ABSTRACT

Norfloxacin For Hepatopulmonary Syndrome: A Pilot Study of a Rare Disease

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Introduction: Hepatopulmonary Syndrome is a rare disease characterized by abnormal gas-exchange and a poor prognosis, with no known effective medical therapy. A rat model and preliminary human data suggest that this disease may be caused by intestinal bacterial overgrowth, systemic endotoxemia and increased nitric oxide. Methods: We conducted a pilot crossover randomized controlled trial of norfloxacin versus placebo over four weeks, in seven subjects with HPS or a milder condition called pre-HPS, with a primary outcome of alveolar-arterial oxygen gradient (AaDO₂). Results: There was no trend toward improved AaDO₂, this outcome and other intermediate outcomes were highly variable, and results suggested that a longer treatment course might be necessary. We identified multiple obstacles to recruitment. Conclusion: We believe that a full-scale study of norfloxacin therapy for HPS will require 1) a six-month therapeutic period, 2) more specific HPS diagnostic criteria for clinical and study populations, and 3) creative recruitment maneuvers.
ACKNOWLEDGEMENTS

Many individuals have played an important role in the success of this particular study, in the establishment of a hepatopulmonary syndrome clinical program at St. Michael’s Hospital, and in guiding my career choices and opportunities.

I’d like to express my sincere gratitude to:

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<tr>
<td>6MWD</td>
<td>six-minute walk distance</td>
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<td>AaDO₂</td>
<td>alveolar-arterial oxygen gradient</td>
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<td>ABG</td>
<td>arterial blood gas</td>
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<td>baseline dyspnea index</td>
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<tr>
<td>CBD</td>
<td>common bile duct</td>
<td></td>
</tr>
<tr>
<td>CE</td>
<td>contrast echocardiogram</td>
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<td>CF</td>
<td>Cystic Fibrosis</td>
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<tr>
<td>CF TDN</td>
<td>Cystic Fibrosis Therapeutics Development Network</td>
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<td>CME</td>
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<td>CO</td>
<td>cardiac output</td>
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<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
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<td>CoV</td>
<td>coefficient of variation</td>
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<td>clinical research network</td>
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<td>CRQ</td>
<td>Chronic Respiratory Disease Questionnaire</td>
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<td>DLCO</td>
<td>diffusion lung capacity for carbon monoxide</td>
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<td>EA</td>
<td>endotoxin activity</td>
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<td>Eastern Cooperative Oncology Group</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>EURORDIS</td>
<td>European Organization for Rare Disorders</td>
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<td>FEV₁</td>
<td>forced expiratory volume in one second</td>
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<td>FMD</td>
<td>flow-mediated vasodilatation</td>
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<td>forced vital capacity</td>
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<td>intrapulmonary vascular dilatation</td>
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<td>LT</td>
<td>liver transplantation</td>
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<td>MAA</td>
<td>macroaggregated albumin</td>
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<td>MCID</td>
<td>minimal clinically important difference</td>
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<td>model for end-stage liver disease</td>
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<td>PaO₂</td>
<td>partial pressure of arterial oxygen</td>
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<td>PIM</td>
<td>pulmonary intravascular macrophage</td>
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<td>QR</td>
<td>quinolone resistant</td>
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<td>QTc</td>
<td>corrected QT interval</td>
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<td>randomized controlled trial</td>
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<td>RVSP</td>
<td>right ventricular systolic pressure</td>
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<td>SBP</td>
<td>spontaneous bacterial peritonitis</td>
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<td>standard deviation</td>
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<td>TDI</td>
<td>transitional dyspnea index</td>
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<tr>
<td>TGH</td>
<td>Toronto General Hospital</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>TPR</td>
<td>total peripheral resistance</td>
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<td>University of Toronto</td>
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CHAPTER 1: INTRODUCTION

1. Background: Hepatopulmonary Syndrome
The first recorded description of hypoxemia with hepatic dysfunction was by Flückiger in 1884, when he described a 37-year-old woman with syphilitic cirrhosis who developed clubbing and cyanosis and had distended pulmonary blood vessels on post-mortem examination \(^1\). Though several inter-current case reports identified a similar association, it was not until nearly a century later, in 1977, that Kennedy and Knudson described a patient with alcoholic cirrhosis, exertional hypoxemia and orthodeoxia, and drew an analogy to the well identified hepatorenal syndrome, to coin the term “hepatopulmonary syndrome” \(^2\).

i. Definition
The hepatopulmonary syndrome (HPS) is defined by a triad of features: (1) liver dysfunction or portal hypertension, (2) intrapulmonary vascular dilatations (IPVDs) and (3) abnormal gas-exchange, defined by an alveolar-arterial oxygen gradient (AaDO\(_2\)) > 20 mm Hg \(^3\text{-}^5\) (Table 1).

Table 1: Definition of the Hepatopulmonary Syndrome

<table>
<thead>
<tr>
<th>1. Liver Dysfunction or Portal Hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Cirrhosis, or</td>
</tr>
<tr>
<td>- Non-cirrhotic portal hypertension (e.g. portal vein thrombosis, nodular regenerative hyperplasia, Budd-Chiari Syndrome), or</td>
</tr>
<tr>
<td>- Acute fulminant hepatitis, or</td>
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<td>- Allograft rejection</td>
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<table>
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<tr>
<th>2. Abnormal Gas-Exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Alveolar-arterial oxygen gradient (AaDO(_2)) &gt; 20 mm Hg (at rest, in the standing position) (partial pressure of alveolar oxygen calculated with the ideal alveolar gas equation)</td>
</tr>
</tbody>
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<table>
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<tr>
<th>3. Intrapulmonary Vascular Dilatations</th>
</tr>
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<tbody>
<tr>
<td>- Contrast echocardiography delayed shunt (&gt;3 heartbeats), or</td>
</tr>
<tr>
<td>- Technetium-99m-labelled macroaggregated albumin ((^{99m})TeMAA) scan increased shunt ((\geq 6%))</td>
</tr>
</tbody>
</table>
ii. Epidemiology
HPS is a well recognized complication of liver disease of varying causes. Though HPS has been reported in cases of non-cirrhotic portal hypertension with normal synthetic liver function (e.g. nodular regenerative hyperplasia), the commonest cause remains cirrhosis, though no specific etiology nor severity of cirrhosis has been found to be correlated with the incidence or severity of HPS. Among cirrhotic subjects awaiting orthotopic liver transplantation (LT), approximately 70% complain of dyspnea, 34-47% have IPVDs, and 10-24% have HPS \(^3,6-11\). Given that chronic liver disease and cirrhosis affect greater than 400 000 people and result in over 27 000 deaths annually in the United States, the societal burden of this rare disease is significant \(^12\).

iii. Natural History and Prognosis
The natural history of HPS is dismal, with the majority of patients suffering progressive hypoxemia over time \(^13\). Furthermore, this progressive decline in gas-exchange often occurs despite stable liver function \(^14\). The first reported survival statistics in HPS looked retrospectively at a cohort of 22 patients, demonstrating a mean survival of only 2.5 years after diagnosis \(^14\). The only prospective study of the natural history of HPS evaluated a cohort of 111 patients with cirrhosis, of whom 27 (24%) had HPS. Median survival was 10.6 months in HPS patients compared to 40.8 months in non-HPS patients; differences were similar when groups were stratified by severity of liver disease and when liver transplanted patients were eliminated \(^6\). Finally, among HPS subjects, those with a PaO\(_2\) ≤ 60 mm Hg have been noted to have a significantly higher mortality \(^13\).

iv. Treatment
Liver transplantation (LT) is the only known effective therapy for HPS, with significant improvement in oxygenation observed in 70-100% of survivors within one year of transplantation \(^15\). However, there are no clear predictors of the extent or timing of post-operative reversibility of hypoxemia; reports have noted resolution of hypoxemia in as soon as a few days to as long as 14 months post-operatively. Furthermore, LT carries an elevated peri-transplant mortality of 16-29% in HPS subjects \(^13,15,16\). Moreover, this risk increases incrementally with the degree of pre-operative hypoxemia, with the strongest
predictors of post-operative mortality being a partial pressure of arterial oxygen ($\text{PaO}_2 \leq 50 \text{ mm Hg}$) alone or in combination with an MAA shunt fraction $\geq 20\%$ (Table 1) $^{16}$. In fact, the only prospective study in the literature reports a peri-transplant mortality of 67% in those subjects with a pre-transplant $\text{PaO}_2 \leq 50 \text{ mm Hg}$ $^{16}$. Despite this increased peri-transplant mortality, it has been shown that HPS subjects with a $\text{PaO}_2 \leq 60$ and $\leq 50 \text{ mm Hg}$ have significantly better five-year survival rates with LT compared to supportive therapy $^{13}$. As a result, the United Network for Organ Sharing (UNOS) has recommended that HPS patients with a $\text{PaO}_2 < 60 \text{ mm Hg}$ be prioritized with a goal of transplantation within three months of listing $^{17}$. Finally, within the last decade, increasing interest and expertise has led to the availability of living-related LT in many centers. Though data in HPS is limited to case reports, it appears as though this treatment is as effective as cadaveric LT for HPS $^{18-20}$. In summary, since hypoxemia is progressive in HPS and pre-operative prognosis and LT outcomes are closely linked to the severity of hypoxemia, HPS patients should be listed for transplantation as early as possible.

It must be noted, however, that there are a number of other important caveats regarding LT for HPS. Firstly, there have been numerous reports of early postoperative refractory hypoxemia requiring prolonged mechanical ventilation and techniques such as Trendelenberg positioning $^{16,21,22}$. Also, a number of centers have noted increased postoperative infections and anastamotic bile duct leaks, suspected to be on the basis of delayed wound healing due to hypoxemia $^{21,23}$. In addition, numerous early postoperative thrombotic complications including portal vein and hepatic artery thrombosis have been reported, in the context of polycythemia from chronic hypoxemia $^{21,23}$. Furthermore, there are reports of post-transplant recurrence of HPS as a result of graft dysfunction, and there has been a report of post-transplant resolution of hypoxemia followed by development of progressive pulmonary hypertension $^{24,25}$. Finally, numerous reports have shown that decreased pulmonary diffusion capacity in HPS does not improve post-transplant, despite an improvement in oxygenation $^{25-27}$. These issues highlight the difficult and unpredictable post-operative course seen with LT in HPS patients.
2. **Pathophysiology and Study Rationale**

Autopsy series in HPS subjects have described alveolar septal capillaries and pre-capillary areas dilated to a diameter of 60-80 μm, compared to a normal diameter of 7-15 μm. Efforts to decipher the pathophysiology of HPS have focused on the etiology of these intrapulmonary vascular dilatations, which are the basis for gas-exchange abnormalities.

