System to train the algorithm for the ^{13}C-Methacetin Breath Test (MBT) in assessment of Portal Hypertension in Patients with Compensated Liver Cirrhosis
Protocol Reference Number:	CSPH-EX-0414
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Phase of Development:	Phase II (training)
Sponsor:	**Exalenz Bioscience Ltd.** 4 Hama'ayan Street Modi'in, Israel 71700 Tel: +972-8-9737500 Fax: +972-8-9737501
Principle Investigators:	a. Dr. Juan Carlos García Pagán, Barcelona, Spain b. Dr. Agustín Albillos, Madrid, Spain c. Dr. Christophe Bureau, Toulouse, France d. Dr. Didier Lebrec, Dr. Pierre Ratou, Paris (Clichy), France e. Dr. Guadalupe Garcia-Tsao, New Haven , CT , USA f. Dr. Velimir Luketic, Richmond, VA, USA g. Dr. Annalisa Berzigotti, Bern, Switzerland h. Dr. Manal Abdelmalek, Durham, NC, USA
Institutions:	a. Hospital Clinic, , Barcelona, Spain. b. Hospital Santiago Ramón y Cajal, Madrid, Spain c. Hopital Purpan, Toulouse, France d. Hopital Beaujon, Paris (Clichy), France e. Yale University School of Medicine, New Haven, CT / VA Connecticut Health Care System, USA f. Virginia Commonwealth University Medical Hospital, VA, USA g. Inselspital University Hospital, Bern, Switzerland h. Duke University, Durham, NC, USA

Investigated disease:	Clinically Significant Portal Hypertension (CSPH) in patients with compensated liver cirrhosis
Combination Product	BreathID® *MCS* system together with ^{13}C-labeled Methacetin
Comparator:	HVPG - Hepatic Vein Portal Pressure Gradient
Primary Objective:	The primary objective of the study is to develop an algorithm and its cut-off to detect CSPH, defined as HVPG ≥10mmHg, based on the MBT.
Primary Efficacy Endpoint:	Binary diagnosis of CSPH, defined as HVPG ≥10 mmHg, correlated with an MBT result to be developed.
Secondary:	1. Binary diagnosis of portal hypertension with HVPG ≥ 12mmHg 2. Binary diagnosis of portal hypertension with HVPG ≥ 20mmHg 3. Correlation of MBT with HVPG readings 4. Assess the effect of beta blockers or other confounding factors on the Correlation of MBT with HVPG readings 5. Examine the effect of a single small (<3cm) HCC (hepatocellular carcinoma) on the correlation of MBT with HVPG tracings
Safety Endpoints:	Safety will be assessed by all adverse and serious adverse events occurring in all subjects enrolled throughout the duration of the study.
Study Design:	A multicenter, non-randomized, blinded, cross-sectional comparative exploratory study comparing various MBT measures to HVPG. This study will be used to train an algorithm using MBT measures and to select a cut-off to determine presence or absence of CSPH as defined by HVPG ≥ 10mmHg.

Study Population[a]**:** At least 200 consecutive compensated cirrhotic patients, defined as cirrhotic patients without complications[b] and meeting all inclusion/exclusion criteria will be enrolled on a walk-in basis. If beta blockers or HCC or other confounding factors affect the correlation between MBT and HVPG the sample size may be enlarged following interim analysis.

Inclusion Criteria:

1. Adult men or women (≥18 years of age)
2. Patient (or legal guardian) can sign an Informed Consent Form
3. Known chronic liver disease with cirrhosis confirmed by either:
   a. liver biopsy or
   b. clinical (palpable left lobe, splenomegaly) and laboratory (platelets <150,000/mm^3 or albumin< 3.8g/dL, or INR >1.3) evidence of cirrhosis and/or
   c. imaging studies by abdominal sonography, computer assisted axial tomography, or magnetic resonance imaging, showing a nodular liver and/or enlarged spleen and/or portosystemic collaterals with portal vein patency and/or minimal ascites, and/or colloid shift on a colloid-isotope liver-spleen scan or measurements of liver stiffness suggestive of cirrhosis
4. Europe: Indicated to undergo HVPG testing
   US: Consented for HVPG
5. Can tolerate an overnight (8-hour) fast
6. For patients treated with statins: They have to be on a stable dose for at least 4 weeks prior to any study related tasks (MBT or HVPG measurement)
7. For patients treated with beta blockers: They have to be on a stable dose for at least 6 weeks prior to any study related tasks (MBT or HVPG measurement)

[a] Most patients to be enrolled in the proposed study will be from centers in Europe. Based on expert opinion in the field of hepatology, the population to be enrolled from Spain and France is representative of patients with chronic liver disease in the United States. The management of patients in the United States is based upon and derived from the experience and guidelines developed in Barcelona (Spain). Patients in these countries have similar etiologies and prognosis and are being used interchangeably in multicenter clinical trials using HVPG testing

[b] See eligibility criteria.

8. For patients who stopped their treatment with beta blockers: Their last dose should be at least 6 weeks prior to any study related tasks (MBT or HVPG measurement)

   Note: In case patient has HCC, breath test has to be performed on the day of HVPG measurement.

Exclusion Criteria:

1. Decompensated cirrhosis as clinically defined by the occurrence of any of the following: ascites, hepatic encephalopathy, variceal bleeding (active upper GI bleeding) or hepatorenal syndrome
2. Renal failure (creatinine > 2.5 mg/dl)
3. Known acute renal tubular disease
4. Known hypotension (Systolic Pressure <100mmHg)
5. Hypocoagulablity defined as PT >6 and INR >2.3.
6. Congestive heart failure (assessed clinically as NIHA >2)
7. Known pulmonary hypertension (right ventricular systolic pressure > 45 mm Hg)
8. Uncontrolled diabetes mellitus (HBA1C >9.5gr%)
9. Concurrent prednisone or immunosuppressive treatment, if therapy and/or response to treatment are not stable for at least 3 months.
10. Documented hepatocellular carcinoma lesion larger than 3cm and/or multifocal lesions and/or evidence of vascular invasions
11. Gastric bypass surgery or extensive small bowel resection
12. Total parenteral nutrition
13. Any organ transplant recipient
14. Pregnant or breast feeding
15. Allergy to acetaminophen and/or other related medications.
16. Documented drug-related concurrent hepatotoxicity or drug-related silent steatosis or drug-related fibrosis (e.g. amiodarone, methotrexate and tamoxifen)
17. Uncontrolled malabsorption or diarrhea
18. Documented non-cirrhotic PHT, partial/complete portal venous occlusion, hepatic venous occlusion, previous PHT surgery, or placement of a transjugular intrahepatic portosystemic shunt (TIPS)
19. Primary or secondary biliary cirrhosis, primary or secondary sclerosing cholangitis, hepatic sarcoidosis, or other cholestatic disorders
20. Subjects unable to perform the MBT within 7 days of HVPG procedure.

21. Subject should not have taken any of the following for at least 48 hours prior to the breath test: Acyclovir , allopurinol, carbamazepine, cimetidine, ciprofloxacin, daidzein, (herbal) disulfiram, echinacea, enoxacin, famotidine, fluvoxamine, methoxsalen, mexiletine, montelukast, norfloxacin, phenylpropanolamine, phenytoin, propafenone, rifampin, terbinafine, ticlopidine, thiabendazole, verapamil, zileuton or any medication that might interfere with Methacetin metabolism or might affect CYP 1A2
22. Subject should not have taken amiodarone within the last 30 days prior to the breath test or HVPG procedure. Splenomegaly secondary to inflammation/infection or hematologic malignancy.
23. Last dose of HCV treatment received within the past 3 years

On the day of the MBT:

1. 8 hours fasting prior to MBT including all PO morning medications
2. No Smoking on the day of the MBT
3. No caffeine within 24 hours prior to MBT
4. No paracetamol (acetaminophen) based medications within 24 hours prior to MBT
5. No general anesthesia or sedation within 24 hours prior to MBT
6. No alcohol within 24 hours prior to MBT
7. Patient should be symptom free of any prior intervention (e.g. biopsy)

Listing of Study Procedures:

1. IC and eligibility
2. Baseline and Physical Examination
3. HVPG procedure[c]
4. *MBT* - ^{13}C-Methacetin Breath Test (within 7 days of HVPG)

   Note1: The MBT may be performed prior to HVPG as long as it is done within 7 days.

   Note2**:** If patient is using a stable dose of beta blockers both the MBT and the HVPG procedures should be performed while continuing the stable dose even on the morning of each test.

   Note3**:** If patient has HCC, breath test has to be performed on the day of HVPG measurement.

[c] The measurement will be performed in a uniform way including following a standardized procedure to measure HVPG pressures (see Appendix II), either before or after the MBT, with certain limitations detailed in the protocol.

Rules of discontinuations (Subject and/or Study):

1. Withdrawal of patients from the study if they have a CTCAE (Common Terminology Criteria for Adverse Events) Grade 3 or higher regardless of whether the event is attributed to the drug unless it is caused by an accident that could not reasonably be attributable to the drug (CTCAE: Common Terminology Criteria for Adverse Events Version 4.0).
2. Discontinuance of study, if ≥ 2 patients on Methacetin develop the same CTCAE Grade 3 event or 1 patient develops a CTCAE Grade 4 or higher event due to the breath test.

Data Analysis / Statistics: A ROC analysis will be used to determine the optimal parameter and cut-offs for required performance measures sensitivity and specificity.

All efficacy analysis will include the point estimates of sensitivity, specificity, PPV and NPV with respective exact 95% confidence intervals. The respective Positive and Negative Predictive Values (PPV and NPV) will be presented as a function of possible CSPH prevalence and will include the prevalence of the enrolled population.

4 VISIT SCHEDULE OVERVIEW

Screening (Baseline)

- Informed Consent
- Inclusion/exclusion criteria
- Safety urine sample (pregnancy)*
- Medical/disease history
- Concomitant medications
- Eligibility screening for HVPG

Day 1-7

- Physical examination and vital signs
- HVPG procedure
- ^{13}C-methacetin breath test
- Concomitant medications
- Adverse event reporting

5 BACKGROUND

Exalenz Biosciences Ltd. has developed diagnostic breath test products consisting of combinations of a medical device and various ^{13}C-labeled diagnostic substrates for gastrointestinal and liver applications.

The proposed device is a molecular correlation based spectrometer, using a patented technology based on specific light source emissions and absorption of $^{13}CO_2$ and $^{12}CO_2$ gases. This technology is already implemented in a similar device already approved for marketing in Europe and cleared in the USA. The ^{13}C-labeled substrates (in this case ^{13}C-Methatecin) are metabolized by the target organ under investigation, producing $^{13}CO_2$ which in turn leads to changes in $^{13}CO_2/^{12}CO_2$ ratio in patient's exhaled breath over time. These ratio changes are displayed in real time on the device's screen and printed at end of test.

The rate and pattern of the $^{13}CO_2/^{12}CO_2$ ratio curve reflect substrate metabolism, i.e. target organ's metabolic capacity.

The aim of the Company in the field of liver diseases is to provide a non-invasive, point-of-care, breath test to assess disease severity using ^{13}C-Methacetin.

5.1 Portal Hypertension and Hepatic Decompensation

Cirrhosis results from continuous liver injury and repair, and is defined pathologically as hepatic regeneration constrained into a disorganized nodular architecture by bridging bands of fibrosis.[1, 2] Early cirrhosis showing no functional abnormality is said to be "compensated".[3] However, as fibrosis worsens and further distorts liver architecture, the naturally highly compliant liver sinusoids exhibit increasing resistance to blood flow. These sinusoidal changes are marked by loss of the fenestrae (pores), sub-endothelial collagen deposition and by contractile myofibroblasts derived from activated stellate cells[4] as well as by nodules.[1, 5] Progressive impairment of liver performance follows.

Increased intrahepatic vascular resistance raises sinusoidal and subsequently portal venous pressure. When the pressure gradient between the portal and systemic venous systems rises, patients are said to be afflicted by Portal Hypertension (PHT). The rise in pressure causes the opening and enlargement of existing porto-systemic connections and the creation of new porto-systemic collateral veins (neovascularization). The shunting vasculature is known as varices when they line mucosal surfaces of the esophagus, stomach and elsewhere in the gastrointestinal tract.[6] An additional shunting effect is present within the liver by the formation of intrahepatic shunts which in-fact are bypassing the functional liver. PHT may result in the transudation of extracellular fluid from the liver into the peritoneal cavity (ascites), and may also be responsible

for both the opening and enlargement of existing porto-systemic connections and the creation of new porto-systemic collateral veins (neovascularization) that are known as varices when they line mucosal surfaces of the esophagus, stomach and elsewhere in the gastrointestinal tract.[6] When ascites is present or varices bleed, the cirrhotic patient is said to be "decompensated", which correlates with increased patient morbidity and mortality.[3] Progressive PHT may lead to splenomegaly and subsequently systemic vasodilatation as well as changes in systemic hemodynamics affecting the heart (hyperdynamic circulation) and blood flow to vital organs, notably the kidneys, lungs and brain.[7, 8] Whereas PHT and its complications may be recognized clinically and/or with non-invasive imaging, actual portal pressure can only be measured invasively.