Observational and experimental data suggest that the vasodilatation of HPS may be related to systemic endotoxemia secondary to underlying cirrhosis, and mediated by nitric oxide (NO) (Figure 1). Theories about the role of NO in pulmonary vascular dilatation have emerged from a well-established literature supporting the role of NO in the systemic and splanchnic vasodilatation that characterize the hyperdynamic circulatory state of cirrhosis. In fact, portal hypertension is characterized by small bowel bacterial overgrowth associated with bacterial translocation across the intestinal wall and resulting systemic bacteremia with endotoxemia, as well as a hyperdynamic circulation characterized by a decreased systemic vascular resistance. In 1991, Vallance and Moncada proposed that nitric oxide, which is associated with both endotoxemia and vasodilatation, may mediate this relationship. These researchers demonstrated that porcine aortic endothelial cells exposed to bacterial lipopolysaccharide expressed an inducible form of nitric oxide synthase (iNOS) which resembles the iNOS found in human macrophages. They postulated that the endotoxemia of cirrhosis leads to vascular endothelial macrophage recruitment, overexpression of iNOS, elevated local NO production, vasodilatation, and a hyperdynamic circulation. In support of this theory, Laffi, et.al. later demonstrated that circulating mononuclear cells taken from cirrhotic subjects with endotoxemia and a hyperdynamic circulation had significantly higher NO synthase activity than those taken from healthy subjects.

Subsequently, a study in human subjects sought to establish the role of bacterial translocation and NO in the hyperdynamic circulatory state of cirrhosis by using forearm blood flow as a marker of hyperdynamic state. This study demonstrated the presence of elevated basal forearm blood flow in cirrhotic subjects compared to controls, which was
reversible with administration of the antibiotic norfloxacin, presumably through a mechanism of decreased gut bacterial translocation. Furthermore, the administration of N-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NO synthase which results in decreased endothelial NO levels, was found to decrease forearm blood flow in cirrhotic subjects more so than in controls, and this difference disappeared after norfloxacin administration \(^{36}\). These findings support the role of NO as an intermediary between bacterial translocation and vascular dilatation in cirrhotic subjects.

Subsequently, a more extensive study demonstrated that selective intestinal decontamination with a four-week course of norfloxacin caused a significant decrease in systemic endotoxin levels, and a corresponding increase in systemic vascular resistance \(^{37}\). However, it should be noted that this study used a crossover design with treatment and placebo arms, but did not implement a wash-out period between four-week treatment courses. Hence, the results may be biased by carry-over effects. The authors justified this design by the short half-life of norfloxacin (2.5-8 hours); however, they failed to recognize that the downstream biological effects, from gut bacterial load changes to pulmonary macrophage recruitment and iNOS upregulation in the lungs, may take much longer to reverse.

Turning now to the pulmonary circulation, scientists have focused on exhaled breath nitric oxide (NO) levels in exploring the possible role of NO in intrapulmonary vascular dilatation. Initial studies demonstrated an elevated rate of exhaled NO production among cirrhotic subjects compared to controls \(^{38-40}\), as well as among HPS subjects compared to controls \(^{41}\). Based on these observations, in 1995, Cremona, et.al. proposed that local pulmonary vascular NO overproduction was the cause of the IPVDs which characterize HPS, and lead to hypoxemia \(^{41}\). Indeed, a subsequent study confirmed the presence of a decreasing gradient of exhaled NO in subjects with HPS, non-hypoxemic cirrhotics, and normal volunteers, respectively. Furthermore, exhaled NO production correlated significantly with alveolar-arterial oxygen gradient (AaDO\(_2\)) \(^{42}\). Unfortunately, in this study, patients with HPS were not matched with the non-HPS cirrhotic subjects, leaving the observed relationship susceptible to confounding; furthermore, non-hypoxemic cirrhotic subjects were not tested for intrapulmonary vascular dilatations. Finally, in a
“before-and-after” observational study, cirrhotic subjects who had liver transplantation (LT) had postoperative decreases in exhaled NO levels to near-normal values, correlating with an improvement in their AaDO₂s ⁴³.

In a culmination of these theories, in 2001, a single human case of resolution of HPS after treatment with a four-week course of norfloxacin was reported ⁴⁴. Overall, though the NO hypothesis is intriguing, it must be noted that the human data is subject to limitations. First, existing studies have study design limitations, as noted earlier, and second, no study has systematically studied the effects of bowel decontamination on oxygenation, the major predictor of mortality in this population.
Figure 1: Pathophysiologic Model for HPS
Portal hypertension is associated with bacterial translocation across the gut wall, resulting in systemic endotoxemia and bacteremia. In the systemic circulation, this leads to systemic vasodilatation, and the characteristic hyperdynamic circulatory state of cirrhosis. In the pulmonary circulation, this causes recruitment of pulmonary intravascular macrophages, which upregulate iNOS and release NO locally, causing the IPVDs that characterize HPS.

However, animal data do support this theory. In fact, an elegant rat model of bile duct ligation-induced cirrhosis and HPS has provided critical insight into the origins of increased pulmonary vascular NO and the pathogenesis of HPS.

Firstly, it has been demonstrated that rats with HPS have bacterial translocation across the intestinal wall, resulting in a chronic, low-grade systemic endotoxemia. Next, it has been established that these rats have extensive pulmonary intravascular macrophage (PIM) recruitment, demonstrated both histologically in rat lung explants, and in vivo.
by pulmonary radioactive colloid scintigraphy. Endotoxemia appears to mediate this PIM sequestration by stimulation of macrophage and endothelial adhesion molecules, through release of the cytokine tumor necrosis factor alpha (TNF-α), which is also a powerful inducer of iNOS. Indeed, these rats had increased pulmonary iNOS protein expression, and elevated exhaled NO levels. Also, treatment with a nonspecific NOS inhibitor (L-nitro-L-arginine methyl ester) decreased exhaled NO and prevented the development of HPS. Finally, bile duct-ligated rats treated immediately with a five-week course of norfloxacin had significantly lower levels of endotoxemia, PIM load, and activity and expression of lung iNOS, associated with a reduction in the severity of HPS.

Next, it was demonstrated that HPS rats had increased hepatic production and plasma levels of endothelin-1 (ET-1), another important mediator of vascular physiology. ET-1 levels were then correlated both with severity of HPS (by AaDO2), and with pulmonary levels of endothelial nitric oxide synthase (eNOS), an NO-producing enzyme expressed constitutively in the pulmonary vasculature. On the basis of these results, certain authors postulated that the increased pulmonary vascular NO in HPS is produced primarily by eNOS (as opposed to iNOS), and stimulated by increased ET-1 levels. Subsequently, it was shown that ET-1 dependent eNOS stimulation is mediated by the endothelin B (ETB) receptor on the luminal side of pulmonary endothelial cells. This receptor was shown to be selectively upregulated in cirrhotic rats, secondary to the increased pulmonary vascular shear stress of a hyperdynamic circulation. Finally, it was shown that selective ETB receptor blockade in vivo decreased pulmonary endothelial eNOS and improved HPS.

In comparing these two postulated mechanisms, it remains unclear whether iNOS or eNOS plays a more important role in the NO production that leads to IPVDs in the rat model of HPS. However, recent data has suggested that these two mechanisms are not mutually exclusive, and may in fact both be linked by endotoxemia and TNF-α. As noted, in the bacterial translocation theory of HPS, systemic endotoxemia increases TNF-α, which upregulates iNOS production and local NO release by PIMs. However,
endotoxemia can also cause a direct increase in ET-1 levels, either by direct action in the liver, or through TNF-α \(^{57}\); furthermore, both endotoxin and TNF-α can each directly enhance eNOS activity \(^{58}\). This suggests that endotoxemia and TNF-α release may be common triggers for both iNOS- and eNOS-mediated NO-releasing pathways. In support of this theory, a study of the non-specific TNF-α blocker pentoxifylline in experimental HPS demonstrated a decrease in PIM recruitment, endothelial ET\(_B\) receptor expression, eNOS expression and activation, and an improvement in the severity of HPS \(^{59}\). Overall, it is likely that both iNOS and eNOS contribute importantly to the development of IPVDs in HPS.

In summary, the relationship between endotoxemia/hyperdynamic circulation and increased vascular NO levels, as well as the therapeutic effects of norfloxacin on these phenomena are well established in humans. Additionally, the association between HPS/poor oxygenation and increased exhaled NO levels is well established. However, the effect of norfloxacin on oxygenation had never been studied in subjects with HPS. A randomized controlled trial (RCT) of norfloxacin therapy was necessary to evaluate the efficacy of norfloxacin therapy in HPS.

3. **Overall Study Significance**

HPS is a disease that carries a high morbidity and an alarmingly high mortality. LT is the only effective treatment but is associated with significant peri-operative mortality in these subjects. The body of literature presented above has built a compelling case for the role of gut bacterial translocation and secondary pulmonary NO overproduction in the pathophysiology of HPS. A sophisticated rat model and a human case report have supported the potential for norfloxacin, a widely available, inexpensive and non-toxic antibiotic, to mitigate these effects and improve oxygenation, which is the major predictor of both morbidity and mortality in HPS. Given the dismal prognosis of this disease, the biological plausibility of the hypothesis, and the minimal foreseeable deleterious consequences of the intervention, the rationale for a formal test of this theory is strong.
This thesis outlines a pilot study of norfloxacin therapy carried out as a necessary first step towards an RCT. The study employed a crossover design with a four-week washout period and a randomized order of treatment.

4. **Study Objectives**

   i. To evaluate the magnitude and standard deviation of the change in AaDO₂ with norfloxacin treatment in subjects with HPS, to enable accurate sample size calculations for a future large randomized controlled trial of norfloxacin for the treatment of HPS

   ii. To evaluate subject recruitment and retention, in order to determine the feasibility of a future large randomized controlled trial of norfloxacin for the treatment of HPS

   iii. To qualitatively evaluate the usefulness of a number of measures that have never been utilized in this subject population (six-minute walk distance (6MWD), baseline dyspnea index (BDI), transitional dyspnea index (TDI), and Chronic Respiratory Disease Questionnaire (CRQ))

   iv. To evaluate the hypothesized role of alveolar NO (measured by exhaled NO) as an intermediary in the relationship between norfloxacin administration and change in AaDO₂
CHAPTER 2: DESIGNING THE PILOT RANDOMIZED CONTROLLED TRIAL

1. Pilot study: Concepts

This study was designed as a pilot study for an eventual multi-center randomized controlled trial (RCT) of norfloxacin therapy for the hepatopulmonary syndrome (HPS). As noted, norfloxacin has never been studied for this specific therapeutic application nor in this specific population. As a result, there exists no prior reported experience to guide study design, recruitment logistics, choice of instruments, choice of outcomes, samples size calculations, and resource requirements. Thus, a pilot study was seen as an essential first step in laying the foundations for a later RCT. Although a number of disease-specific study outcomes were included (Chapter 3), we did not anticipate any statistically significant changes in these outcomes in this pilot study.

In fact, a pilot study should be considered a small preparatory investigation that is not intended to directly investigate or test the research hypotheses of interest, but rather to understand multiple facets of the research process related to testing these hypotheses. Unfortunately, features and objectives of pilot studies lack a uniform definition, and the term “pilot study” is often applied inappropriately. For example, in the pharmaceutical industry, this term is often used to designate a “non-pivotal study,” while medical investigators often use it to designate a study that is simply not of full-scale. Lancaster et.al. reported a lack of literature providing methodological guidance as to what constitutes a pilot study, a low rate of publication of pilot studies, and lack of an editorial policy regarding the publication of pilots among high-impact journals. In light of these numerous ambiguities, it is worth examining the conceptual features and appropriate uses of pilot studies in general.

To begin, pilot studies may be used to provide important quantitative data required for planning a full-scale study. Particularly, pilot studies of RCTs involving novel interventions provide an estimation of the standard deviation (SD) of the primary outcome measure, enabling sample size calculations for the larger trial. In certain cases,
pilots may even be used to guide the selection of the most appropriate primary outcome measure based on its reliability, and characteristics and feasibility of the measurement instrument involved. Furthermore, certain aspects of a planned data analysis can be tested in a pilot study, potentially revealing important limitations in the proposed future analysis 62. Finally, pilots allow for assessment of the adequacy of allotted resources, including human resources and financial budgets, and identification of hidden costs 63.

Furthermore, in treating the pilot as a “dummy run,” researchers can test various qualitative aspects of the study protocol. Firstly, all equipment and materials can be assessed for performance, and unforeseen glitches can be detected. For example, new data entry systems, data collection forms and questionnaires can be evaluated for comprehensiveness, ease of use and clarity, and competing testing procedures can be compared (e.g. use of a self-administered questionnaire versus a one-on-one interview) 64. Furthermore, research staff can be assessed for their consistency in administering these various tests and procedures 63. Also, pilots may expose ambiguities in recruitment criteria (i.e. potential “loopholes” between inclusion and exclusion criteria), and test practical aspects of the randomization procedure (e.g. whether, in a sealed envelope randomization, envelopes can be kept safely yet remain accessible to researchers, and whether there are unforeseen risks of surreptitious unblinding). Next, pilot experience in subject recruitment, consent, adherence, and drop-out rates, as well as subject impressions about the acceptability of an intervention can be used to develop new tactics to improve these aspects, and are of critical importance in guiding feasibility calculations for the larger trial 60. Researchers may even wish to include a protocol for qualitative evaluation of research methods in their pilot work, including interviews with subjects and research staff 63.

Aside from these specific objectives, pilot studies offer a number of ancillary benefits to researchers. Firstly, RCTs are costly and time consuming, and when an area of research is new and undefined, funding is often difficult to attract, especially for novice researchers. Pilots help to convince reviewers and funding agencies that the research is feasible and worthwhile, and that the applicant is competent and knowledgeable in this
specific area. Furthermore, the novice researcher can use the pilot project as an opportunity for training in both general and disease-specific aspects of the research process. Similarly, pilot work allows for training of all research personnel in proper administration of the intervention and proper use of the various measurement tools involved in outcome assessment. Finally, the pilot run establishes communication and collaboration between the research team, nursing, administrative staff, and any other involved allied-health care workers.

In conclusion, pilot studies are an important tool that allow for timely corrections, revisions and improvements to study design, instruments, measurements and procedures before deployment in a costlier larger scale study. Also, they empower researchers with planning experience and justification in seeking support for future studies from major funding organizations and other stakeholders.

2. Crossover design: Concepts
This study was designed as a crossover randomized controlled trial, due to the limited number of available subjects. A crossover design is one in which half of the participants are randomly assigned to start with a control period, and then switch to an active treatment period, while the other half do the opposite. Given that each subject acts as his or her own control, the possibility of confounding by covariate imbalance is minimized (assuming that covariates remain constant between treatment periods). Also, this approach significantly reduces random error, resulting in narrower confidence intervals. Most importantly for rare diseases like HPS, this design allows for a study with close to the same statistical power as a parallel group study, while requiring half the number of subjects. Furthermore, given the severity of, and poor prognosis with, HPS, severely ill subjects may not have been willing to participate in a study if they faced the risk of being randomized to a placebo arm; a crossover design guaranteed that all participants would receive both treatments. For these reasons, a crossover design was thought to be ideal for the pilot phase of this study.
Unfortunately, crossover studies are subject to several limitations, which we considered and addressed in study planning. Firstly, crossover studies require participants to be available for twice as long as would be necessary for a parallel group study, rendering such studies more susceptible to drop-outs. Since our study was particularly susceptible to drop-outs due to liver transplantation (LT) or death, we limited the duration of each treatment period to four weeks. Next, this design is susceptible to “carry-over” or residual effects, occurring when the effect of a treatment given in the first time period persists into the second time period, and distorts the effect of the second treatment. We addressed carry-over effects by randomizing the order of treatment and by implementing a four-week “washout period” between treatment courses; unfortunately, this also prolonged the duration of the study.

Next, crossover designs are susceptible to confounding bias from a “period effect” – a difference in outcome attributable to the particular period of time in which the treatment was received (e.g. during a particular season in a disease with seasonal variability). We addressed this potential bias by using the above-described two-treatment, two-period, randomized crossover design. As a result of this design, any temporal change that might favour norfloxacin over placebo in one group would favour placebo over norfloxacin in the other group, and cancel out in the treatment comparison. In this study, the only anticipated period effect was a possible gradual increase in alveolar-arterial oxygen gradient (AaDO₂) with time, due to disease progression. Based on prior literature, the median decline in partial pressure of arterial oxygen (PaO₂) in HPS patients is 6.3 mm Hg per year; assuming that an increase in AaDO₂ parallels this decline, we expected an average increase in AaDO₂ of 1.6 mm Hg over the study period due to disease progression alone. As a result of this effect, subjects could have had a higher baseline AaDO₂ in the second treatment period.

Finally, we planned to assess the magnitude and significance of both of carry-over and period effects in statistical analyses (“Planned Analysis,” see later).
3. Overview of Study Design
This was a single-university center (University of Toronto, St. Michael’s Hospital), randomized, controlled, crossover study. The intervention was exposure to norfloxacin (400 mg po bid) for a four-week period, compared to an identical placebo. All subjects received both norfloxacin and the placebo medication, with a four-week washout period between treatments and a randomized order of treatment (Figure 2).

Figure 2

Figure 2: Overview of Study Design
4. **Randomization Procedure**

Eligible subjects were recruited by the “respirology research coordinator,” a designated study coordinator based at St. Michael’s Hospital (SMH). On visit one of the study, the research coordinator provided the hospital’s research pharmacist with a unique subject identifier for documentation purposes, and the pharmacy provided a four-week supply of either norfloxacin or an identical placebo. On visit four, after initial treatment and washout phases (eight weeks total), the pharmacy provided a four-week supply of the alternate agent (crossover) (Figure 2). The pharmacist allocated treatment order according to a pre-determined, computerized block randomization scheme using randomly permuted block sizes. The pharmacist was the only person aware of the treatment allocation throughout the study.

5. **Study sample**

i. **Inclusion criteria**

a) HPS:

- evidence of portal hypertension (esophagogastric varices or portal hypertensive gastropathy identified on esophagogastrroduodenoscopy, and/or varices seen on computerized tomography scan or ultrasound, and/or splenomegaly with no other explanation, and/or ascites with no other explanation, and/or hepatic vein wedge pressure > 12 mm Hg)

- intrapulmonary vascular dilatations (IPVDs) diagnosed on contrast echocardiogram (CE)

- \( \text{AaDO}_2 > 20 \text{ mm Hg} \) on standing, room air arterial blood gas (ABG)
ii. Exclusion criteria

a) Significant pre-existing respiratory disease (“Potential Sources of Bias,” see later):
   - forced expiratory volume in one second (FEV1) < 70% of predicted, or
   - forced vital capacity (FVC) < 70% of predicted, or
   - FEV1/FVC < 0.7*

b) Inability to perform pulmonary function tests

c) Pulmonary hypertension:
   - echocardiographic estimated right ventricular systolic pressure (RVSP) ≥ 50 mm Hg, or
   - right heart catheterization mean pulmonary artery pressure > 25 mm Hg

d) Inadequate echocardiographic window to allow for accurate transthoracic CE

e) Antibiotic use within the last one month

f) Norfloxacin intolerance:
   - known allergy or intolerance to norfloxacin or other fluoroquinolones
   - history of tendon rupture associated with norfloxacin or other fluoroquinolones
   - glucose six-phosphate dehydrogenase deficiency (possibility of hemolytic reactions with norfloxacin)
   - known prolongation of the corrected QT interval (QTc), subjects taking QTc prolonging drugs, subjects with uncorrected hypokalemia, clinically significant bradyarrhythmias or acute myocardial ischemia
   - pregnancy (norfloxacin contraindicated)

g) Age < 18 or > 70

h) Expected death/transplantation within three months (treating physician’s judgment)

i) Lactose intolerance (placebo contains lactose)

j) A history of cigarette smoking within one month (may affect exhaled nitric oxide (NO) levels)

k) Current use of exogenous nitrates (may increase exhaled NO levels)

* = criterion amended to FEV1/FVC < 0.65 after start of study (“Recruitment,” Chapter 3)
6. Outcomes