5.2 Hepatic Venous Pressure Gradient (HVPG) assessing Portal Hypertension

Sinusoidal pressure can be estimated by catheterizing the hepatic veins (most commonly right hepatic vein via transjugular access). The pressure is then measured in the hepatic venous radicles once when the vein is open and a second time when it is occluded.[9, 10] The difference between the free (unoccluded) and wedged pressure is the hepatic venous pressure gradient (HVPG), which gives a reliable estimate of sinusoidal portal pressure. (The hepatic venous pressure method does not detect presinusoidal portal hypertension, e.g. due to portal vein obstruction).

HVPG > 5 mm Hg defines PHT, whereas HVPG ≥ 10mm Hg is recognized as "clinically significant portal hypertension (CSPH)", as it is the pressure threshold that predicts many of the complications of cirrhosis.[6, 11] As shown in a recent review based on the recommendations of the AASLD (American Association for the Study of Liver Disease) and EASL (European Association for the Study of the Liver), supported by over 80 references,[12] increments in HVPG are significantly associated with liver decompensation and patient death. Increments in HVPG occuring over time are positive predictors of the development of varices, variceal hemorrhage, non-variceal complications and death.

For a better understanding of HVPG and its utility, a citation from the current American Association for the Study of Liver Disease (AASLD) Guidelines[13] is quoted:

> *"The method for assessing portal pressure is the wedged hepatic venous pressure (WHVP) measurement, which is obtained by placing a catheter in the hepatic vein and wedging it into a small branch or, better still, by inflating a balloon and occluding a larger branch of the hepatic vein. The WHVP has been shown to correlate very closely with portal pressure both in alcoholic and non-alcoholic*

> *cirrhosis.[14] The WHVP is always corrected for increases in intra-abdominal pressure (e.g., ascites) by subtracting the free hepatic vein pressure (FHVP) or the intra-abdominal inferior vena cava pressure, which act as internal zeroes. The resultant pressure is the hepatic venous pressure gradient (HVPG), which is best accomplished with the use of a balloon catheter, usually taking triplicate readings and, when measured with a proper technique, is very reproducible and reliable.[10] Since it is a measure of sinusoidal pressure, the HVPG will be elevated in intrahepatic causes of portal hypertension, such as cirrhosis, but will be normal in pre-hepatic causes of portal hypertension, such as portal vein thrombosis. The normal HVPG is 3-5 mmHg. The HVPG and changes in HVPG that occur over time have predictive value for the development of esophagogastric varices,[15, 16] the risk of variceal hemorrhage,[7, 17, 18] the development of non-variceal complications of portal hypertension,[7, 19, 20] and death.[3, 18, 20, 21] Single measurements are useful in the prognosis of both compensated and decompensated cirrhosis, while repeat measurements are useful to monitor response to pharmacological therapy and progression of liver disease. Limitations to the generalized use of HVPG measurement are the lack of local expertise and poor adherence to guidelines that will ensure reliable and reproducible measurements,[10] as well as its invasive nature."*

The reproducibility of HVPG in skilled hands was recently assessed in a study in which repeated HVPG measurements were performed between 20 and 720 minutes from baseline demonstrating a mean change in HVPG of only 0.4%.[22] In addition, another recent study showed excellent inter-observer agreement ($r = 0.98$) in tracing interpretation.[12] A study performed in post-transplantation recurrent hepatitis C showed HVPG to be more accurate in predicting the occurrence of a decompensation event than liver biopsy.[23] Based on these results, HVPG is regarded as the "standard of care" for assessment of the severity of liver disease.[12]

Currently, HVPG measurement is considered the standard of care for measuring portal hypertension.[13, 24-29]

Many peer-reviewed publications support the use of HVPG as a valuable tool for evaluating liver disease severity and prognosis.[30] HVPG measurements have been discussed in approximately 600 publications, showing high correlation with the degree of liver disease severity, development of complications and patient survival. HVPG has been called *"the best and only surrogate biomarker in Hepatology"*.[26] Recent comprehensive reviews even refer colloquially to the HVPG measurement as the "*gold standard"* for the determination of CSPH.[25, 31] In a recent review[25] leading experts in the field stated:

> Bosch J, Abraldes JG, Berzigotti A, Garcia-Pagan JC[(30)]: *"Measurement of hepatic venous pressure gradient (HVPG) and upper GI endoscopy are considered the **gold standards** for portal hypertension assessment in patients with cirrhosis. However, both types of investigation are invasive and HVPG measurement is routinely available and/or performed with adequate standards only in expert centers.*
>
> Castera L, Pinzani M, Bosch J:[(25)] *There is thus a need for non-invasive methods able to predict, with acceptable diagnostic accuracy, the progression of portal hypertension ...."*

An editorial in *Hepatology* by other leading hepatologists stated:

> Burroughs AK, Thalheimer U:[(27, 32)] *"At present, HVPG measurement is the best surrogate of portal pressure and also the best single prognostic marker available for patients with cirrhosis."*
>
> Another review by Triantos CK, Nikolopoulou V, Burroughs AK [(32)] concluded that: *"The prognostic and therapeutic value of HVPG is established beyond portal hypertensive bleeding for which there are some clinical surrogates. [e.g. the development of gastro-oesophageal varices and bleeding, ascites, hepatorenal syndrome and hepatic encephalopathy] HVPG measurement should now be part of everyday clinical practice."*

Furthermore, it is clinically accepted that reduction of portal pressure is the ultimate goal in the treatment of patients with cirrhosis.[(13)] HVPG has been shown to be highly correlative with the response to portal pressure-lowering drugs and their beneficial effects on liver disease complications. These studies demonstrate the ability of HVPG to predict the clinical course of these patients, as well as to enable follow-up of treatment in patients with chronic liver disease. Hepatology experts concluded[(33)] HVPG can serve as a valid tool for follow up of disease progression in patients with chronic liver disease.

There are over 40 clinical trials listed in clinicaltrial.gov, including several drug development studies in the US approved by FDA (NCT: 00737594, 01644656, 01714609) as well as multiple Phase 4 studies (01769040, 00563602, 00596414 00570973, 00570622, 00188045) in which HVPG is the primary outcome for evaluating portal hypertension.

A recent review[(30),] by Jaime Bosch, M.D., summarizes the clinical value of single and repeated HVPG measurements, including stepwise HVPG cut-off values and changes in time, with repeat measurements [(7, 15, 17, 19, 34-42)] and further supports the cut-off: *≥10mmHg of CSPH* as a predictor

"for the risk of developing varices, ascites, clinical decompensation, and hepatocellular carcinoma (HCC), and the risk of decompensation after liver resection for HCC".

The following table (Table 1) summarizes some of the clinical indications and the change in management as a result of HVPG measurements:

Table 1 - Selected literature based utilities of HVPG measurements

HVPG - MBT	CLINICAL UTILITY		
PATIENT POPULATION	**INDICATION**	**CHANGE IN MANAGEMENT**	**REFERENCES**
Patients with cirrhosis	Patients at risk for varices and bleeding (based on HVPG ≥10 mmHg) to determine necessity for upper endoscopy and frequency of endoscopy.	1. May avoid the need for Endoscopy in low risk patients 2. Define frequency of Surveillance for varices and their management	(16, 43) (44, 45) (46-48)
Patients with chronic liver disease and varices being treated with beta blockers	Response to Beta Blocker	1. Dose adjustment for responders 2. Stop therapy and possibly refer to alternative therapy (e.g. banding) for non-responders	(18, 20, 43)
Patients with HCC	Determine the risk of resection (HVPG<10)	Performance of surgery	(49, 50)
Patients at risk for HCC (Cirrhosis or HBV)	Assess the risk for developing HCC	Enhance HCC surveillance with more expensive imaging studies	(11)
Patients with cirrhosis treated in the community	Classify patients into low and high risk groups (HVPG ≥10)	Refer higher risk group to transplant center although MELD is not elevated	(44)
Patients with cirrhosis and acute bleeding	Determine if urgent TIPS should be performed	Performance of TIPS procedure	(40, 41)

5.3 HVPG ≥10 mmHg defines Clinically Significant Portal Hypertension

As mentioned above, HVPG can distinguish sub-stages within compensated and decompensated cirrhosis. HVPG > 5 mm Hg defines portal hypertension (PHT), whereas HVPG ≥ 10 mmHg is

widely recognized as "clinically significant portal hypertension (CSPH)",[48] as it is the pressure threshold that predicts many of the complications of cirrhosis.[6, 11, 33] As shown in a recent review,[22] including over 80 references, HVPG of 10 mmHg and higher is significantly associated with liver decompensation, hepatocellular carcinoma (HCC) and patient death.

5.4 Unmet need for a noninvasive tool for assessment of CSPH

Although HVPG is highly clinically valuable,[26] and the method used to measure HVPG is accurate, reproducible and reasonably safe,[15] limitations to the generalized use of HVPG are a lack of local expertise and poor adherence to guidelines to ensure reliable and reproducible measurements.[10, 13] Moreover, HVPG is an invasive intravascular procedure involving imaging, contrast media injection and catheterization. Hence HVPG is limited to large medical facilities requiring resources beyond the hepatology clinic and point of care physician and holds significant added risk to the patients. Berzigotti et al. in their recent review [31] determined that HVPG is the best method to assess CSPH, but they also cite the limitations of the procedure, especially in the context of therapy monitoring:

> *"HVPG measurements are not available in all centers, the technique is invasive and some patients are unwilling to be submitted to it. This is even more relevant when the repetition of the procedure is needed to monitor treatment response. These issues have raised interest to non-invasively determine when CSPH is present, so allowing defining a patient at risk of developing portal hypertension-related complications."* [31]

Hence, HVPG is an impractical test for everyday clinical use. Serial HVPG measurements are likely to be useful but not practicable in clinical practice. Furthermore, key opinion leaders (KOL) have commented that many more HVPG measurements would be done if it was not for the practical drawbacks and limitations since it is felt to be a good predictor of complications of cirrhosis.[12, 22] It is clearly desirable and necessary to have an equally reliable surrogate test that is non-invasive.

Moreover, although HVPG is clinically highly valuable,[26] even in experienced hands, complications can occur including large hematomas of the neck or groin being the most common (as reported in a leading Spanish center that regularly measures HVPG with a complication rate of 2.3%).

5.5 ^{13}C-Breath Tests

Breath testing with ^{13}C-labeled substrates provides a safe, non-invasive means for evaluating hepatic impairment as it pertains to liver metabolic function. ^{13}C is a stable, non-radioactive isotope, which can be incorporated into a specific location within a test substrate so it can be metabolized into $^{13}CO_2$ by the liver. Ideally, the ^{13}C-compound would be administered orally, rapidly absorbed and exclusively metabolized by the liver. ^{13}C-Methacetin has been identified as such a substrate.

5.5.1 ^{13}C-Methacetin Breath Tests - MBT

Methacetin had been available and described in literature for over 100 years. Within the last 30 years its use for Breath testing has been described as well. No risks have been reported in the literature using much higher doses of Methacetin than currently proposed to be used in this protocol. No adverse events have been reported in performing *^{13}C-Methacetin Breath Test* (MBT) using the proposed device or substrate to date including in more vulnerable populations. Additional safety assessments will be made during this clinical study.

Methacetin metabolism involves an initial O-demethylation process carried out exclusively by hepatic cytochrome CYP450 Iso-enzyme 1A2 producing two products: O-demethylated Methacetin, similar to acetaminophen, and formaldehyde.[51-56] Formaldehyde is then transformed into $^{13}CO_2$, through additional fast enzymatic steps. The rate limiting factor in this process is the O-demethylation performed by the liver cytochrome CYP450.[57] More details regarding the Methacetin metabolism are presented in section 8.2.

The ability of MBT to assess liver's function had been demonstrated in several applications. The ^{13}C-Methacetin metabolism performed exclusively by the liver and resulting in $^{13}CO_2$ exhaled as described in section 8.2. In a study using MBT for the prediction of postoperative outcome after hepatectomy,[58] results demonstrated 64 patients showing close correlation between MBT results and liver volume (as measured by CT volumetry) during regeneration ($r=0.94$, $p<0.0001$). MBT was the only predictor of outcome, namely liver failure and/or mortality for these patients prior to surgery. The concept of MBT as an overall liver function test, has also been demonstrated in rats,[59] where MBT correlated with liver mass following partial hepatic resection; as the liver mass increased with regeneration, so did the MBT values. Furthermore, MBT was assessed for the hepatic specificity of Methacetin metabolism in 5 patients undergoing liver transplantation, by injecting ^{13}C-Methacetin intravenously at the *anhepatic* stage (just after removal of the liver) and before reperfusion of the graft.[58] Liver function was assessed with an MBT, and a clear increase in $^{13}CO_2$ was observed after reperfusion, as can be seen in *Figure* 1. This clearly demonstrated that the metabolism of Methacetin was solely liver-dependent.

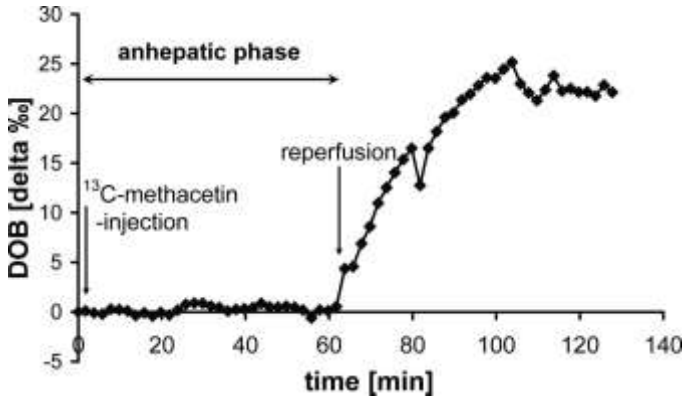

Figure 1: ^{13}C-Methacetin Breath Test during Orthotopic Liver Transplant

It has been further demonstrated that MBT can be used to assess liver reserve and function before and after transplantation[(60)] and for pre-operative assessment of liver reserve to determine the probability of success.[(61)] The same group has shown utility of the MBT in predicting prognosis in the ALF population.[(62)]

Furthermore, several large clinical trials have shown the correlation of MBT with severity of disease in cirrhotic patients compared to other tests such as CTP, MELD and ICG.[(63-67)]

Several factors may affect ^{13}C measurements in cirrhotic patients. These include: alteration in blood flow, both intra-hepatic and shunting[(30)] of blood due to portal hypertension thereby decreasing the rate of metabolism; the presence of fibrotic barriers in peri-sinusoidal spaces causing a possible delay the release of CO_2 into the blood; damage to hepatocytes which further decreases the metabolism of Methacetin. Accordingly, the Methacetin breath test can potentially be a tool for overall assessment of liver disease, portal hypertension and their consequences.

5.6 Rationale to use the MBT for assessment of portal hypertension

Tests of true hepatic function that rely on the metabolism of administered exogenous compounds have not yet been widely adopted in clinical practice because they have been cumbersome to perform and acquisition of results is slow. The recent development of the *BreathID®* analyzer offers a unique opportunity to demonstrate a metabolism-based test showing agreement with HVPG.

Low values of Methacetin metabolism may be suggestive of patients who are likely to have increased portal pressure. In advanced liver disorders, the liver has reduced ^{13}C-Methacetin metabolic capacity therefore the MBT values are low. Several MBT output variables (Percentage

Dose Recovery rate and Cumulative Percentage Dose Recovered - PDR/CPDR values at different time points) could be used to assess CSPH, as they were shown to be proportionally lowered in the presence of CSPH in two independent studies and in a meta-analysis.

Exalenz completed a study performed in eight large US centers and three OUS centers entitled "Pivotal study to evaluate the efficacy and safety of the BreathID® system for detection of cirrhosis using the ^{13}C-Methacetin Breath Test MBT" (G080107) during which a subset of 21 patients with MBT data had undergone HVPG at the time of their liver biopsy. When comparing MBT variables and HVPG, significant correlations were noted.. For example, a statistically significant inverse relationship (r=-0.75) was found between the PDR values at 45 minutes (PDR45) and HVPG values as depicted in ***Figure 2***. It can be concluded that the correlation does not seem to be affected by the use of non-selective beta-blockers or the presence of varices. It was also observed that ascites was observed in those patients with CSPH.

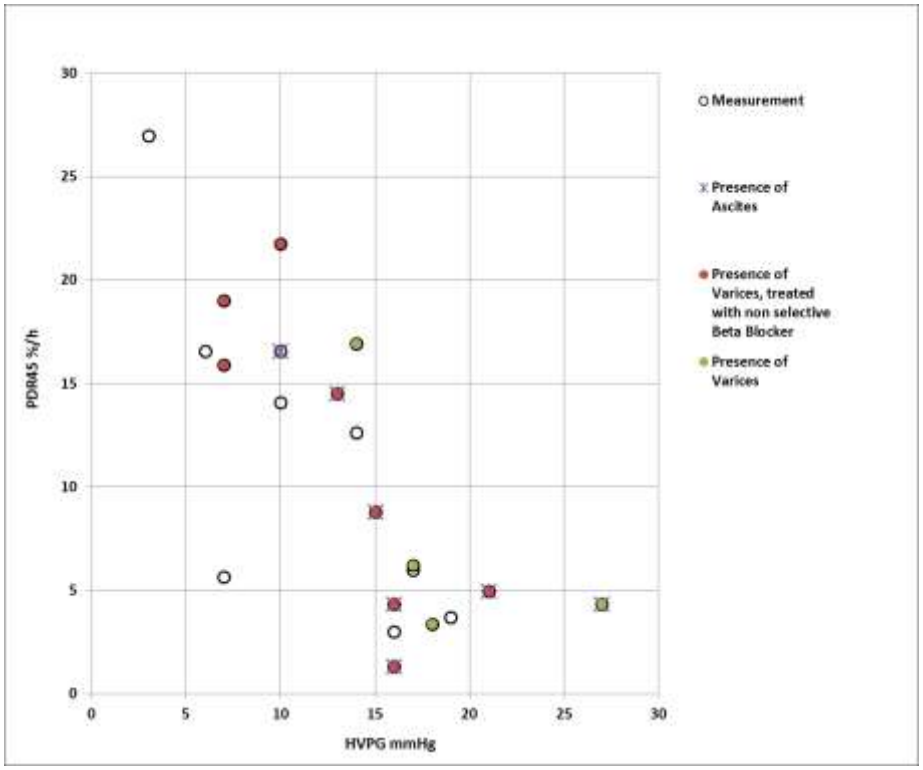

Figure 2: *Correlation between HVPG and MBT measure (PDR45)*

In addition, using the Receiver Operating Characteristic (ROC) Area Under the Curve (AUC) to assess the ability of an MBT variable to identify CSPH, demonstrated the Cumulative Percent Dose Recovered at 15 minutes (CPDR15) MBT parameter distinguished HVPG ≥10mmHg (CSPH) from lower HVPG values (non-CSPH). The AUC of the MBT parameter (CPDR15) was 0.89 (95% CI [0.72-1.00], $p < 0.0001$)[68] (see ***Figure 3***). The patient population tested included 5 non-CSPH subjects and 16 subjects with CSPH.

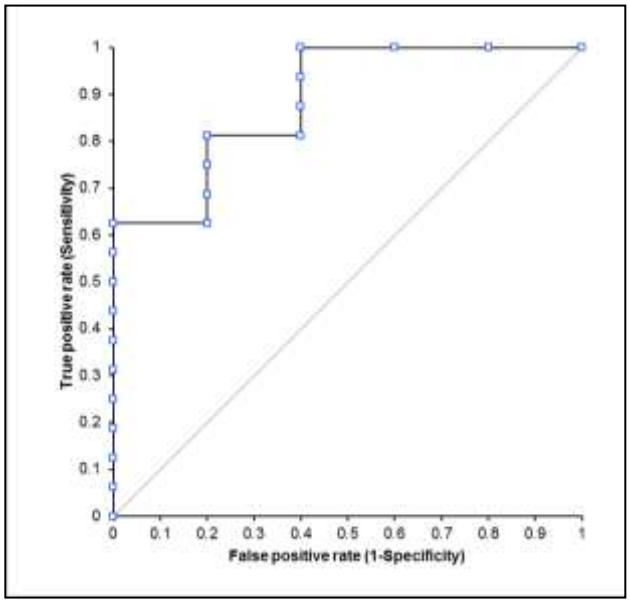

Figure 3: *ROC curve for the detection of CSPH as measured by MBT Measure CPDR15*

Based on these findings, an additional study was conducted in India to compare MBT to HVPG in 31 subjects clinically identified as cirrhotic. All subjects underwent both tests within 2 days of each other. The results showed a statistically significant correlation of approximately r= -0.6 for most MBT variables with CSPH. The ROC AUC remained at 0.90 for CPDR15 (Cumulative PDR at 15 minutes) (see ***Figure 4***). There were, however only two subjects with HVPG measures lower than 10mmHg.

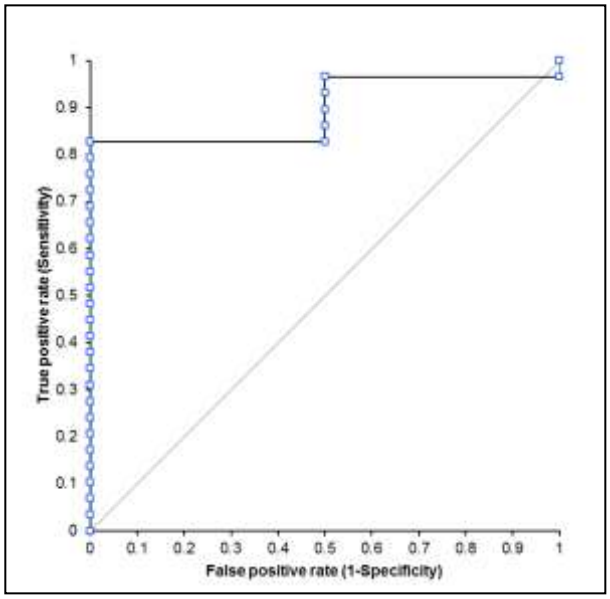

Figure 4: *ROC curve for the detection of CSPH as measured by MBT Measure CPDR15 in Indian data*

The Company conducted a meta-analysis of all accumulated data (in the US and in India) including several patients from an additional study including cirrhotic patients conducted under IDE G080227, who had HVPG done as part of their clinical care. A total of 58 subjects were included in this analysis to assess the correlation between MBT and HVPG as well as the ability of the MBT to identify CSPH (51 with CSPH and 7 without CSPH). The analysis demonstrated a statistically significant negative correlation between HVPG and MBT (r=-0.57) as seen in ***Figure 5***.

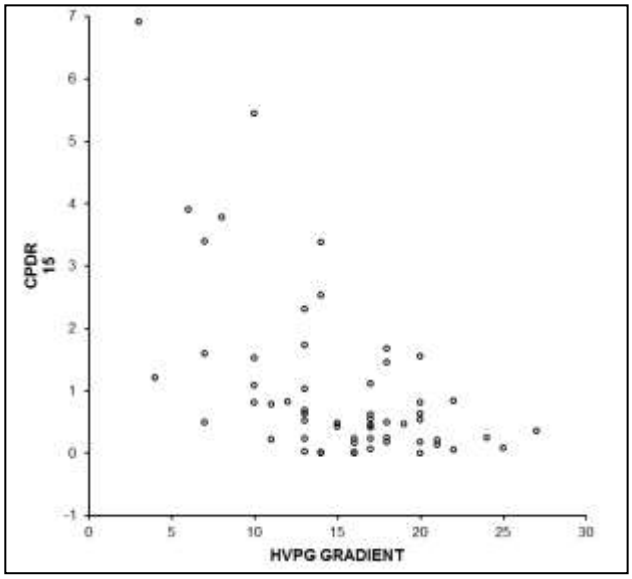

Figure 5: *Correlation between HVPG and MBT measure CPDR15 on 58 patients*

The AUC in this meta-analysis remained at 0.88 for CPDR15. The ability of CPDR15 to assess CSPH can be seen in a boxplot in ***Figure 6***.

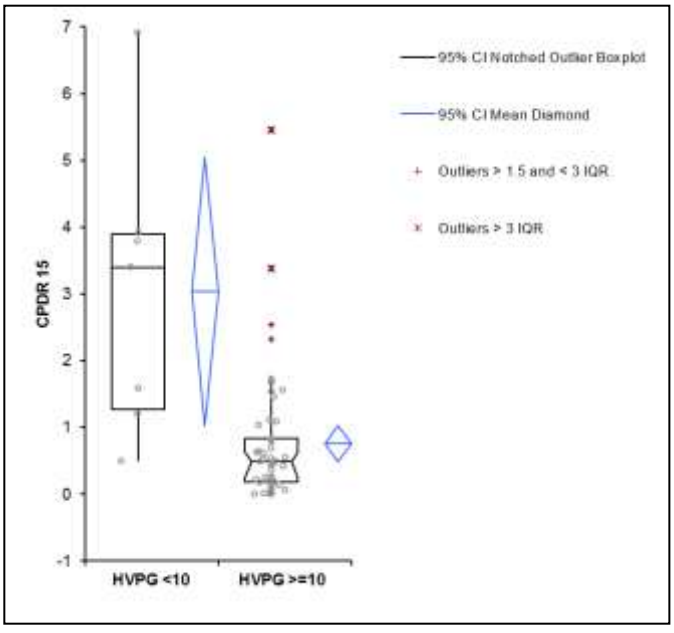

Figure 6: *Boxplot showing the ability of CPDR15 to assess CSPH in 58 patients*

The aim of the proposed protocol is to collect prospective clinical data and complete the statistical assessments to identify the most appropriate, sensitive and specific measurement and/or algorithm to use to identify patients at high risk for de-compensation as defined by HVPG ≥ 10mmHg as the basis for the device labeling requested.

The company's current focus is to discriminate between the presence and absence of CSPH, defined as HVPG ≥ 10mmHg in the cirrhotic patient as a dichotomous test. As noted above, this will provide a simple non-invasive repeatable method of determining the onset/presence of CSPH, thereby contributing to the efficient identification and management of patients with advanced liver disease.

It had been proposed that additional endpoints will be evaluated as well, including: the dichotomous discrimination of HVPG lower or greater than 20 mmHg and the assessment of response to treatment (beta blockers) of patients with proven CSPH acutely, during the test and / or at steady state of the prescribed treatment.

6 INTENDED USE / INDICATION FOR USE

The BreathID® System ^{13}C-Methacetin Breath Test (MBT) is to be used for the identification of clinically significant portal hypertension (CSPH), as defined by an Hepatic Venous Pressure Gradient (HVPG) ≥ 10 mmHg in patients with liver cirrhosis independent of disease etiology, without a history of clinical decompensation (variceal bleeding, uncontrolled ascites, hepatic encephalopathy and/or hepatorenal syndrome).