The primary endpoint was the difference in the change in AaDO2 between treatment and placebo groups over the treatment course. Secondary endpoints included difference in the change in PaO2, exhaled NO, diffusion lung capacity for carbon monoxide (DLCO), six-minute walk distance (6MWD), cardiac output (CO), total peripheral resistance (TPR), RVSP (on echocardiogram), endotoxin levels, model for end-stage liver disease score (MELD score, based on creatinine, bilirubin and INR), baseline dyspnea index (BDI)/transitional dyspnea index (TDI), and Chronic Respiratory Disease Questionnaire (CRQ) scores.

i. Assessment of Outcomes (Figure 2)

Once randomized, subjects had initial assessment at visit one (zero weeks), as follows:

a) ABG (PaO2, AaDO2)

b) Pulmonary function tests: exhaled NO, DLCO, 6MWD

c) Blood pressure (BP), echocardiogram, arterial tonometry: CO, TPR, RVSP

d) Blood tests: INR, bilirubin, creatinine (MELD score), endotoxin level

e) Questionnaires: BDI/TDI, CRQ

f) Safety checks: history and physical exam by study physician, serum β-hcg (females with child-bearing potential), ekg (for QTc), complete blood count (for new cytopenias/hemolytic anemia), liver enzymes (ALT, AST, ALP, bilirubin), albumin

All of these measures were repeated after four weeks (end of first treatment course) (visit three), eight weeks (before start of next treatment course) (visit four), and 12 weeks (end of second treatment course) (visit six). In addition, ABG and exhaled NO alone were tested after two weeks (visit two) and 10 weeks (visit five) (midway through each treatment period). Finally, plasma was frozen at 0, 4, 8, and 12 weeks and stored for future measurement of variables. Candidate variables for future measurement include: endothelin-1, nitrates and nitrites, TNF-α, vasoactive intestinal peptide, serotonin, prostacyclin, atrial-natriuretic factor, glucagon, calcitonin gene-related peptide, substance P, interleukins (IL-1, IL-6), and platelet activating factor.
7. Potential Sources of Bias

i. Bias Due to Knowledge of Treatment Allocation

Bias may occur in any trial of therapy if participants and or research personnel become aware of treatment allocation and alter their behaviour or reporting in a systematic way (in accordance with their personal beliefs). In this study, though the primary endpoint (AaDO2) was objective and robust to knowledge of allocation (‘Assessment Tools and Potential Measurement Biases,’ see later), this bias could have affected certain subjective secondary outcomes such as questionnaires. This was minimized by blinding at each of the following levels: subjects, research coordinator, physicians, and personnel assessing outcomes (pulmonary function technicians, echocardiographers, lab technicians). Furthermore, the randomly permuted block randomization protected against allocation bias (‘Randomization Procedure,’ see earlier). In addition, the research coordinator was blinded to subject outcomes, such that even if she became aware of the allocation code, future allocation would not be biased by knowledge of preliminary study results.

ii. Crossover Bias

Next, there was potential for crossover bias, whereby subjects in the placebo arm might be prescribed norfloxacin (or a similar spectrum antibiotic) for other indications, or subjects in the norfloxacin arm might be noncompliant, in both cases biasing results toward the null hypothesis. Though neither of these problems could be predicted or avoided, they were careful documented; all new medication prescriptions were documented at weekly intervals by telephone or in clinic (Appendix 1: ‘Telephone Follow-Up Log’ and Appendix 2: ‘Clinic Safety Log’) and compliance was assessed with pill counts at the end of each treatment period.

iii. Bias Due to New Medical Conditions

As noted earlier, in a crossover design, treatment groups are identical by definition, minimizing the potential for confounding. However, confounding might still occur by chance, by the differential distribution between treatment and placebo arms, of new medical conditions that result in a change in the primary outcome (in this case, AaDO2). In this study, potential causes included any new primary lung disease (e.g. acute
inflammatory pneumonitis, pneumonia, pleural effusion, atelectasis, pulmonary embolism), or congestive heart failure (i.e. any cause of an increase in AaDO₂). As a result, any new medical conditions were documented thoroughly at weekly intervals (Appendix 1: “Telephone Follow-Up Log” and Appendix 2: “Clinic Safety Log”). Although none of these specifically occurred in this study, initial plans called for data from any participant who developed a condition affecting AaDO₂ to be removed from the main analysis and used for descriptive purposes only.

iv. Assessment Tools and Potential Measurement Biases

In general, measurement bias was minimized by blinding outcome assessors, as described, and by having a standard time point and protocol for outcome assessment. Important assessment tools used and their potential measurement biases are discussed below.

a) Arterial Blood Gas

The AaDO₂ (primary outcome) was determined from the ABG; this outcome was chosen because it is highly objective and robust to subject and investigator knowledge of allocation, and because it is more sensitive to changes in pulmonary gas-exchange than PaO₂ alone²⁶,⁶⁸. When ABG analysis is performed according to standard laboratory procedure within 10 minutes of arterial puncture, this test provides an accurate measure of oxygenation. However, precision of measurements and degree of intra-subject variability in AaDO₂ have never been reported, and were areas of concern. Prior studies have shown high intra-subject variability in PaO₂ in ICU subjects. Thorson, et.al. found a mean coefficient of variation (CoV) of 5.1% and a mean percentage change of 7.9% between first and last ABGs, when six ABGs were taken over 50 minutes in stable ICU subjects⁶⁹. Similarly, Sasse, el.al. demonstrated a CoV of 6.1% when 13 ABGs were performed over one hour, in ICU subjects⁷⁰. Given that AaDO₂ is less sensitive to changes in ventilation than PaO₂, we believed that the variability of AaDO₂ would be lower than these, but definitive data were lacking.
b) Chronic Respiratory Questionnaire (CRQ)

The outcome measure that was most subjective was the Chronic Respiratory Questionnaire (CRQ); this rendered it susceptible to bias if either subjects or the questionnaire administrator became aware of treatment allocation. However, this outcome was chosen because it is an important measure of health-related quality of life, and has been well validated in large numbers of subjects with various respiratory diseases, with established threshold values for a minimal clinically important difference.

71, 72.

c) Contrast Echocardiogram (CE)

There was a possibility of misclassification bias resulting from the CE used to detect the IPVDs that characterize HPS. Contrast echocardiography has been shown to be extremely sensitive for IPVDs; however, 34-47% of cirrhotic patients have IPVDs, and not all of these patients also have the elevated AaDO2 required for a diagnosis of HPS 3, 6-10. Little is known about the subset of patients with IPVDs that do not have an AaDO2 > 20 mm Hg (called “pre-HPS” patients). It is likely that IPVDs in pre-HPS patients are caused by a similar mechanism to those in HPS, but that their extent and/or severity are simply insufficient to cause gas-exchange abnormalities. However, in subjects with IPVDs, any other pulmonary disorder that causes an increase in AaDO2 to above the threshold value of 20 mm Hg would result in a diagnosis of HPS, even though the underlying cause of abnormal gas-exchange would be different than that hypothesized for HPS (“Pathophysiology and Study Rationale,” Chapter 1). Accordingly, in order to isolate “true” HPS subjects, many authors have advocated exclusion of subjects with significant concurrent cardio-respiratory disease. In this study, we minimized this misclassification bias by setting exclusion criteria for significant concurrent respiratory disease, based on the results of pulmonary function testing (“Study Sample,” see earlier).

Next, there was also a possibility of misclassification bias resulting from ambiguous test results. Echocardiographic detection of contrast bubbles in the left atrium after exactly 3 beats constitutes a “grey zone” between an intrapulmonary and an intracardiac shunt 5, 10. As a result, patients with an intracardiac shunt might occasionally be misdiagnosed with
IPVDs, and mislabeled as HPS or pre-HPS patients. As in the prior example, inclusion of these subjects in the study would bias results toward the null hypothesis, as they would not be expected to respond to norfloxacin therapy. In order to minimize these errors, we made efforts to standardize subject position and phase of the respiratory cycle at the time of contrast injection and image analysis. Furthermore, all CE’s were performed at SMH, where operators have extensive experience in this procedure, with previously demonstrated minimal inter-operator variability.

d) Exhaled NO Test
Several studies have shown poor intra-and inter-subject reproducibility of exhaled NO measurements in normal controls, cirrhotic subjects, and subjects with HPS. The only prior study employing the same measurement technique as that used in this study reported an inter-subject CoV of 13.9% among cirrhotic subjects. A smaller study that followed normal subjects over time demonstrated an intra-subject CoV of 15.8% at seven days, and 16.8% at 23 days. In our study, subject variability was minimized by standardization of body position, technique, and expiratory flow rate (since exhaled NO concentration is flow-rate dependent); also, consecutive measurements were performed until two results within 10% of each other were obtained, and these two values were averaged to yield the final value. Furthermore, inter-observer variability was minimized by standardized training of pulmonary function personnel and instrument variability was minimized by daily calibration of the NO sensor with known gas concentrations. Finally, since there is a degree of subjectivity in the determination of actual exhaled NO plateau values, as noted earlier, all pulmonary function personnel were blinded to subject treatment allocations.

Other secondary endpoints were chosen because they are also surrogate markers in support of the pathophysiologic model for HPS; the most objective and clinically relevant tools available were chosen.
8. **Norfloxacin: Safety Considerations and Assessment of Safety**

Norfloxacin was chosen as the active drug in this study because it had been the agent of choice in prior rat studies of HPS and human studies of the hyperdynamic circulation of cirrhosis (Chapter 1), and was considered to be safe based on extensive prior clinical experience in cirrhotic subjects. In fact, norfloxacin has been studied extensively for both primary and secondary prophylaxis of spontaneous bacterial peritonitis (SBP), and for prophylaxis in the context of variceal hemorrhage in cirrhotics.\(^{77-79}\)

Prior studies have suggested few adverse events with this medication. Though prophylactic norfloxacin therapy has been shown to increase the rate of emergence of quinolone-resistant stool flora, this has not been associated with any deleterious clinical consequences\(^{80-86}\). Furthermore, hypersensitivity reactions and other side-effects from this medication are rare.\(^{77-79, 85, 87}\)

In addition, the study protocol included several safety monitoring measures. Firstly, subjects received a weekly telephone call from either the research coordinator or a study physician, during which a standardized safety form was completed. This form detailed all adverse events, specific medication complications, co-interventions, and any new medical problems (Appendix 1: “Telephone Follow-up Log”). A similar form was completed by the study physician after history and physical examination at 0, 4, 8, and 12 weeks (Appendix 2: “Clinic Safety Log”). Furthermore, the protocol called for detailed reports of any adverse events to be forwarded to the research ethics board (REB) at the time of occurrence, and annually thereafter (Appendix 3: “Adverse Event and Cointervention Log”). Other safety measures included a complete blood count and ekg before and after each treatment cycle, monitoring for rare hematological side effects and QT prolongation, respectively. In addition, subjects were given an information form (Appendix 4: “Patient Information Form”) detailing important complications of drug therapy, with a 24-hour telephone number for questions or concerns. Subjects were assessed the same day or next day by a study physician if there was any suspicion of toxicity. Given these precautions, and the fact that this medication has been used safely for many years in SBP prophylaxis and remains a commonly used medication for other
indications (e.g. urinary tract infections), we did not feel that a data safety monitoring board was required.