7 PROPOSED CLINICAL ALGORITHM USING THE MBT

The company's goal is to develop a point-of-care device for the identification of clinically significant portal hypertension (CSPH).

The company proposes to train a selected MBT-based variable that can reliably identify clinically significant portal hypertension (defined as HVPG ≥ 10mmHg) in patients with compensated liver cirrhosis. This will enable the use of a dichotomous measure from MBT to assess CSPH by means of rule-in or rule-out or both.

8 MBT SHORT DESCRIPTION

8.1 MBT Overview

The *MBT* is a non-invasive test for assessing liver metabolic capacity to metabolize ^{13}C-Methacetin in order to assess presence of CSPH. The *BreathID® MCS System* consisting of the *BreathID® MCS* device and a test kit containing a breath collection cannula and a non-radioactive isotope ^{13}C-Methacetin solution, which measures and computes the ratio between $^{13}CO_2$ and $^{12}CO_2$ in the patient's exhaled breath.

For more details please refer to the study Investigator Brochure.

The device is based on an FDA cleared device (510k#: K130524) for assessment of *H. pylori* infection in the stomach utilizing ^{13}C-Urea as a substrate for the bacterial urease. Performance and safety of the device utilizing the ^{13}C-Methacetin substrate for assessment of liver function, have been studied in thousands of patients worldwide, including in a large US pivotal study – under IDE G080107 – of over 400 patients with chronic liver disease from 11 participating sites (including 141 patients with biopsy proven cirrhosis),[69] which validated the safety of the MBT.

8.2 ^{13}C-labelled Substrate: ^{13}C-Methacetin

The ^{13}C-labeled Methacetin has been selected as the test substrate. ^{13}C-Methacetin is a white, crystalline powder bearing the chemical name [N-(4-Methoxy phenyl) acetamide]. ^{13}C-Methacetin is rapidly absorbed and metabolized exclusively by hepatic microsomal function oxidase via O-demethylation, mainly by cytochrome P450 1A2, into acetaminophen (also known as paracetamol, Tylenol™, and n-acetyl-p-aminophenol or APAP) and formaldehyde.[51-56] Formaldehyde is then transformed, through two successive oxidative steps, into $^{13}CO_2$. The rate limiting factor in this process is the O-demethylation performed by the liver cytochrome CYP450.[57] The general reaction is described in the following scheme:

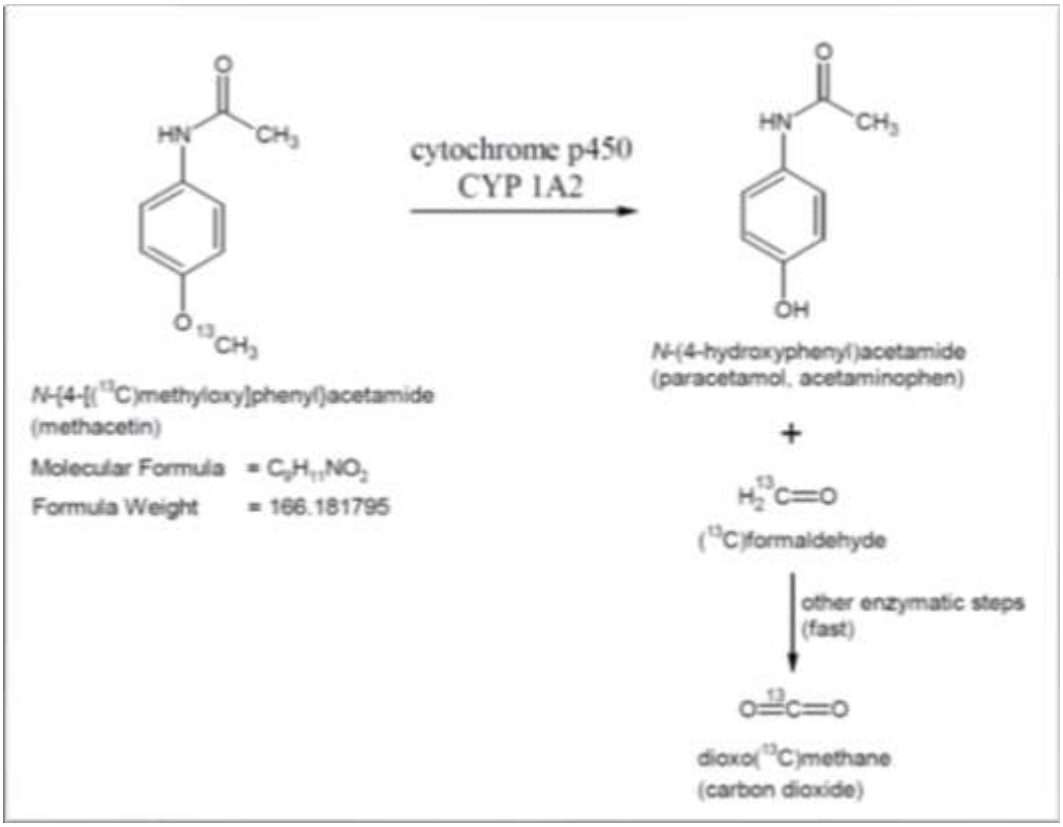

Figure 7: Oxidative Cleavage of Methacetin to Acetaminophen and Formaldehyde

The resultant $^{13}CO_2$ can be measured in the exhaled breath. The amount of metabolized Methacetin indicates the capability of the liver to accomplish one of its main physiological tasks and has been shown to correlate with liver fibrosis and cirrhosis.[66, 70-72]

^{13}C-Methacetin meets all of the qualifications for a liver function test substrate: It is a non-toxic small molecule, is administered orally, is rapidly absorbed and is exclusively metabolized by the liver. Furthermore, ^{13}C can be easily synthesized into a key location within this molecule. No adverse events have been reported when using this substance, including in vulnerable populations, and the compound remains stable over time.

8.2.1 ^{13}C-Methacetin Safety

The ^{13}C-Methacetin substrate is a well-known diagnostic reagent that has been described in the literature and used for over 100 years in general and for over 30 years specifically for breath testing by leading researchers around the world (US, Europe and Japan). As previously mentioned, there have been no reports of any complications with the use of this substance. The

main metabolite of Methacetin is acetaminophen which has wide routine clinical use at much higher doses than orally administered dose of ^{13}C-Methacetin of 75mg used in this study.

In current literature regarding pre-clinical and clinical studies performed with Methacetin, results from a total of 2987 individuals (2021 chronic liver disease patients including critically ill patients and 966 controls) were reported in 73 publications, including neonates,[73] infants,[74] healthy adults, adults with various forms of liver disease (including toxic injury), pregnant women[75] and the elderly - with no reports of adverse events. MBT has also been used in patients with Acute Liver Failure. Some of these data were developed utilizing the Exalenz device and substrate, while the majority were performed with other similar systems.

Regarding internal unpublished data using our device and Methacetin, 1466 chronic liver patients (of these 278 cirrhotic - some before transplant) and 74 healthy controls/subjects without liver disease, have also been tested. No adverse events related to the substrate were experienced by the subjects

Toxicology testing results in animals and other information support the safe use of Methacetin in humans. Based on the acute toxicity studies in mice and rats where relatively high LD50 values of 1190mg/kg were administrated,[76] the Methacetin dose administered in human breath tests in adults of 75 mg, or approximately 1mg/kg, has a safety ratio in excess of 1000-fold.

Many studies using ^{13}C-Methacetin for liver function assessment have been published. Representative references have been cited that support the conclusion that ^{13}C-Methacetin is a "safe" molecule and that it has been used on high-risk population groups such as the elderly, neonates or pregnant women.[60, 65, 77, 78] This provides further assurance that the ^{13}C-Methacetin substrate is appropriate to use in breath tests intended for liver function assessment.

8.3 MBT Components

The following are the components of the *MBT System*: The *BreathID® MCS* device, the ^{13}C-Methacetin substrate and the sampling kit containing breath collection cannulae.

The *BreathID® MCS* device will be provided by Exalenz Bioscience Ltd. The cannulae, accessories and the test substrate will be supplied in proper packaging and will bear all required labeling based on applicable regulatory requirements.

The solution administered orally to the patient is a 0.05% solution of ^{13}C-Methacetin in purified water, supplied in amber thermoplastic polyester (PET) bottles with a child resistant plastic stopper (75mg of ^{13}C-Methacetin in 150mL purified water). All bottles are for single use only and must be used solely for per-protocol investigational product administration. Each bottle

contains a single test dose. The solution is manufactured at Taro Pharmaceutical Industries, Ltd., 14 Hakitor St., Haifa Bay 26110, Israel and will be provided under the responsibilities of Exalenz Bioscience Ltd, 4 Ha` Ma'ayan St., Modiin, 71700, Israel.

All study test components must be stored in an environment according to the Investigator Brochure and product labeling guidance.

9 STUDY DESIGN

This study is a multicenter, non-randomized, blinded, cross-sectional comparative exploratory study comparing various MBT measures to HVPG.

This study will be used to train an algorithm using MBT measures and to select a cut-off to determine presence or absence of CSPH as defined by HVPG ≥ 10mmHg.

10 STUDY OBJECTIVES

10.1 Efficacy Objective

The primary objective of the study is to develop an algorithm and its cut-off to detect CSPH, defined as HVPG ≥10mmHg, based on the MBT.

10.2 Safety Objective

All adverse events, serious adverse events (SAE) and suspected unexpected serious adverse reactions (SUSAR) will be reported according to local regulations. The actual reporting is discussed in section 14. No breath-test related adverse events are expected. For more information please refer to the Investigator Brochure.

10.3 Secondary Objectives

1. To assess the ability of the MBT model to diagnose portal hypertension with HVPG ≥ 12mmHg
2. To assess the ability of the MBT model to diagnose portal hypertension with HVPG ≥ 20mmHg
3. To assess the correlation of MBT with HVPG readings

4. To Assess the effect of beta blockers or other confounding factors on the correlation of MBT with HVPG readings
5. Examine the effect of a single small (<3cm) HCC (hepatocellular carcinoma) on the correlation of MBT with HVPG tracings

10.4 Efficacy Endpoints

10.4.1 Primary efficacy endpoint

Binary diagnosis of CSPH, defined by HVPG ≥10 mmHg, correlated with an MBT result to be developed.

The relationship will be assessed on both sides of the definitive HVPG value to obtain a clinically significant MBT model/algorithm and its cut-off to be validated in a separate study.

10.4.2 Secondary efficacy endpoint

1. Binary diagnosis of portal hypertension with HVPG ≥ 12mmHg
2. Binary diagnosis of portal hypertension with HVPG ≥ 20mmHg
3. Correlation of MBT with HVPG readings
4. Assess the effect of beta blockers or other confounding factors on the correlation of MBT with HVPG readings
5. Examine the effect of a single small (<3cm) HCC (hepatocellular carcinoma) on the correlation of MBT with HVPG tracings

10.5 Safety Endpoints

Adverse Event reporting.

11 SUBJECT SELECTION

The study population will be essentially enrolled on a consecutive basis of able and willing subjects meeting all inclusion/exclusion criteria with clinically, imaging or biopsy proven liver cirrhosis without decompensation events in the past.

Most patients to be enrolled in the proposed study will be from centers in Europe with the addition of site(s) in the US. Based on expert opinion in the field of hepatology, the population to be enrolled from Spain and France is representative of patients with chronic liver disease in the

United States. It is presumed patients in both countries have similar etiologies and prognosis and are being used interchangeably in multicenter clinical trials using HVPG testing.[7, 8, 18]

11.1 Inclusion Criteria

1. Adult men or women (≥18 years of age)
2. Patient (or legal guardian) can sign an Informed Consent Form.
3. Known chronic liver disease with cirrhosis confirmed by either:
    a. liver biopsy or
    b. clinical (palpable left lobe, splenomegaly) and laboratory (platelets <150,000/mm^3 or albumin< 3.8g/dL, or INR >1.3) evidence of cirrhosis and/or
    c. imaging studies by abdominal sonography, computer assisted axial tomography, or magnetic resonance imaging, showing a nodular liver and/or enlarged spleen and/or portosystemic collaterals with portal vein patency and/or minimal ascites, and/or colloid shift on a colloid-isotope liver-spleen scan or measurements of liver stiffness suggestive of cirrhosis
4. Europe: Indicated to undergo HVPG testing
   US: Consented for HVPG
5. Can tolerate an overnight (8-hour) fast
6. For patients treated with statins: They have to be on a stable dose for at least 4 weeks prior to any study related tasks (MBT or HVPG measurement)
7. For patients treated with beta blockers: They have to be on a stable dose for at least 6 weeks prior to any study related tasks (MBT or HVPG measurement)
8. For patients who stopped their treatment with beta blockers: Their last dose should be at least 6 weeks prior to any study related tasks (MBT or HVPG measurement)

Note: *In case patient has HCC, breath test has to be performed on the day of HVPG measurement.*

11.2 Exclusion Criteria:

1. Decompensated cirrhosis as clinically defined by the occurrence of any of the following: ascites, hepatic encephalopathy, variceal bleeding (active upper GI bleeding) or hepatorenal syndrome
2. Renal failure (creatinine > 2.5 mg/dl)
3. Known acute renal tubular disease
4. Known hypotension (Systolic Pressure < 100mmHg)
5. Hypocoagulablity defined as PT >6 and INR >2.3
6. Congestive heart failure (assessed clinically as NIHA >2)

7. Known pulmonary hypertension (right ventricular systolic pressure > 45 mm Hg,)
8. Uncontrolled diabetes mellitus (HBA1C >9.5gr%)
9. Concurrent prednisone or immunosuppressive treatment, if therapy and/or response to treatment are not stable for at least 3 months
10. Documented hepatocellular carcinoma lesion larger than 3cm and/or multifocal lesions and/or evidence of vascular invasions
11. Gastric bypass surgery or extensive small bowel resection
12. Total parenteral nutrition
13. Any organ transplant recipient
14. Pregnant or breast feeding
15. Allergy to acetaminophen and/or other related medications.
16. Documented drug-related concurrent hepatotoxicity or drug-related silent steatosis or drug-related fibrosis (e.g. amiodarone, methotrexate and tamoxifen).
17. Uncontrolled malabsorption or diarrhea
18. Documented non-cirrhotic PHT, partial / complete portal venous occlusion, hepatic venous occlusion, previous PHT surgery, or placement of a transjugular intrahepatic portosystemic shunt (TIPS)
19. Primary or secondary biliary cirrhosis, primary or secondary sclerosing cholangitis, hepatic sarcoidosis, or other cholestatic disorders
20. Subjects unable to perform the MBT within 7 days of HVPG procedure
21. Subject should not have taken any of the following for at least 48 hours prior to the breath test: Acyclovir , allopurinol, carbamazepine, cimetidine, ciprofloxacin, daidzein, (herbal) disulfiram, echinacea, enoxacin, famotidine, fluvoxamine, methoxsalen, mexiletine, montelukast, norfloxacin, phenylpropanolamine, phenytoin, propafenone, rifampin, terbinafine, ticlopidine, thiabendazole, verapamil, zileuton or any medication that might interfere with Methacetin metabolism or might affect CYP 1A2
22. Subject should not have taken amiodarone within the last 30 days prior to the breath test or HVPG procedure
23. Splenomegaly secondary to inflammation/infection or hematologic malignancy.
24. Last dose of HCV treatment received within the last 3 years.