A literature review of the overall safety of norfloxacin in cirrhotic subjects is presented in Appendix 5.

9. Recruitment Strategy
An initial recruitment strategy for this study was focused mainly toward the Toronto General Hospital (TGH) pre-liver transplantation (LT) waiting list and clinic, from where it was expected that the majority of subjects would be recruited. This clinic represents a well-defined, closely followed and tested group of subjects with a high proportion of subjects meeting the inclusion criteria. Also, subjects on the transplant list were thought to be more likely to be willing to participate, given that they often have significant symptoms as a result of abnormal gas-exchange, and treatment could directly impact their quality of life. An additional advantage was that given their close ties to the medical system, these subjects were thought to be unlikely to be lost to follow-up (<1% historical loss to follow-up rate, personal communication, Medical Director of Liver Transplantation, Dr. Les Lilly).

It is standard practice for all subjects being evaluated for LT to have a screening ABG before listing, and all results are posted on a regularly updated pre-transplant database. Given that all of these subjects have portal hypertension by definition, subjects with an AaDO₂ > 20 mm Hg on screening ABG fulfill 2/3 syndromic criteria for HPS, and were thought to have a high likelihood of having the disease. Under current clinical protocols, this database is screened every three months and all new patients with AaDO₂ > 20 mm Hg are referred to the SMH HPS Clinic, where a full assessment includes a CE to detect the presence of IPVDs and thereby confirm the diagnosis of HPS. Accordingly, the recruitment strategy was based on the current clinical referral strategy, with all patients on the TGH pre-transplant list with AaDO₂ > 20 mm Hg being referred for clinical evaluation in the SMH HPS Clinic, and potential recruitment thereafter. An
initial screen of the pre-transplant database was undertaken at the inception of the study, along with usual screening at three-month intervals thereafter.

Next, as part of the routine protocol at the SMH HPS Clinic, all evaluated subjects are asked for consent to enter the SMH HPS Database (a database containing demographic information and test results). This database was established, in part, to facilitate recruitment to studies involving HPS subjects, and the consent includes a clause that subjects may be contacted for ongoing studies. Accordingly, all recruitment to the study proceeded directly from the SMH HPS Database. Once a diagnosis of HPS was made, study eligibility was determined by the treating physician by examining the database according to inclusion and exclusion criteria. If eligible, subject contact information and an eligibility criteria checklist (Appendix 6: “Eligibility Checklist”) was relayed to the respirology research coordinator. The coordinator then contacted subjects by telephone to provide an introduction to the study, and organized a follow-up visit for further information and completion of a written consent if the subject was agreeable. All eligible subjects were recorded in an enrollment log, and in cases of non-participation, given reasons were recorded (Appendix 7: “Enrollment Log”).

Furthermore, efforts were made to attract referrals from general gastroenterology and hepatology clinics within the University of Toronto (UoT) academic hospitals, where other HPS patients might be followed (“Rare Disease Research Strategies in the Study of HPS,” Chapter 5). Also, preliminary plans for recruitment at other centers were made, in case of recruitment difficulties. In the process of preparing this protocol, peers from two other centers: the Mayo Clinic in Rochester, Minnesota, and McGill University in Montreal, Quebec, had been involved. Although both sites expressed an interest in recruiting subjects locally, in order to avoid logistic complications, we limited recruitment to our own center for this pilot phase. However, in the event of a lower-than-expected recruitment rate, or higher-than-expected drop-out rate from our center, we planned to recruit subjects from one or both of these sites. Finally, one of the study investigators (Dr. Marie E. Faughnan) has an academic appointment and maintains a
monthly clinic at the University of Montreal, which is a large liver transplantation center; this center was considered another potential source of outside recruitment, if necessary.

10. Recruitment Feasibility (Figure 3)
As noted, the recruitment strategy called for the majority of subjects to be recruited from the SMH HPS Clinic, based on clinical referral from the TGH pre-transplant waiting list, numbering approximately 350 subjects at the time of study design. A conservative estimate based on the literature suggested an expected rate of screening AaDO₂ > 20 mm Hg of 35%, and a rate of positive contrast echocardiography (and thus HPS) of 45% among those with AaDO₂ > 20 mm Hg\(^{3,7}\), for a final HPS prevalence of 16%\(^{3}\), or 56 subjects. An additional 10 subjects were expected from non-transplant clinic referrals, for a total of 66 subjects. Assuming a 30% loss due to exclusion criteria (details below), 46 eligible subjects remained. Assuming a conservative recruitment rate of 50%, 23 subjects would be available for the study, and estimating a 10% drop-out rate, 20 subjects would complete the study. Additionally, as noted earlier, should recruitment statistics have fallen short of estimations, or should drop-out rates have exceeded estimations, plans called for other centers to be contacted for recruitment (Figure 3).

In terms of specific exclusion criteria, the pre-transplant database and the relevant literature were examined prior to the study in order to assess the impact of each exclusion on recruitment. The most relevant exclusions were the following: 1) FEV1/FVC < 0.7, expected to exclude 20% of subjects (based on a chart review in 40 subjects on the TGH pre-transplant database), 2) antibiotic use within the last one month, expected to exclude 20% of subjects (mainly due to prophylaxis of SBP or variceal hemorrhage) (personal communication, Medical Director of Liver Transplantation, Dr. Les Lilly), and 3) pulmonary hypertension, expected to exclude 5% of subjects\(^{5,88}\). Given that many of these patients had overlapping exclusions, an overall 30% loss due to exclusion criteria was expected.
11. Sample Size Calculation
   i. Theory

Since the sample size was fixed in this pilot study (20 subjects), the unknown variable that was calculated was the difference in the magnitude of change in AaDO₂ between treatment groups that could be detected with a given power. An accurate calculation of this would require knowledge of the standard deviation of the change in AaDO₂ with
norfloxacin therapy; the difference in change between norfloxacin and placebo could then be tested in a paired t-test. However, given that this treatment had never been studied in this patient population, this information was not available in the literature.

However, the inter-subject variance in AaDO₂ among HPS subjects had been reported in a number of studies, and had a median value of 256 (median SD of 16 mm Hg)⁴,⁶,⁹,¹³,²⁶. Using this information, we calculated the variance of the difference in AaDO₂ between norfloxacin and placebo groups at any single point in time. Next, this information was used to calculate what magnitude of difference between four-week (end of treatment) AaDO₂ values (between norfloxacin and placebo treatments) could be detected with any given power (using a sample size calculation for a paired t-test). Assuming that the washout period was adequate, and baseline (week zero) AaDO₂ values were similar in the two treatment arms, this difference would estimate the difference in the change in AaDO₂ between week zero and week four, between treatment arms (which was the actual primary outcome).

As detailed below, this calculation required an estimation of the covariance of end-of-treatment AaDO₂ in norfloxacin and placebo, which in turn required an estimation of the correlation of end-of-treatment AaDO₂ between norfloxacin and placebo. In this case, a coefficient of correlation of 0.6 was assumed; this was believed to be conservative, given the fact that these AaDO₂ values were being measured in the same patients under two different treatment conditions, and were thus expected to be highly correlated. Figure 4 demonstrates the relationship between the assumed correlation and the calculated detectable difference for the given sample size of 20 subjects.
Figure 4: Relationship Between Assumed Correlation and Detectable Difference

Relationship between coefficient of correlation for correlation of end-of-treatment AaDO₂ between norfloxacin and placebo, and detectable difference between the treatments (sample size 20, SD 14.3 mm Hg, two-sided significance level 5%, power 80%). The dashed vertical line corresponds to an assumed coefficient of correlation of 0.6.

ii. Calculations

The basic formula establishing the variance of the difference in AaDO₂ between norfloxacin and placebo treatments (at a single point in time), is as follows:

\[ \text{Variance of the difference in AaDO}_2 \text{ (placebo – norfloxacin) = } \]
\[ \text{Variance in AaDO}_2 \text{ (on placebo) + Variance in AaDO}_2 \text{ (on norfloxacin)} - 2\times \text{Covariance (placebo and norfloxacin)} \]

Next, in estimating the covariance of AaDO₂ (placebo and norfloxacin), the following formula was applied:

\[ \text{Covariance (placebo and norfloxacin) = } \]
\[ \text{Correlation (placebo and norfloxacin) } \times \text{SD (placebo) } \times \text{SD (norfloxacin)} \]
Assuming that the variance of AaDO2 would be the same in norfloxacin and placebo treatments, and using the values noted earlier:

\[
\text{Covariance (placebo and norfloxacin)} = (0.6)(16)(16) = 153.6
\]

Applying this value to (1):

\[
\text{Variance of the difference in AaDO}_2 \text{ (placebo – norfloxacin)} = (256) + (256) - 2(153.6) = 204.8
\]

Finally, given that the SD is defined as the square root of the variance,

\[
\text{SD of the difference in AaDO}_2 \text{ (placebo – norfloxacin)} = \sqrt{204.8} = 14.3 \text{ mm Hg}
\]

Given a total of 20 subjects and a SD of the difference in AaDO2 between norfloxacin and placebo treatments (at the end of treatment) of 14.3 mm Hg, the probability was 80 percent that the study would detect a treatment difference at a two-sided five percent significance level, if the true difference between the treatments was 9.5 mm Hg. Varying detectable treatment differences with required sample sizes, at various power levels, are displayed in Figure 5.
Figure 5: Detectable Treatment Difference by Sample Size, Varying Power

Sample size required to detect varying treatment differences between norfloxacin and placebo treatments, at varying power levels. The dashed vertical line represents conditions in the current pilot study (sample size 20, SD 14.3 mm Hg, two-sided significance level 5%, power 80%, and treatment difference 9.5 mm Hg)

12. Planned Analysis
The analysis plan called for the change in AaDO₂ (primary outcome) to be compared between norfloxacin and placebo treatments with the use of a repeated measures analysis of variance test. Two treatment periods were to be defined according to chronology, and subjects were to be divided into groups according to the order in which the two treatments (placebo or norfloxacin) were received. This is illustrated below:

<table>
<thead>
<tr>
<th>Group 1:</th>
<th>A in period 1</th>
<th>B in period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2:</td>
<td>B in period 1</td>
<td>A in period 2</td>
</tr>
</tbody>
</table>

A= norfloxacin
B = placebo
Accordingly, the model was to include a term for the interaction between treatment and period (to assess for any carry-over effect), and a term for the interaction between group and treatment (to assess for any period effect).