11.3 On the day of the MBT:

1. 8 hours fasting prior to MBT including all PO morning medications except for beta blockers
2. No Smoking on the day of the MBT

3. No caffeine within 24 hours prior to MBT
4. No paracetamol (acetaminophen) based medications within 24 hours prior to MBT
5. No general anesthesia or sedation within 24 hours prior to MBT
6. No alcohol within 24 hours prior to MBT
7. Patient should be symptom free of any prior intervention (e.g. biopsy)

11.4 Consenting

Patients' and/or their representative will sign a consent form prior to study participation which must be signed and witnessed.

The consent will include willingness to allow acquisition and collation of blood, clinical and imaging data taken on entry to the study and incorporate all other data from time of admission until the patient's termination of the study.

11.5 Safety Termination of Subjects or Study

1. Withdrawal of patients from the study if they have a CTCAE Grade 3 or higher regardless of whether the event is attributed to the drug unless it is caused by an accident that could not reasonably be attributable to the drug (CTCAE: Common Terminology Criteria for Adverse Events Version 4.0).
2. Discontinuance of study, if ≥ 2 patients on Methacetin develop the same CTCAE Grade 3 event or 1 patient develops a CTCAE Grade 4 or higher event due to the breath test.

11.6 Early Withdrawal from the Study

Patients, their relatives, their representatives or the patients' physician may withdraw the patient from the study at any time if they feel the study is not in the patients' best interest, or they may be withdrawn by the investigator or sponsor for safety, behavioral, or administrative reasons. All withdrawals must be fully documented.

If the patient withdraws from the study and also withdraws consent for the disclosure of future information, no further evaluations should be performed and no additional data should be collected. Previously acquired data will be analyzed. This will not have any effect on the treatment that the patient receives.

11.7 Expected Duration of Recruitment

The study is planned for a duration of approximately 12-18 months

12 STATISTICAL CONSIDERATIONS

12.1 Study Design and Objectives

This is a multicenter, non-randomized, blinded, cross-sectional comparative exploratory study comparing various MBT measures to HVPG.

This study will be used to train an algorithm using MBT measures and to select a cut-off to determine presence or absence of CSPH as defined by HVPG ≥ 10mmHg.

The modeled algorithm will be validated in a separate study

12.2 Primary endpoints

Binary diagnosis of CSPH, defined as HVPG ≥10 mmHg, correlated with an MBT result to be developed.

The relationship will be assessed on both sides of the definitive HVPG value to obtain a clinically significant MBT model/algorithm and its cut-off to be validated in a separate study.

12.3 Secondary efficacy endpoint

1. Binary diagnosis of portal hypertension with HVPG ≥ 12mmHg
2. Binary diagnosis of portal hypertension with HVPG ≥ 20mmHg
3. Correlation of MBT with HVPG readings
4. Assess the effect of beta blockers or other confounding factors on the correlation of MBT with HVPG readings
5. Examine the effect of a single small (<3cm) HCC (hepatocellular carcinoma) on the correlation of MBT with HVPG tracings

12.4 Safety Endpoints

All Adverse Events.

12.5 Sample Size estimation

As presented in section 5.6 above the MBT parameter CPDR15 was found to have an AUC of about 0.9. Two hundred (200) subjects of which at least 50 will be positive for CSPH and at least 50 will be negative for CSPH will enable the estimation of a more conservative AUC of 0.85 with a half width of the 95% confidence interval of 0.1, if the ratio of positive to negative subjects is as high as 3:1 and as low as 1:8 (1 pos. per 7 neg.).

During the study the correlation between MBT and HVPG will be assessed periodically in order to see if the intake of beta blockers, HCC or any other confounding factors have an effect on the breath test result. If the population using beta blockers, HCC or if some other confounding factor shows a significantly different correlation than the rest of the population, the study population may be increased up to 400 patients.

12.6 Analysis sets

All subjects enrolled to the study.

12.7 Statistical Analysis

12.7.1 General Considerations

Statistical analyses will be performed using SAS® v9.3 or higher (SAS Institute, Cary NC, USA).

If any statistical tests are performed, they will be two-sided. The required significance level of findings will be equal to or lower than 5%. Where confidence limits are appropriate, the confidence level will be 95%.

Baseline values are defined as the last valid value prior to study treatment start.

All statistical analyses of safety and performance measures will be descriptive in nature. Continuous variables will be summarized by a mean, standard deviation, minimum, median and maximum, and categorical variables by a count and percentage. Confidence intervals will be provided where relevant.

If multiple measurements are taken in a single patient, statistics described below will be appropriately modified to accommodate the within patient correlation.

12.7.2 Demographic and Other Baseline Characteristics

Demographic, medical and clinical history variables will be tabulated. Continuous variables will be summarized by a mean, standard deviation, minimum, median and maximum, and categorical variables by a count and percentage.

12.7.3 Disposition of Subjects

The numbers of patients who were enrolled will be provided, as well as the reasons for all enrollment discontinuations, grouped by major reason (e.g., lost to follow-up, adverse event, poor compliance). A list of discontinued patients, protocol deviations, and patients excluded from the efficacy analysis will be provided as well

12.7.4 Safety Analysis

The events related to study drug and/or device AE's rate will be presented along with a 95% exact binomial two sided 95% confidence interval. The analysis of all adverse events will include incidence tables and will include analyses by severity, relationship to device or drug and baseline variables.

12.7.5 Performance Analysis

A model/algorithm will be developed for the differentiation between subjects with and without CSPH, defined as HVPG ≥10mmHg. It will be developed using either logistic regression or neural network or any other known methodology. Using a cut-point on the score a binary diagnosis of CSPH (Y/N) will be obtained. The minimal criterion for sufficient efficacy is an Area under the Receiver Operating Curve (AUC) ≥0.85 in this patient population. A potential clinical utility was designated for the algorithm/model output; where this algorithm could aid the clinician in ruling-out CSPH. This will be translated into finding a model prediction cut-point that provides a high sensitivity (true positive rate) and at least moderate specificity (true negative rate), which in turn achieves a high negative predictive value (NPV). High NPV is considered to be near 90% (i.e. 88%) thus sensitivity should be at least 90% and specificity should be at least 50%. Cross validation methods will be used to test algorithm stability.

12.7.6 Interim Analysis

This study is an open label study for training purposes. The data may be assessed prior to achieving the full sample size. This will be done specifically in order to assess the effect of beta blockers, HCC, or any other confounding factor on the correlation between MBT and HVPG. If beta blocker, HCC or if some other confounding factor shows significantly different correlation than the rest of the population, the study population may be increased up to 400 patients.

12.7.7 Pooling

Subgroup analysis of the primary performance endpoint by center via a regression model will be used to evaluate the poolability of the results. If the variable by center analysis is found significant, the reason for this will be further explored and rationalized. This evaluation may include demographic features, symptoms at presentation, clinical and treatment history, and center comparability in the features found to be associated with the primary efficacy variable

12.7.8 Handling of Missing Data

The study variables cannot be evaluated for patients for whom MBT device results or HVPG results are not available and therefore these subjects will be left out of the efficacy analysis.

Demographic clinical characteristics and safety data of patients with missing data will be compared to patients with complete data.

12.7.8.1 Procedure

After all the relevant data will be entered into the database, a soft lock to the database will be performed. The study statistician will perform the assessment of the primary and safety endpoints.

12.7.8.2 Decision Rules

Stop the study in case of severe safety concern.

13 STUDY PROCEDURES

13.1 General

1. The study will be conducted in compliance with this protocol, with GCP standards, and the applicable regulatory requirements.
2. This study will be cross-sectional, where patients will be enrolled, if possible, in a consecutive manner so as to prevent any bias. All patients with cirrhosis and without clinical signs of complications should be screened and reason for non-suitability documented.
3. Patients' relative or other representative will sign a consent/assent form prior to study participation.
4. A CRF will be filled out for each patient and all data will be collated incorporating all demographics, detailed clinical history, physiological variables, radiological data, biochemical data and organ support provided (cardiovascular, respiratory, renal, hepatic, neurological) and all baseline characteristics.
5. HVPG procedure will be performed in a standardized fashion in all study centers (see section 13.4 and Appendix II).
6. In addition, the tracings will be read at a centralized location by an independent expert reader. This independent analysis will be done by an expert reader blinded to the breath test results.
7. The *MBT* will be performed within 7 days (prior or post) of the HVPG procedure using a single dose of 75mg ^{13}C-Methacetin. Note that the same

policy should be applied with regards to the use of beta blockers as for the HVPG procedure for those patients that are treated with beta blockers. In case patient has a HCC, breath test has to be performed on the day of HVPG measurement.

8. If patient is using a stable dose of beta blockers, both the MBT and the HVPG procedures should be performed while continuing the stable dose even on the morning of each test.
9. All investigators will be blinded to the *MBT* score results until the end of the study recruitment. The *MBT* results will therefore not affect the current patient management.
10. Safety follow up will be done during the entire period of the study, and any adverse events will be recorded according to GCP ICH guidelines. Section 16.6 relates to the reportable and non-reportable SAEs.
11. Results of the individual blood tests and other clinical assessments, standard prognostic criteria and clinical condition of the patient will be collected in the CRF.
12. The raw data will be saved on the *BreathID® MCS* device and will be downloaded to a flash memory stick and uploaded to a computer for further analyses.
13. All concurrent medication will be recorded. Certain drugs will be pre-recorded in the CRF.
14. Patients' survival will be ascertained 30 days following their last breath test.
15. The breath test procedure is described in detail in the main protocol and it requires minimal operator and patient participation. *BreathID® MCS* automatic breath collection eliminates virtually all breath collection related errors, as described in section 13.3 and Appendix III.

13.2 Masking procedures

In order to ensure unbiased interpretation of assay results, the following steps for masking will be taken:

- The authorized medical staff performing the HVPG procedure will be blinded to the breath test results.
- The principal investigator or co-investigator(s) will not be blinded to the results of HVPG and other diagnostic tests, since he may be the one performing the procedure and needs to evaluate the eligibility of the subject for treatment and/or possible monitoring as part of standard of care management.
- The blinding procedure is applicable only for the output of the *BreathID® MCS* device. The breath is collected and analyzed by the *BreathID® MCS* device and the results are printed out automatically at the end of the test. Hard copies of the device printouts will be stored with the CRFs as the source data.
- All HVPG tracing print outs will be stored as a hard copy or downloaded to a computer at the end of participation of each subject in the study, so that an independent analysis by a third party may be done.

13.3 Breath Test Procedure

Please refer to Appendix III and the IB for the MBT procedure

13.4 Principles of the Hepatic Venous Pressure Gradient (HVPG)

In order to standardize the measurements of the HVPG in the study the main procedure steps, derived from the common standards of care practice[10], are described below in Appendix II.

Under conscious sedation and with non-invasive vital sign monitoring (ECG, arterial blood pressure and pulse oximetry) the right jugular vein (or the femoral or antecubital vein) is catheterized, a venous introducer is placed and a balloon-tipped catheter is advanced under fluoroscopic control into the inferior vena cava and a hepatic vein, to measure pressures. This is a moderately invasive technique that is only being performed as part of the clinical evaluation of the patient (and not solely for the purpose of the clinical trial) in common with other routine procedures that are carried out in patients with chronic liver injury.