Furthermore, exploratory analyses were to use the same model to look for differences in changes in AaDO₂ between weeks zero and two and weeks two and four, between treatment and placebo arms. These analyses would serve to generate hypotheses about the timing of any observed norfloxacin effect on AaDO₂. Similar analyses were to be performed for each of the secondary outcome variables. Also, in order to examine the proposed biological mechanism, a correlation (Pearson’s correlation coefficient) would be sought between the change in the primary outcome variable and that in the main secondary outcome variable, exhaled NO level.

Finally, a purely exploratory subgroup analysis was to compare subjects with a mildly elevated AaDO₂ (20-30 mm Hg), to those with AaDO₂ > 30 mm Hg, in order to assess for a differential treatment effect by severity of disease.
CHAPTER 3: RESULTS

1. Recruitment
   i. Screening

   After study approval, we first assessed study eligibility and recruitment among subjects already being followed at the St. Michael’s Hospital (SMH) HPS Clinic. We successfully recruited one HPS subject who was not on the TGH pre-liver transplantation (LT) waiting list, though the remainder of assessed subjects was already on the list.

   Approximately one month later, after the majority of existing SMH HPS Clinic subjects had been assessed for eligibility, testing slots and clinical time were available for new referrals. Accordingly, we undertook the planned initial screening of the TGH pre-transplant waiting list in early November 2006 (“Recruitment Strategy,” Chapter 2). In order to allow for a direct comparison to the recruitment plan (“Recruitment Feasibility,” Chapter 2), subjects who were already being followed at the SMH HPS Clinic and may have been assessed before the first screening, but were also on the TGH pre-transplant waiting list as of November, were included in the initial waiting list screening data below.

a) Initial Screening for Referrals (November 2006) (Figure 6)

In the initial screening for referrals, we identified 310 patients on the pre-transplant waiting list (11.4% less than the anticipated figure of 350, Figure 3, Chapter 2). Of these, 181 had no available ABGs on the pre-transplant database (these patients lived outside of Toronto and had ABGs done at local hospitals). Given that the study entailed six visits over a 12-week period, we decided that these patients had a low likelihood of successful recruitment, and they were not pursued (this was an additional study exclusion that was added at this point; “Recruitment,” Chapter 5). One-hundred and twenty-nine patients remained, and 55 of these (43%) had AaDO$_2$ > 20 mm Hg. These 55 patients represented 18% of the original pre-transplant waiting list, compared to an anticipated figure of 35% (Figure 3, Chapter 2). However, we discovered that another six of these patients were from outside of Toronto, and we excluded another six patients due to the presence of an alternate cause for hypoxemia (a confounder to the diagnosis of HPS; “Potential Sources of Bias,” Chapter 2). The remaining 43 patients were considered for referral to the SMH
HPS clinic; seven had LT or died before they could be seen in clinic, six were either unreachable (three), refused to be seen (two), or awaiting OHIP coverage (one), six were believed by their treating physician to be too ill to endure frequent travel for the study (“Recruitment,” Chapter 5), and seven (16%) had study exclusions (total 26/43, or 60% eliminated). This left 17 potentially recruitable patients, whom we saw for clinical evaluation at the SMH HPS clinic. On initial ABG at SMH, 9/17 (52.9%) had $\text{AaDO}_2 \leq 20$ mm Hg, excluding HPS by definition; by diagnosis, 5/17 had HPS, 4/17 had pre-HPS (positive contrast echocardiogram (CE) with $\text{AaDO}_2 \leq 20$ mm Hg), and 8/17 had cirrhosis only (negative CE). Of the five HPS patients, two died before they could be recruited and the remaining three were recruited, however one was excluded after randomization due to a new finding of right ventricular systolic pressure (RVSP) $> 50$ mm Hg on the first visit echocardiogram (an exclusion criterion).

b) Second Screening for Referrals (February 2007) (Figure 7)
Three months after the initial screening, we undertook a subsequent screening for patients newly added to the pre-transplant waiting list. Of all newly added patients, 22 patients had $\text{AaDO}_2 > 20$ mm Hg. Of these, three were discovered to be from outside of Toronto, and another four had an alternate cause for hypoxemia. Of the remaining 15 patients, two had LT before they could be seen in clinic, three were either unreachable (two), or refused to be seen (one), one was felt by his treating physician to be too ill to endure frequent travel for the study, and one had a study exclusion. This left eight potentially recruitable patients, whom we saw for clinical evaluation at the SMH HPS clinic. On initial ABG at SMH, 2/8 (25%) had $\text{AaDO}_2 \leq 20$ mm Hg, excluding HPS by definition; by diagnosis, 2/8 had HPS, 1/8 had pre-HPS, and 5/8 had cirrhosis only. Of the two HPS patients, one refused, citing language barrier and lack of help for travel, and one was recruited.

c) Third Screening for Referrals (May 2007) (Figure 8)
Three months later, we undertook another screening. Of all patients newly added to the pre-transplant waiting list, 14 patients had $\text{AaDO}_2 > 20$ mm Hg. Of these, four had an alternate cause for hypoxemia, and another four were eliminated due to a “borderline”
AaDO$_2 \leq$ 22 mm Hg on TGH ABG. This was a new exclusion criterion that was added due to limited resources for clinical assessment of patients (“Recruitment,” Chapter 5) and the observation in previous screening rounds that most patients had a lower AaDO$_2$ on SMH ABG than on TGH ABG, rendering these “borderline” subjects a very low yield group (“Intra-Subject Variability of AaDO$_2$,” Chapter 5). Of the six remaining patients, one had LT before he/she could be seen in clinic, two were too ill to endure frequent travel for the study, and two had study exclusions. This left one potentially recruitable patient, whom we saw for clinical evaluation at the SMH HPS clinic, and had a diagnosis of pre-HPS.
Figure 6

* = see “Amendments to Improve Recruitment,” later

Figure 6: Initial Screening for Referrals (November 2006)
Figure 7: Second Screening for Referrals (February 2007)
Figure 8: Third Screening for Referrals (May 2007)
ii. Amendments to Improve Recruitment

a) Change in FEV1/FVC Cutoff

In October 2006, upon assessment of subjects on the SMH HPS Database, we noted that an otherwise eligible HPS subject had an FEV1/FVC ratio of 0.68, which constituted a study exclusion (“Study Sample,” Chapter 2). However, given that this subject did not have a clinical diagnosis of obstructive lung disease, we reasoned that the FEV1/FVC cutoff of < 0.7 was too strict, unnecessarily excluding some subjects with no significant pre-existing respiratory disease. As a result, we amended the exclusion criteria to change the cutoff value for FEV1/FVC to < 0.65. After later applying this new criterion to all three screening rounds, five subjects were rendered newly eligible as a result of this amendment. However, only one of these subjects was recruited (three subjects did not have HPS or pre-HPS and one died before he/she could be recruited).

b) Inclusion of Pre-HPS Subjects

In the SMH HPS Database, pre-HPS subjects were noted to have mean exhaled NO levels similar to those of HPS subjects, and significantly higher than normal cirrhotic subjects (unpublished data, presented in poster form at the American Thoracic Society International Conference 2007, Appendix 8). On this basis, we hypothesized that IPVDs in pre-HPS likely had the same pathophysiologic basis as those in HPS (“Pathophysiology and Study Rationale,” Chapter 1). In March 2007, after the second round of screening for recruitment, very poor study recruitment was evident, and we decided to amend inclusion criteria to allow for recruitment of subjects with pre-HPS and an elevated exhaled NO level (>12.6 ppb, or one standard deviation (SD) above the mean value in a cohort of cirrhotic subjects with no IPVDs, unpublished data). Though this represented an important change in the study population, and a change in AaDO2 (primary outcome) was unlikely in these subjects (given their normal baseline levels), we believed that these subjects would provide an important “proof of principle” for the proposed pathophysiologic model.

In applying these new criteria to the first screening round, four pre-HPS subjects were identified, two were eliminated due to exhaled NO level < 12.6 ppb, and the other two
were successfully recruited (Figure 6). In the second screening round, one pre-HPS subject was eligible, but refused to participate due to inconvenience from frequent travel requirements (Figure 7). In the final screening round, one pre-HPS subject was eligible, but refused to participate due to frail health and difficulty traveling (Figure 8).

c) Recruitment from Other Centers

Original recruitment plans for the study included the possibility of recruiting subjects from the Mayo Clinic in Rochester, Minnesota, as well as from McGill University and the University of Montreal, in Montreal, Quebec, in the event of a lower-than-expected recruitment rate (“Recruitment Strategy,” Chapter 2). Upon approaching these potential collaborators in February 2007, we determined that it would take a minimum of six months for resources and approvals to be in place for study enrolment to begin locally, and that exhaled NO measurement would not be available at these centers. For these reasons, we adopted an alternate strategy which consisted of clinical assessment of eligible HPS subjects from other centers at SMH, with recruitment to the SMH HPS Database, followed by local recruitment to the study. Accordingly, subjects would travel to SMH for study visits, and visits 2 and 10 were omitted for subject convenience. In March, 2007, we amended the study to allow for subjects from other centers to be recruited under these conditions.

Given the logistics of cross-border travel and health insurance coverage, the Mayo Clinic was not considered a feasible center for this strategy. Among Montreal centers, McGill University had no HPS patients for potential recruitment, and the University of Montreal had three eligible patients; in June 2007, one of these patients was recruited.

iii. Role of Research Ethics Board Approval Time in Lost Recruitment

The initial study required ethics approvals from both the SMH and the University of Toronto (UoT) Research Ethics Boards (REBs), while subsequent amendments required only SMH REB approvals. For amendments, given that any REB-requested clarifications were provided within 0-3 days, times until approval closely approximate total REB response times. Table 2 details submission and approval dates for all REB submissions,
Table 2: REB Approval Delays and Recruitment Consequences

<table>
<thead>
<tr>
<th>REB Submission</th>
<th>Date of Submission to REB (d/m/y)</th>
<th>Date of Approval by REB (d/m/y)</th>
<th>Time Until Approval (days)</th>
<th>Lost Recruitment</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMH REB Initial Study Approval</td>
<td>17/05/2006</td>
<td>18/07/2006</td>
<td>62</td>
<td>2 subjects died and 2 subjects had LT while awaiting initial approval</td>
</tr>
<tr>
<td>UoT REB Initial Study Approval</td>
<td>19/07/2006</td>
<td>28/09/2006</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>(expedited)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMH REB Change in exclusion criteria: FEV1/FVC &lt; 0.65</td>
<td>17/10/2006</td>
<td>10/11/2006</td>
<td>24</td>
<td>1 subject died while awaiting approval</td>
</tr>
<tr>
<td>SMH REB Change in inclusion criteria: pre-HPS</td>
<td>07/03/2007</td>
<td>05/04/2007</td>
<td>29</td>
<td>None</td>
</tr>
<tr>
<td>SMH REB Change in inclusion criteria: Outside of Toronto recruitment</td>
<td>23/03/2007</td>
<td>01/06/2007</td>
<td>70</td>
<td>2 subjects at University of Montreal had LT while awaiting approval</td>
</tr>
</tbody>
</table>

2. Demographics and Baseline Characteristics

Subjects in this study (seven) were all male, had a mean age of 61 +/- 6 years, were predominantly in Childs-Pugh Classes B and C, and had predominantly alcoholic and hepatitis C-related cirrhosis (Table 3). In contrast, in the largest published HPS series to date, Swanson, et.al. described 61 HPS subjects with 51% males and a younger mean age of 50.6 +/- 12.6 years. Disease severity and etiology were similar to those in our group, with subjects predominantly in Childs-Pugh Classes B (46%) and C (51%), having
predominantly alcoholic (33%), cryptogenic (16%), and hepatitis C (13%)-related cirrhosis.

Table 3: Demographics and Baseline Characteristics

<table>
<thead>
<tr>
<th>Subject #</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Etiology of Liver Disease</th>
<th>Childs-Pugh Score (class)</th>
<th>Diagnosis Prior to Study</th>
<th>AaDO$_2$ (mmHg) (V1)$^b$</th>
<th>PaO$_2$ (mmHg) (V1)$^b$</th>
<th>Exhaled NO (ppb) (V1)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>M</td>
<td>NASH$^a$</td>
<td>8 (B)</td>
<td>HPS</td>
<td>21.8</td>
<td>87</td>
<td>19.8</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
<td>M</td>
<td>Hepatitis C</td>
<td>5 (A)</td>
<td>HPS</td>
<td>16.0$^c$</td>
<td>84</td>
<td>10.1</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>M</td>
<td>Alcoholic</td>
<td>7 (B)</td>
<td>Pre-HPS</td>
<td>13.8</td>
<td>95</td>
<td>13.4</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>M</td>
<td>Hepatitis C</td>
<td>7 (B)</td>
<td>Pre-HPS</td>
<td>11.0</td>
<td>89</td>
<td>12.6</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>M</td>
<td>Alcoholic</td>
<td>8 (B)</td>
<td>HPS</td>
<td>38.0</td>
<td>76</td>
<td>10.6</td>
</tr>
<tr>
<td>6</td>
<td>57</td>
<td>M</td>
<td>Alcoholic</td>
<td>11 (C)</td>
<td>HPS</td>
<td>16.8$^c$</td>
<td>92</td>
<td>9.6</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>M</td>
<td>Alcoholic</td>
<td>7 (C)</td>
<td>HPS</td>
<td>70.8</td>
<td>43</td>
<td>8.1</td>
</tr>
<tr>
<td>Mean +/- SD</td>
<td>61 +/- 6</td>
<td>-</td>
<td>-</td>
<td>7.6 +/- 1.8 (1A/4B/2C)</td>
<td>-</td>
<td>26.9 +/- 21.3</td>
<td>80.9 +/- 17.8</td>
<td>12.0 +/- 3.9</td>
</tr>
</tbody>
</table>

a = non-alcoholic steatohepatitis
b = value on study visit 1 (at SMH)
c = AaDO$_2$ values indicating a change from HPS diagnosis prior to study (based on a clinical ABG at SMH) to pre-HPS diagnosis on study visit 1 (“Intra-Subject Variability of AaDO$_2$,” Chapter 5)

3. Primary Outcome: AaDO$_2$

i. Graphical Exploration

No obvious trend in change in AaDO$_2$ was seen with either norfloxacin or placebo. See figures 9a, 9b, 10, and 11, next page.
**Figure 9a:** AaDO₂ vs. time, norfloxacin-placebo sequence (5 subjects). **Figure 9b:** AaDO₂ vs. time, placebo-norfloxacin sequence (2 subjects). Area between dotted lines represents the washout period.

**Figure 10:** Mean AaDO₂ vs. time, by sequence. Error bars represent standard error of the mean. Period between week 4 and week 8 represents the washout period.
ii. Mean Differences and Repeated Measures Analysis of Variance Model
The mean change in AaDO2 between week zero and week four in the norfloxacin and placebo treatment arms was 1.1 +/- 4.7 mm Hg and -6.0 +/- 12.9 mm Hg, respectively (Table 4). The mean difference in the change in AaDO2 between norfloxacin and placebo was 7.2 +/- 14.7 mm Hg over four weeks of therapy. A repeated measures analysis of variance (ANOVA) model showed no significant treatment (p=0.13), carry-over (p=0.32), or period effects (p=0.23).

In further exploratory analyses, the mean difference in the change in AaDO2 between norfloxacin and placebo was 3.7 +/- 7.2 mm Hg between zero and two weeks, and 4.2 +/- 10.2 mm Hg between two and four weeks, with p=0.15 and p=0.30 for treatment effect in a repeated measures ANOVA model, respectively.

iii. Other analyses
Among subjects with HPS at first study visit (subjects one, five, seven), the mean difference in the change in AaDO2 between norfloxacin and placebo was 16.3 +/- 14.0 mm Hg, compared to 0.4 +/- 12.7 mm Hg among subjects with pre-HPS at first study visit (subjects two, three, four, and six).
In a pre-specified subgroup analysis, HPS subjects with initial $\text{AaDO}_2 > 30$ mm Hg (subjects five and seven) had a difference in the change in $\text{AaDO}_2$ between norfloxacin and placebo of $16.8 \pm 19.8$ mm Hg, compared to $-1.5 \pm 14.8$ mm Hg in HPS subjects with initial $\text{AaDO}_2 < 30$ mm Hg (subjects one, two and six), over four weeks of therapy.

Finally, an exploratory analysis looked for delayed norfloxacin effects in subjects treated with norfloxacin first (five subjects). The mean change in $\text{AaDO}_2$ between zero and eight weeks (after four weeks of norfloxacin therapy and four weeks of washout) was $-3.6 \pm 5.2$ mm Hg, between zero and 10 weeks (after four weeks of norfloxacin therapy, four weeks of washout, and two weeks of placebo) was $-5.3 \pm 10.4$ mm Hg, and between zero and 12 weeks (after four weeks of norfloxacin therapy, four weeks of washout, and four weeks of placebo) was $-5.8 \pm 9.3$ mm Hg.

iv. Variability of $\text{AaDO}_2$

The overall (all data) mean $\text{AaDO}_2$ was $21.6 \pm 19.3$ mm Hg (coefficient of variation (CoV) 89.2%). The intra-subject CoV ranged from 2.8 to 70.2% (mean 41.8%) during the period of the study. Two of five subjects with $\text{AaDO}_2 > 20$ mm Hg calculated on an SMH ABG prior to recruitment (pre-study diagnosis of HPS) had $\text{AaDO}_2 \leq 20$ mm Hg on the first study visit ABG (technically, changing to a diagnosis of pre-HPS) (Table 3). Also, during the course of the study, another two of the remaining three HPS subjects had at least one study ABG with $\text{AaDO}_2 \leq 20$ mm Hg. As demonstrated in earlier graphs and analyses, these decreases were not attributable to norfloxacin therapy, as they occurred across both treatments. This high degree of variability in $\text{AaDO}_2$ was seen across all time points, as demonstrated in Figure 12.
Figure 12

**Box Plot of AaDO2 values vs. time (weeks on therapy).** Rectangular boxes correspond to upper and lower quartiles, middle line represents the median, plus sign represents the mean, whiskers indicate minimum and maximum data values.

4. **Secondary Outcome: Exhaled NO**

i. **Graphical Exploration**

No obvious trend in change in exhaled NO was seen with either norfloxacin or placebo. See figures 13a, 13b, 14, and 15, next page.
Figure 13a: Exhaled NO vs. time, norfloxacin-placebo sequence (5 subjects). Figure 13b: Exhaled NO vs. time, placebo-norfloxacin sequence (2 subjects). Area between dotted lines represents the washout period.