Free hepatic venous pressure is measured by maintaining the tip of the catheter "free" in hepatic vein, 2-4 cm from its opening into the vena cava. Wedge hepatic venous pressure is measured by occluding the hepatic vein by inflating a balloon at the tip of the catheter. The wedged hepatic venous pressure is measured until the value remains stable over 30-40 seconds. All measurements are performed in triplicate, permanent tracings are captured on a computer or chart recorder. The difference between the free and wedged hepatic venous pressures is the HVPG. Adequate occlusion of the hepatic vein is confirmed after pressure measurement, by slowly injecting 5cc of contrast into the vein that should show a typical "wedged" pattern without reflux of the contrast or its washout through communications with other hepatic veins.

Major complications have been limited to local injury at the puncture site, including leakage, hematoma and rarely an arteriovenous fistula or Horner syndrome. Ultrasonographic guidance reduces the risk of procedural complications. Passage of the catheter through the right atrium might cause supraventricular dysrhythmias that are self-limited in more than 90% of cases. Although coagulation disorders are common in patients with cirrhosis, only severe thrombocytopenia (platelets <20,000/mm^3) calls for platelet transfusion. The procedure entails very little discomfort, is carried out under moderate conscious sedation that does not influence HVPG measurement. The procedure's acceptability is comparable to that of upper gastrointestinal endoscopy.

Complications of the HVPG measurement will be reported as anticipated Adverse Events. The test procedure in this protocol is the MBT. The HVPG should be performed for clinical cause according to local standards of care; the HVPG will not be performed for the purpose of enrolling a patient under this protocol. For the purpose of the study, in US centers the HVPGs is considered an investigational procedure since HVPG is not used as common practice in the US.

13.4.1 Laboratory Tests

The patients will routinely undergo laboratory tests in preparation for their HVPG procedure. All liver related blood tests will be recorded along with other available laboratory information (as described below). If more than one blood collection is performed on a given day, the blood test results at the time closest to the time of the breath test will be recorded.

Recent (most recent, but within 6 months): blood test results will be recorded **if available**

Blood tests used as part of the pre-procedure safety evaluation for the HVPG procedure should be within 2 months (or sooner, if indicated by the patient's co-morbid conditions).

1. aPTT, fibrinogen
2. Hepatic panel (AST, ALT, alkaline phosphatase, GGTP, total bilirubin, direct bilirubin, serum albumin and total protein)

3. Renal and electrolyte panel (BUN, creatinine, electrolytes (Na+ and K+), total calcium, and PO_4 (phosphate corrected), magnesium
4. Blood count (WBC, Hemoglobin, Platelets)
5. Ammonia – arterial (preferred) or venous (if venous must be sampled from central vein not femoral or peripheral vein)

Additionally, assessments of the following variables will be made (not necessarily on days of breath test, but most recent within the past 6 months):

1. Ultrasound, CT (liver outline, vasculature, evidence of fat etc.), imaging of major hepatic vessels, pancreas, guts, and liver volume results if calculated; a single assessment of these variables when available.
2. Clinical data including: previous medical history, presentation, etiological factors, special diets and likely diagnosis on entry and update as available.
3. Habits and history of alcohol, substance and smoking will be recorded.
4. Liver Biopsy – if undertaken
5. Etiology, including etiological screening tests and diagnosis

13.5 Investigational Product Handling

The Investigator and Research Pharmacist (if relevant) will be provided with *Investigational Product Handling Guidelines* that will provide details regarding the packaging and labeling requirements, receipt of investigational product, dispensing and accountability procedures, preparation instructions, storage and stability of Investigational product, disposition of Investigational product, with the following forms required: Proof of receipt form, Temperature logs, Accountability logs, Investigational product and material transfer/ disposition form and pharmacy staff identification log (if applicable).

Local forms may be authorized for use after being approved by the Sponsor or his assigned representative.

13.6 Investigational Product Accountability

The Investigator and Study Pharmacist (if relevant) are responsible for ensuring that all study supplies received at the site are inventoried and accounted for throughout the study. The dispensing of study substrate to the subject must be documented in the respective accountability form. The study Investigational Product must be handled in strict accordance with the protocol and the container label and will be stored in a limited access area or in a locked cabinet under

appropriate environmental conditions. Unused study materials must be available for verification by the sponsor's site monitor during on-site monitoring. The destruction of unused study materials (both, expired or unexpired) will be documented on the return/disposition form. The Sponsor will authorize to destroy excess supplies on site according to local policy. In this case, before proceeding, the site must seek authorization from the Sponsor using the return/destruction form and this must also be documented on the Study Supply Return Form.

Study substrate should be dispensed under the supervision of the investigator, a qualified member of the investigational staff, or by hospital clinical pharmacist.

14 ETHICS & REGULATORY CONSIDERATIONS

14.1 Ethics & Regulatory Approvals

The study will be conducted in both the United States of America and Europe (Spain and France). As such, regulatory requirements that are relevant for all countries will be discussed in this section.

The study will be conducted in compliance with the principles of the Declaration of Helsinki (1996), the principles of GCP and in accordance with all applicable regulatory requirements.

This protocol and related documents will be submitted for review to all relevant ethics committees delegated to approve the study at their respective sites. Any amendment to the protocol may be proposed by the principal investigator. The amendment must be submitted to the Sponsor and, when approved to the relevant IRB or EC. When applicable, the implementation of the amendment will take place only once approved by the appropriate ethics committee.

Annual progress and safety reports and a final report at conclusion of the study will be submitted by Exalenz Bioscience (or on behalf of the Sponsor) to the applicable regulatory bodies within the timelines defined in the Regulations.

15 INVESTIGATOR RESPONSIBILITIES

The investigator is responsible for ensuring that the clinical study is performed in accordance with the protocol, current ICH guidelines on GCP, and applicable regulatory requirements. GCP is an international ethical and scientific quality standard for designing, conducting, recording, and reporting studies that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety, and well-being of study subjects are

protected, consistent with the principles that originated in the Declaration of Helsinki, and that the clinical study data are credible.

The investigational devices will bear an identification number and their accountability will be filed in the Investigator's Study File. Study supplies logged in will be kept by the investigator or the delegated persons in a secure place. All supplies (device, substrate and cannulae) will be used for this study only. After completion of the study, the device, drug and all unused accessories must be returned to Exalenz Bioscience Ltd. as per their request or alternatively, destroyed according to local regulations after receiving explicit authorization by Exalenz to do so and provide Exalenz with a confirmation.

The Principal Investigator will act as custodian for the study data. Patient data will be anonymized and all the anonymized data will be stored on a password protected computer. All data will be compliant with CFR and GCP.

16 SAFETY CONSIDERATIONS

16.1 Adverse Event Definitions

An adverse event (AE) is any undesirable or unintentional event that occurs in a patient or clinical investigation subject, whether or not considered related to the investigational product; this includes clinically significant changes in laboratory values. As this study is not therapeutic in nature, there will be no therapeutic failures to report.

Regardless of severity or relationship to the study investigational product, all diagnoses, symptom(s), sign(s) or finding(s) with a start date after the first study test has begun (Breath test/EGD) and until the last study procedure is being completed will be documented, assessed by the investigator and recorded in the subject's CRF.

A surgical procedure that was planned prior to the first study test by any physician treating the subject should not be recorded as an AE.

16.2 Serious Adverse Event (SAE), Unanticipated Adverse Device Effect (UADE) and Suspected Unexpected Serious Adverse Reactions (SUSAR)

A serious adverse event (SAE) is an event that is either:

a) Fatal
b) Life-threatening

Life threatening is defined as an event in which the patient is at risk of death at the time of the event. It does not refer to an event that hypothetically might have caused death if it was more severe.

c) Results in persistent or significant disability/incapacity

Disability is defined as a substantial disruption of a person's ability to conduct normal life functions.

d) Requires or prolongs inpatient hospitalization
e) A congenital anomaly or birth defect.
f) Another serious or important medical event as judged by the investigator.

An Unanticipated Adverse Device Effect (UADE) or a Suspected Unexpected Serious Adverse Reactions (SUSAR) is an SAE, related to the investigational product, for which the nature or severity is not consistent with the expected outcomes of the treatment/testing being offered.

An unexpected adverse event is one that has not been previously observed, or one that is of a specificity or severity not consistent with the current investigator brochure.

As with every procedure risks and discomforts may occur with relations to the Breath ID test. The potential risks/complications and discomforts which may be associated with all study procedures are listed below.

- Allergic and/or other reactions to BreathID reagent
- Aspiration (inhaling) of food or fluids into the lungs
- Discomfort

When an adverse event occurs, the following information and assessments should be documented in the subject's file and reported in the adverse event section of the CRF:

- The signs, symptoms, or diagnosis of the event if available
- The date and time of onset of the event using the 24 hour clock where midnight is 00:00 and noon is 12:00
- The adverse event severity using the criteria outlined below
- The relationship of the event to the study medical device or drug as outlined below
- Describe any action taken regarding study medical device or drug disposition
- List any required therapy, medication, treatment, or diagnostic procedure.

16.3 Relationship to Study Medical Product

An investigator, who is a qualified physician, should assess the relationship to the study medical device and the substrate, based on all information available at the time of assessment.

The following definitions should be used:

- **Not Related:**

1. The event is clearly related to other factors such as the patient's clinical state, therapeutic interventions or concomitant drugs.
2. There is a highly likely alternative explanation.

- **Related:**

1. The event AE is reasonably associated with the use of the investigational product (device or substrate).

16.4 Adverse Event Severity

- **Mild Adverse Events**

A mild adverse event is one that the symptoms are barely noticeable to the patient. It does not influence performance, require drug treatment or prevent the patient from carrying on with normal life activities.

- **Moderate Adverse Events**

A moderate adverse event is one that the symptoms make the patient uncomfortable and causes some impairment to normal life activities. Treatment for symptom(s) may be required.

- **Severe Adverse Events**

A severe adverse event is one that the symptoms cause severe discomforts to the patient and severely limits the patient’s normal daily activities. Treatment for symptom(s) is given.

Note: Serious and severe are not synonymous. A serious adverse event must fulfill the requirements listed in the definition above.

16.5 Adverse Event Reporting

The investigator should report all adverse events in the case report form. The investigator is responsible for the appropriate medical management of all adverse events.

The investigator must report any serious adverse event to the sponsor immediately, within 24 hours of awareness, via email. Full details of the event, severity, outcome (if available) and an assessment of the relationship to the study investigational product must be provided in the report.

All SAEs must be followed up until resolution or stabilization by submission of updated reports. Follow-up SAE reports will be reported according to the same timelines as initial reports, as soon as new significant information becomes available.

All SAEs that fulfill UADE or SUSAR definitions will be reported to the relevant authorities by expedited means.

Notification of the IECs/IRBs and regulatory authorities

Notification to the IECs/IRBs about all relevant events (e.g. SAEs, UADEs, SUSARs) will be performed by the sponsor and/or by the investigator according to all applicable regulations.

The processing and reporting of all relevant events to the regulatory authorities will be done by the sponsor according to all applicable regulations.

If unexpected safety issues are identified, specific amendments will be implemented.

16.6 Study-Specific Exceptions

Disease complications as a result of disease progression is expected and it is not necessary to report them as SUSARs, but they should be reported in the follow-up section of the case report form (CRF).

The following situations and non-fatal toxicities that fulfill the definition of an SAE are excluded from expedited notification on an SAE form, but should be reported in the follow-up section of the CRF form:

- Elevation of bilirubin
- Elevation liver enzymes
- Nausea, vomiting , diarrhea
- Bleeding following line insertion

Although these events are anticipated to occur as part of the disease process, they will be collected and periodically analyzed to assess whether they are occurring at a higher rate than would be expected in this population. If certain events occur at a higher rate than expected for a given stage or severity of the disease, then the adverse event analysis will be reported under

expedited reporting requirements. (FDA Guidance: Safety Reporting Requirements for INDs and BA/BE Studies. http://www.fda.gov/downloads/Drugs/.../Guidances/UCM227351.pdf)

17 ACCESS TO SOURCE DATA AND DOCUMENTS

17.1 Availability of Source Data and Documents

The Investigator will permit study-related monitoring, audits, EC review, and regulatory inspections (where appropriate) by providing direct access to source data and other documents (i.e. patients' case sheets, blood test reports, histology reports etc.).

17.2 Patient Confidentiality

The patient's name and personal data will remain confidential and will not be published in any way. All data will be coded and stored in locked offices or on password protected computers.

18 MONITORING AND QUALITY ASSURANCE

Monitoring of this study will be to ensure compliance with GCP, local regulations and scientific integrity and will be managed and oversight retained by Exalenz Bioscience (the Sponsor) or its assigned representative.

18.1 Data Collection and Monitoring

An eCRF will be completed for each patient. The EDC (electronic data capture) system will be 21 CRF Part 11 compliant and the site staff and all involved parties will be trained on the system prior to patient enrolment.

IRB and EC members and regulatory authorities will be permitted to review study documents at the site according to local and federal regulations.

19 PUBLICATION POLICY AND FINANCE

It is intended that the results of the study will be reported and disseminated at international conferences and in peer-reviewed scientific journals. The policy regarding publications appears in the non-disclosure agreement signed by each study participant prior to signing of the contract.

20 FINANCIAL ASPECTS

The device and compound will be provided by the Sponsor. Funding for regulatory approvals and administration will also be provided by the Sponsor. Funding will also be provided for a study support staff at local sites.

21 STUDY TERMINATION

The study may be terminated after appropriate consultation between the study sponsor and the chief investigator and co investigators. Conditions warranting termination include, but are not limited to:

- Failure of the investigator to enroll patients at an acceptable rate
- Insufficient adherence to protocol requirements
- Failure to adhere to GCP
- Decision by the study sponsor to suspend or discontinue development of the device or kit

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APPENDIX I - PROTOCOL APPROVAL SIGNATURE PAGE

Protocol No: CSPH-EX-0414

Protocol Title: Clinical Study of the BreathID® MCS System to train the algorithm for the ^{13}C-Methacetin Breath Test (MBT) in assessment of Portal Hypertension in Patients with Compensated Liver Cirrhosis

Version: 1.5

Date of Protocol: 21-NOV-2016

Site Name: _____

Principal Investigator: _____

Print name

I have read this protocol and agree to conduct the study as outlined herein and as per GCP and local regulations.

Principal/ Chief Investigator_____ Date_____

APPENDIX II – DETAILED HVPG PROCEDURE

HVPG procedure: Instruction for a correct measurement

Jaime Bosch, MD, PhD, FRCP

Professor of Medicine, Hospital Clínic-IDIBAPS, University of Barcelona

Abbreviations:

HVPG : hepatic venous pressure gradient

WHVP: wedged hepatic vein pressure

FHVP: free hepatic vein pressure

IVCP : inferior vena cava pressure

RAP: right atrial pressure

HVPG: What is it?

HVPG stand for hepatic vein pressure gradient, which is calculated as the difference between the "wedged" and the "free" hepatic vein pressure (**Figure 1**).

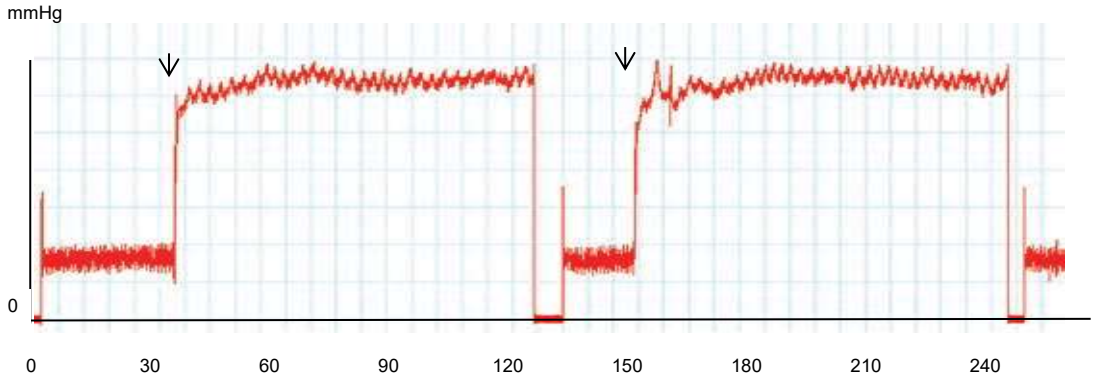


Figure 1. ***Typical measurement of the hepatic venous pressure gradient (HVPG) with a balloon catheter.*** With the balloon deflated, the "free" pressure (FHVP) is recorded. After occluding the hepatic vein by inflating the balloon (*arrows*), the "wedged" pressure (WHVP) is recorded. Between measurements the "zero" pressure is verified by opening the transducer to air. HVPG is calculated as the difference between WHVP (32 mmHg) and FHVP (8 mmHg); in this case it was very high, of 24 mmHg. Note that the two measurements shown in the figure are identical, and that in this patient it took about 45 seconds for WHVP to reach a stable plateau after inflating the balloon.

The wedged hepatic vein pressure is obtained by occluding the lumen of the hepatic vein by inflating a balloon at the tip of the catheter (or by advancing an end-hole catheter as far as it could go into the hepatic vein, a technique that is no longer used as it is less accurate and has more variability that the balloon catheter technique). Once the hepatic vein has been occluded, the static column of blood makes that the pressure sensed by the catheter corresponds to that existing in the hepatic sinusoids. Because of that, the wedged pressure reflects the hepatic sinusoidal pressure. In the normal liver, the hepatic sinusoids are a very uniformly communicated vascular bed. By contrast, in advanced liver disease, especially in cirrhosis, fibrosis and nodule formation makes that the intersinusoidal communications are lost, which makes that when the hepatic vein is occluded, the static column of blood extends beyond the sinusoids and thus reflects portal pressure (**Figure 2**). It has long been known that in different forms of cirrhosis measurement of WHVP equals to the direct measurement of portal pressure, which is much more invasive as requires the transhepatic or surgical catheterization of the portal vein.

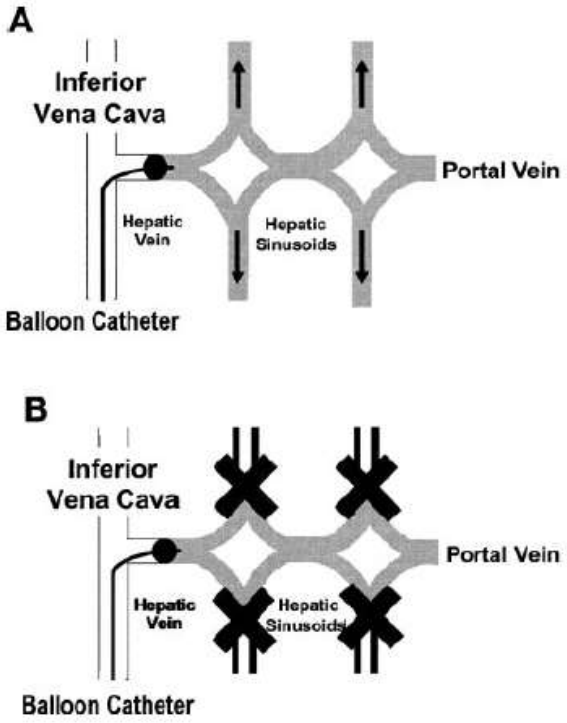
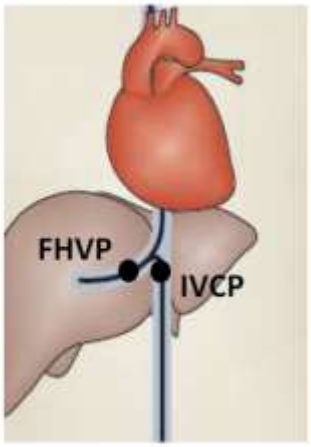


Figure 2. ***Measurement of wedged hepatic venous pressure (WHVP) in the normal and cirrhotic liver***. (**A**) In the normal liver, after occlusion of blood flow in a hepatic vein with a balloon catheter, the pressure of the static column of blood (WHVP) equilibrates through the interconnected sinusoids. Therefore WHVP is a measurement of the hepatic sinusoidal pressure, which is slightly lower than portal pressure (-1 mmHg). (**B**) In the cirrhotic liver, the pressure of the static column of blood created by balloon inflation cannot be decompressed at the sinusoidal level due to the sinusoidal narrowing and disruption of the normal intersinusoidal architecture by fibrosis and nodule formation. Because of this, WHVP equilibrates with portal pressure.

The "free" hepatic vein pressure is obtained by deflating the balloon of the hepatic vein catheter, and is very close to the pressure in the inferior vena cava. If the difference between the free and the IVC pressure is $\geq$ 2 mmHg it usually reflects that the catheter is advanced too much into an irregular hepatic vein. Because of this the free pressure should always be obtained also in a "retired" position, close to the IVC (**Figure 3**, below)



- Inferior vena cava pressure should always be recorded to check if FHVP has been measured correctly.
- FHVP should not be over 2mm Hg above IVCP. If greater (stenosis, compression…), use IVCP to calculate HVPG.
- FHVP should be measured close to its opening into the IVC

Figure 3. ***Free pressures should be measured in a "retired" position, close to the IVC***.

In hemodynamic terms, absolute pressures have much less meaning than pressure gradients, the difference of pressure between two points in a vascular system. The pressure gradient (ΔP) is mathematically related to blood flow (Q) and to vascular resistance (r) by Ohms law in the equation: $\Delta P = R \times Q$.

Expressing pressures as pressure gradients beyond being physiologically the correct way has the practical advantage of being independent of external influences. For instance the presence of ascites or obesity may increase the intra-abdominal pressure; this will equally increase the portal pressure and the IVC pressure (or its equivalents, the wedged and free hepatic vein pressure) but will not affect the pressure gradient. The same happens when the electronic pressure transducer is placed to low or to high with respect to the recumbent patient; this will make all pressures to be increased or decreased, but will not alter the pressure gradient. An important final point regarding pressure gradients is that this should be obtained between the WHVP and FHVP (or IVC), not with the right atrial pressure! Using the right atrial pressure introduces a systematic error that overestimates the true pressure gradient and it has been shown that doing this detract the prognostic value of the measurement. In short, the right atrial pressure should not be used to assess portal pressure or hepatic hemodynamics.

The procedure

Hepatic vein catheterization is a moderately invasive procedure that should be done by well-trained personnel. It is an ambulant procedure, although it is done with a day-hospital admission in most centers (see video "HVPG measurement" in Garcia-Tsao and Bosch, NEJM 2010, also available in You Tube).

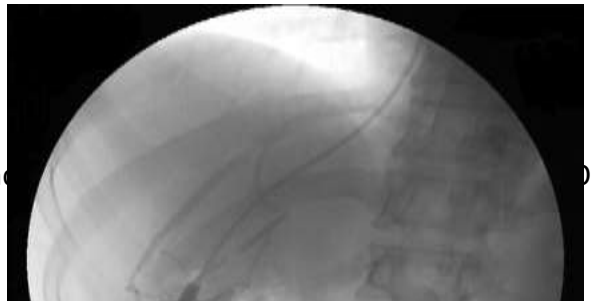
The patients usually accept to have repeat HVPG measurements over the time, as the procedure is usually very well tolerated. The factor more closely related to a less good tolerance is the duration over 45 minutes (which most frequently reflect lack of expertise or need for associated procedures such as a transjugular liver biopsy). The median duration of the procedure is 25 minutes.

The procedure should be done preferably in the morning, after fasting overnight. This is so since there is a circadian variation of the HVPG that increases during the day and decreases over the night. On this rhythm there is a superimposed postprandial increase of HVPG that lasts 1-2 hours. If a patient is to undergo repeat measurements these should always be done at approximately the same hour.

The favorite access points are either the right internal jugular vein or the right femoral vein. The procedure is done under slight conscious sedation. Deep sedation should be avoided as it results in exaggerated deep breathing that cause artifacts precluding accurate pressure measurements. Direct ultrasonography guidance should be used, as it is saves time and reduces the risk of the more common complications (hematomas, local bleeding, or vascular lesions such as arteriovenous fistulae).

After the access vein has been punctured, a guide wire is advanced into the vein allowing the placement of a venous introducer sheath allowing advancing the balloon catheter under fluoroscopic guidance into a hepatic vein. This usually takes less than 5 minutes.

Once the catheter is in the hepatic vein (preferably, the main right hepatic vein) (Figure 2) an adequate occlusion of the hepatic vein should be checked after inflating the balloon by injecting by hand a small amount of iodinated contrast media. This should show the occluded hepatic vein, without reflux of contrast through the occluded vein or through communications with another hepatic vein. If present, these communications prevent an adequate estimation of the portal pressure. This is extremely uncommon in cirrhosis but very frequent in idiopathic portal hypertension or nodular regenerative hyperplasia (**Figure 3**).



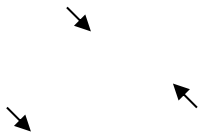


Figure 3. The presence of communicating vessels between different hepatic veins (arrows) prevents an effective occlusion of the vein and precludes a correct measurement of WHVP. This should be checked in every patient. Such communications are very frequent in patients with idiopathic portal hypertension, but very rare in alcoholic, viral or metabolic cirrhosis.

After documenting the correct occlusion of the hepatic vein, the balloon is released and the catheter is thoroughly rinsed with saline or 5% dextrose and connected to a multichannel pressure recorder for the measurement of the wedged and free hepatic venous pressures (see next section).

Pressure measurements

Correct measurement of intravascular pressures requires fulfilling a series of steps and to have adequate equipment. This is available almost in all hospitals, but not all monitors in IR rooms are adequate for accurate measurements of HVPG.

1. Pressure recorder: The adequate pressure recorder should have the following capabilities:

a) Adjusting the pressure scale for a relatively low range 1-40 or 1-50 mmHg. The pressure scale should be printed together with the pressure tracings, and preferably a milimetric grid should also be disclosed.
b) Adjusting the recording speed to low velocity (1-6 mm/second).
c) Capacity of obtaining permanent pressure tracings, either using an on-line printing device, or by generating a printed report from the digitally stored data after completing the procedure.

If the monitor you have is not able of doing this, your center does not qualify for the study.

In addition, a non-invasive monitor for EKG, BP and O2 saturation should be used.

2. Pressure transducer: Nowadays most centers use disposable pre-calibrated transducers that are very reliable.

If you are using another type of transducer or you have doubts on its correct functioning, the transducer should be calibrated against a know pressure. This is done by connecting the transducer to a column of saline of 544 mm height (where it should read 40 mmHg); 272 mm height (where is should read 20 mmHg), and then to 136 mm (where it should read 10 mmHg).

The pressure transducer should be placed in a fixed position that should be set at the mid-axillary line of the recumbent patient. This is the conventional level for the right atrium. Neither the transducer nor the patient should move during the procedure.

3. Balloon catheter: These should have a small (8 – 11 mm inflated balloon diameter), preferably not entirely distal (**Figure 4**).

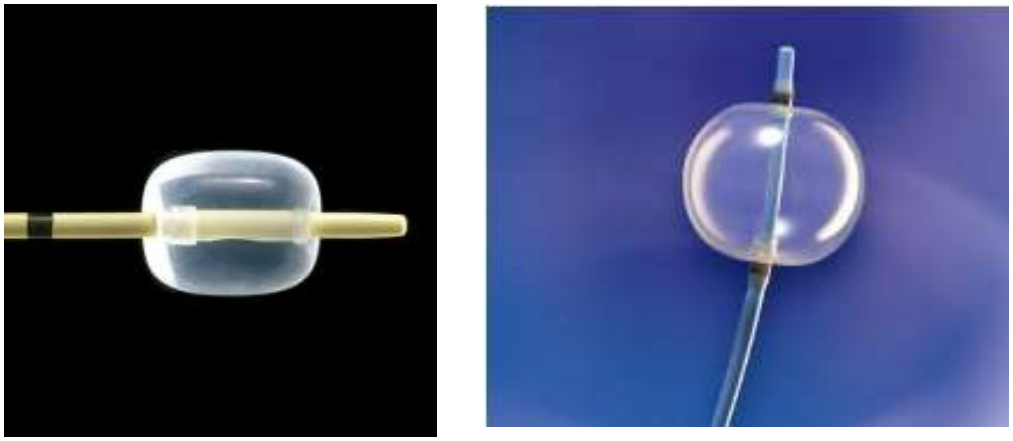


Figure 4. ***Balloon catheters suitable for HVPG measurements***. The balloon is of 8-11 mm in diameter and is not totally distal, so a fully inflated balloon will not occlude the central lumen that will be centered in the axis of the vein.

4. Obtaining the wedged (occluded) hepatic vein pressure (WHVP): After having verified that the catheter is at an adequate position, the hepatic vein is occluded by inflating the balloon while continuously recording the pressure. The hepatic vein pressure will rapidly increase from the free pressure until it finally stabilizes at the final WHVP. This requires usually some 45-60 seconds but sometimes it requires longer periods (**Figures 1 and 5**). The pressure measurement should be continuously recorded for at least 60 seconds (longer if the pressure is still increasing). Because it needs such a long time it is mandatory to run the recorder at very low speed, if not one cannot notice if the

pressure is still increasing or has achieved a plateau. For adequate pressure measurements the patient should be relaxed, asked not to move or talk, and to breathe smoothly.

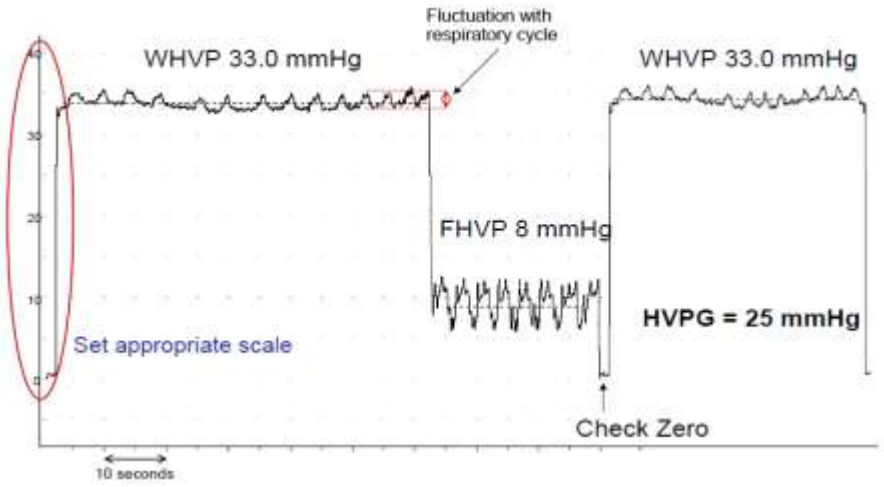


Figure 5. ***Typical HVPG tracing***

Most recorders will allow printing either (or both) the "phasic" oscillating pressure or the "mean" electronically averaged pressure (Figure 6). If possible, both should be recorded. "Mean" pressures are easier to read, but are slow reacting and therefore less adequate to recognize artifacts.

The WHVP should be recorded by triplicate. This allows averaging the values to get a more robust measurement.

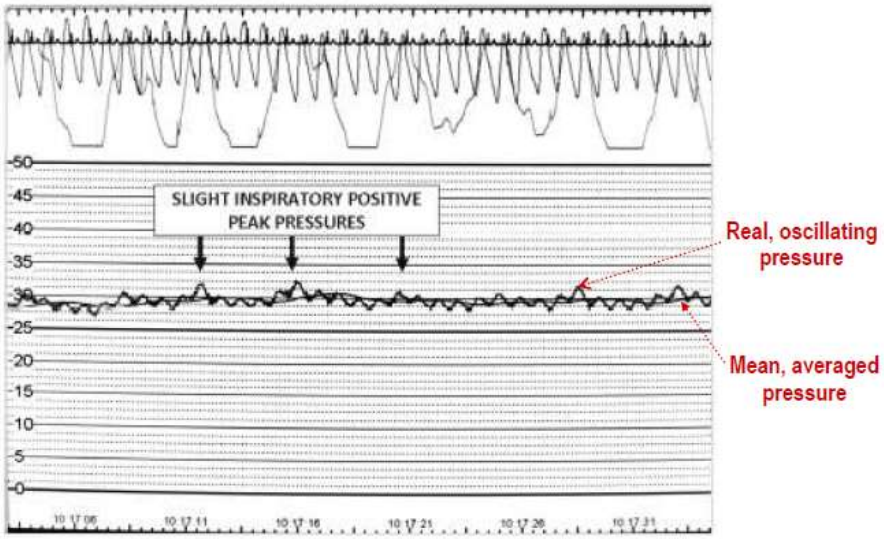


Figure 6. ***Oscillating and "mean" pressures recorded simultaneously***

5. Obtaining the free hepatic vein pressure (FHVP) and the inferior vena cava (IVC) pressure. After completing the measurement of WHVP the catheter should be rinsed, the zero level checked by opening the transducer to atmospheric pressure, and then the FHVP is recorded while the patient is quiet and breathing smoothly. The recording should be run for 20 - 30 seconds.

After obtaining the FHVP the catheter should be slightly withdrawn to obtain another **FHVP at a "retired" position**, with the catheter tip 2-3 cm from the IVC, and from here withdrawn to the **IVC** where pressures are measured again (at the level of the hepatic veins), and finally into the **right atrium**, to measure RAP.

Again, remember that all pressures should be measured by triplicate.

Frequent mistakes

The most frequent mistakes in the measurement of HVPG are due to the fact that the measurement is done at a IR department forgetting to discuss with the radiologist that the main aim of the procedure is to obtain reliable, accurate measurements, with a precision of 0.5 mmHg. The more frequent mistakes are listed and illustrated below:

a) Inadequate scale: many monitors are pre-calibrated for measuring arterial blood pressure. This scale (usually 0 – 150/200 mmHg) is not adequate for measuring HVPG as the deflections would be too small to allow enough accuracy. The preferred scale should be one in which 1 mmHg equals one division of the grid. Figure n shows an example of a correct and of an incorrect measurement.

b) Inadequate speed: it is common to run the recorder at a speed of 25-50 mm/sec. This is adequate for EKG but not for HVPG, as a full study would require strips of about 15 foot length, or show only a few seconds of recording.

c) Inadequate length: a variant of the previous mistake is to run the recording at correct speed but for a too short period, not allowing the pressure to stabilize. This results in underestimation of the true HVPG.

d) Artifacts due to deep respiration are very common if the patient is excessively sedated and almost invariable when using IV fentanyl. Typically this results in a wide variation of all venous pressures during the respiratory cycle, precluding accurate measurements of HVPG (--, Figure). The patient moving, talking or coughing during the measurements may also cause artifacts. Whenever an artifact occurs, this should be annotated in the tracing and the pressure measurement discarded and repeated.

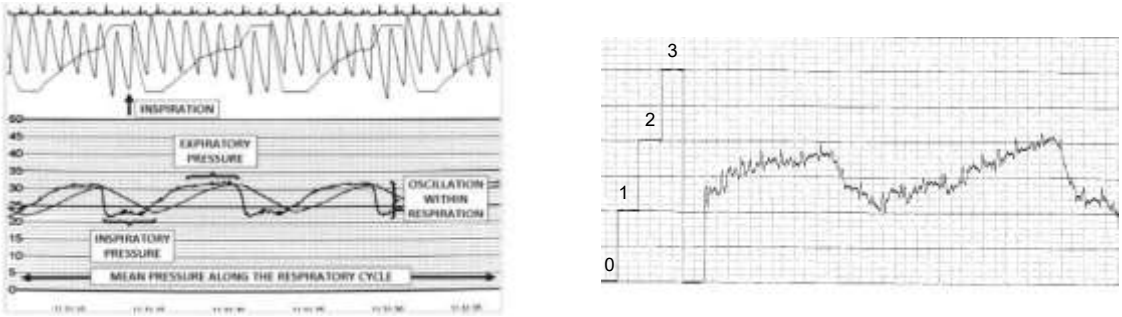


Figure 6. ***Marked respiratory oscillation due to excessive sedation***

Sending the tracings of a HVPG measurement to the central reader.

The original tracings are important source documents that should be kept in the patient's charts. Please, do not send original materials by mail or courier. It is safer, quicker and cheaper to send by e-mail a scanned copy of the tracings as a PDF file.

Each tracing should be clearly identified with the name/code number of the local Principal Investigator (PI); patient's code number and initials, and date.

The tracings should include the calibration used, and 3 sets of measurements of the WHVP (balloon occluded), FHVP, "retired" FHVP, IVC and RAP. Remember to set the recorder at a low speed (1-6 mm/sec), an adequate scale (0-40 or 0-50 mmHg), and to run the measurements at least for 20 seconds for FHVP, IVC and RAP and at least for 60 seconds for WHVP.

APPENDIX III – DETAILED MBT PROCEDURE

Preparation of the study subject

Once enrolled, the patients will perform the breath test if they meet the criteria of the patient population. In preparation for each breath test the patient will be asked to comply with the following instructions:

1. Fast 8 hours prior to the breath test.
2. Refrain from use of caffeine, propranolol, within the last 24 hours. Refrain from use of acetaminophen-related medications (e.g. Tylenol) 24 hours prior to the breath test.
3. Refrain from use of alcohol 24 hours prior to the breath test.

Preparation of ^{13}C-Methacetin

Exalenz Bioscience Ltd. will provide 75 mg ^{13}C-Methacetin doses in a 0.05% solution of ^{13}C-Methacetin in purified water, supplied, in amber thermoplastic polyester (PET) bottles with a child resistant plastic stopper (75mg of ^{13}C-Methacetin in 150mL purified water). No preparation is needed other than pouring the contents of the solution into a cup for ingestion if administered orally.

Performance of the breath test

Only trained personnel (doctors, nurses) will perform the breath test procedure. The actual breath collection is automatically done by the device and is not operator dependent. If the patient was not connected properly with the cannula (e.g. the breath does not reach the device), the *BreathID® MCS* device will prompt the operator to adjust the cannula.

1. Turn on device from switch in rear and allow up to 1 hour for warm-up to complete. To perform the test, ensure that the *BreathID® MCS* console screen shows that the device is in the 'Ready' state.

2. The cannula will be attached to the *BreathID® MCS* device and to the patient. The *BreathID® MCS* device will be acti*vated an*^{d} *wi*ll collect the patient's baselin*e exhale*^{d} *br*eath for approximately 15-20 minutes.

3. The solution ^{13}C-Methacetin is poured into a disposable cup and administrated to the patient. The solution should be administered by a medical practitioner registered on the delegation log or a research nurse if specific training for administration has been given. Immediately after ingestion, the operator will press the "continue" button and activate the

actual measurement. CO_2 production with ^{13}C may be visible within a few minutes in cases with relatively functional livers.

Note: In rare cases the administration of fluids may cause vomiting. If this happens the test should be aborted and repeated the next day. The expected adverse event should be reported in the appropriate CRF section.

4. The patient should remain in a seated position breathing in a normal manner for up to 75 minutes, whilst data are collected.

5. The *BreathID® MCS* device continuously measures and analyzes the patient's exhaled breath in real time. As the ^{13}C-Methacetin is metabolized, the value of the $^{13}CO_2/^{12}CO_2$ ratio in the exhaled breath will change and will be calculated in real time by the *BreathID® MCS* device. The device will prompt the operator to enter patient details such as weight and height, age and gender. The *BreathID® MCS* device will calculate the *MBT* variables.

6. If at any time the device does not detect patient's breath, or if there is any other deviation from the desired test requirements, the device will produce an appropriate warning signal.

7. At the completion of the procedure, the cannula is removed and the patient is disconnected from the *BreathID® MCS* device.

The patient will be under the supervision of the physician or any other qualified medical staff during the entire test.

The operators will be trained how to terminate the breath test early. In the following situations, the *MBT* test will be terminated and a test termination form will be completed:

1. The patient vomits.

2. The patient is inadvertently disconnected.

3. The *BreathID® MCS* device malfunctions (In this case, the operator will complete a technical complaint form in addition to the test termination form and contact Exalenz immediately for further instructions)

In all these cases, the next *MBT* cannot be repeated the same day. An entry will be made in the drug/kit accountability log and the bottles will be kept for inspection by the study monitor.