Figure 14: Mean Exhaled NO vs. time, by sequence. Error bars represent standard error of the mean. Period between week 4 and week 8 represents the washout period.
Figure 15: Mean Exhaled NO vs. time, by treatment. Error bars represent standard error of the mean. Order of treatment has been disregarded in this graph.

ii. Mean Differences and Repeated Measures Analysis of Variance Model
The mean change in exhaled NO between week zero and week four in the norfloxacin and placebo treatment arms was -0.5 +/- 5.5 mm Hg and -0.8 +/- 2.7 mm Hg, respectively (Table 4). The mean difference in the change in exhaled NO between norfloxacin and placebo was 0.3 +/- 7.2 mm Hg, over four weeks of therapy. A repeated measures ANOVA model showed no significant treatment (p=0.64), carry-over (p=0.09), or period effects (p=0.22).

iii. Correlation
Pearson’s correlation coefficient for the correlation between the change in AaDO2 and the change in exhaled NO while on norfloxacin was r = -0.05 (p=0.92).

iv. Variability of Exhaled NO
The overall (all data) mean exhaled NO was 13.6 +/- 4.5 ppb (coefficient of variation (CoV) 32.8%). The inter-subject CoV was 30.0% and the intra-subject CoV ranged from 8.2 to 29.2% (mean 16.7%).
5. **All Outcomes**

There were several other secondary outcome measures in this study (Table 4). Partial pressure of arterial oxygen (PaO₂) changed in the opposite direction to AaDO₂, as expected. Diffusion lung capacity for carbon monoxide (DLCO), a measure of pulmonary gas-exchange, improved slightly with norfloxacin relative to placebo. Six-minute walk distance (6MWD), a measure of functional capacity, also improved with norfloxacin relative to placebo. Systemic hemodynamics were measured both by echocardiography and by radial artery tonometry; both modalities demonstrated a decrease in cardiac output (CO) and an increase in total peripheral resistance (TPR) with norfloxacin relative to placebo, suggesting an improvement in the hyperdynamic circulatory state of cirrhosis. RVSP was measured to ensure that no significant pulmonary hypertension was being induced by norfloxacin, and there was a small increase in echocardiography-estimated RVSP in norfloxacin relative to placebo. Endotoxin levels were measured in order to ascertain the adequacy of selective intestinal decontamination, and though only four subjects could be compared due to loss of samples, there was a small mean decrease with norfloxacin relative to placebo. Finally, Model for End-Stage Liver Disease (MELD) score was included as a measure of the severity of liver disease, and demonstrated a small decrease on norfloxacin relative to placebo. The only effect that was statistically significantly different between norfloxacin and placebo arms (preset alpha 0.05) was the change in CO as measured by tonometry.
### Table 4: Laboratory and Echocardiographic Outcomes Over Four-Week Treatment

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Mean Baseline Values* +/- SD</th>
<th>Mean Change +/- SD on Norfloxacin</th>
<th>Mean Change +/- SD on Placebo</th>
<th>Mean Difference in Change: Norfloxacin-Placebo (95% CIs)</th>
<th>Proportion Having Difference in Change in Hypothesized Direction</th>
<th>Repeated measures ANOVA p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AaDO₂ (mm Hg)</td>
<td>26.9 +/- 21.3</td>
<td>1.1 +/- 4.7</td>
<td>-6.0 +/- 12.9</td>
<td>7.2 (-3.7, 18.1)</td>
<td>2/7 (negative)</td>
<td>0.13</td>
</tr>
<tr>
<td>Exhaled NO (ppb)</td>
<td>12.0 +/- 3.9</td>
<td>-0.5 +/- 5.5</td>
<td>-0.8 +/- 2.7</td>
<td>0.3 (-5.1, 5.6)</td>
<td>1/7 (negative)</td>
<td>0.64</td>
</tr>
<tr>
<td>PaO₂ (mm Hg)</td>
<td>80.9 +/- 17.8</td>
<td>0.3 +/- 4.6a</td>
<td>5.3 +/- 12</td>
<td>-5.0 (-15.0, 5.0)</td>
<td>3/7 (positive)</td>
<td>0.21</td>
</tr>
<tr>
<td>% predicted DLCO (%)</td>
<td>68.3 +/- 15.3</td>
<td>3.4 +/- 7.3</td>
<td>2.6 +/- 7.8</td>
<td>0.9 (-8.6, 10.3)</td>
<td>4/7 (positive)</td>
<td>0.71</td>
</tr>
<tr>
<td>6MWD (m)</td>
<td>412 +/- 77</td>
<td>14 +/- 45</td>
<td>-8 +/- 40</td>
<td>23 (-33, 78)</td>
<td>4/6 (positive)</td>
<td>1.0</td>
</tr>
<tr>
<td>CO ECHO (L/min)</td>
<td>5.2 +/- 1.8</td>
<td>-0.4 +/- 1.7</td>
<td>0.6 +/- 1.4</td>
<td>-1.0 (-2.7, 0.7)</td>
<td>4/7 (negative)</td>
<td>0.11</td>
</tr>
<tr>
<td>CO Tonometry (L/min)</td>
<td>5.8 +/- 0.8</td>
<td>-0.6 +/- 0.6</td>
<td>-0.1 +/- 0.5</td>
<td>-0.5 (-1.1, 0.2)</td>
<td>4/7 (negative)</td>
<td>0.04</td>
</tr>
<tr>
<td>TPR ECHO (dynes-sec-cm⁻⁵)</td>
<td>1410.6 +/- 536.2</td>
<td>-16.3 +/- 278.4</td>
<td>-104 +/- 400.4</td>
<td>87.4 (-236.9, 411.7)</td>
<td>4/7 (positive)</td>
<td>0.10</td>
</tr>
<tr>
<td>TPR Tonometry (dynes-sec-cm⁻⁵)</td>
<td>1186.6 +/- 286.0</td>
<td>77.3 +/- 179.7</td>
<td>70.7 +/- 158.9</td>
<td>6.6 (-156.5, 169.7)</td>
<td>3/7 (positive)</td>
<td>0.10</td>
</tr>
<tr>
<td>RVSP (mm Hg)</td>
<td>31.2 +/- 9.6</td>
<td>2.7c +/- 6.9</td>
<td>-1.0f +/- 7.0</td>
<td>2.8f (-7.8, 13.4)</td>
<td>2/5 (positive)</td>
<td>0.65</td>
</tr>
<tr>
<td>Endotoxin (EA units)</td>
<td>0.278 +/- 0.14h</td>
<td>-0.022 +/- 0.15</td>
<td>0.009 +/- 0.11</td>
<td>-0.034g (-0.27, 0.20)</td>
<td>2/4 (negative)</td>
<td>0.62</td>
</tr>
<tr>
<td>MELD Score</td>
<td>12.1 +/- 1.8</td>
<td>-0.1 +/- 0.9</td>
<td>0.3 +/- 1.1</td>
<td>-0.4 (-1.5, 0.7)</td>
<td>N/A</td>
<td>0.54</td>
</tr>
</tbody>
</table>

* = visit one values in all subjects  
a = r = 0.97 for correlation between PaO₂ at baseline and after four weeks of norfloxacin  
b = corrected for hemoglobin  
c = six subjects  
d = based on echocardiographic measures  
e = based on tonometric measures  
f = five subjects  
g = four subjects  
h = normal < 0.4 EA units, 2/7  
i = normal ≥ 0.4 EA units  

Baseline dyspnea index (BDI) and transitional dyspnea index (TDI) were included as measures of change in respiratory functional capacity with treatment (see Appendix 9 for test details). BDI and TDI scores were slightly higher on placebo than on norfloxacin (Table 5).
Table 5: BDI/TDI

<table>
<thead>
<tr>
<th>Measure</th>
<th>Mean +/- SD on Norfloxacin</th>
<th>Mean +/- SD on Placebo</th>
<th>Mean Difference +/- SD</th>
<th>Repeated measures ANOVA p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDI</td>
<td>5.3 +/- 2.7</td>
<td>5.4 +/- 2.6</td>
<td>-0.1 +/- 0.7</td>
<td>0.76</td>
</tr>
<tr>
<td>TDI (After 4-Week Treatment)</td>
<td>0.4 +/- 0.8</td>
<td>0.6 +/- 1.0</td>
<td>-0.1 +/- 1.5*</td>
<td>0.94</td>
</tr>
</tbody>
</table>

* = two subjects improved by > 1 unit, two subjects declined by > 1 unit, three subjects had no change on norfloxacin compared to placebo.

Finally, the Chronic Respiratory Questionnaire (CRQ) was included to assess health-related quality of life. This scale is divided into four domains: dyspnea, fatigue, emotional and mastery (see Appendix 9 for test details). Baseline scores in each domain were as follows: 4.3 +/- 1.4, 3.9 +/- 1.1, 5.2 +/- 1.5 and 5.0 +/- 0.7 before norfloxacin treatment, and 4.4 +/- 0.9, 3.4 +/- 1.4, 4.8 +/- 0.9 and 4.9 +/- 1.4 before placebo treatment, respectively. All four domain parameters improved slightly with norfloxacin relative to placebo (Table 6).

Table 6: CRQ

<table>
<thead>
<tr>
<th>CRQ Domain</th>
<th>Mean Change +/- SD on Norfloxacin</th>
<th>Mean Change +/- SD on Placebo</th>
<th>Mean Difference in Change Norfloxacin-Placebo (95% CIs)</th>
<th>Repeated measures ANOVA p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyspnea</td>
<td>0.6 +/- 1.0</td>
<td>0.4 +/- 0.6</td>
<td>0.2 (-0.7, 1)</td>
<td>0.87</td>
</tr>
<tr>
<td>Fatigue</td>
<td>0.2 +/- 0.3</td>
<td>0.2 +/- 0.4</td>
<td>0 (-0.3, 0.4)</td>
<td>0.90</td>
</tr>
<tr>
<td>Emotional</td>
<td>0.1 +/- 0.2</td>
<td>0 +/- 0.2</td>
<td>0.1 (0, 0.3)</td>
<td>0.35</td>
</tr>
<tr>
<td>Mastery</td>
<td>0.3 +/- 0.4</td>
<td>0 +/- 0.2</td>
<td>0.2 (-0.2, 0.7)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

a = six subjects

6. Safety, Side-Effects, Compliance and Co-Interventions
i. Safety and Side Effects
No medication-related adverse events were recorded during the study period. One subject developed an ill-defined bilateral knee and lower thigh pain one week after starting norfloxacin, but there were no signs of acute tendonitis or tendon rupture, no signs of rhabdomyolysis (CK levels were normal), and medication was continued without
unblinding. Symptoms resolved gradually over 2-3 weeks, and were not believed to be medication-related. The same subject developed diarrhea for a two-day period during placebo therapy. The subject related this to initiation of oral nystatin therapy; stool Clostridium difficile toxin assay was negative, symptoms resolved spontaneously, and unblinding was not required. Another subject developed three episodes of diarrhea during the washout period, six days after having completed his/her course of norfloxacin without side effects. This was self-limited and unblinding was not necessary.

ii. Medication Compliance
Subjects had no medication intolerability. Pill counts after each treatment cycle revealed two missed placebo pills in one subject, one missed norfloxacin pill in one subject, and eight missed placebo pills in one subject.

iii. Co-Interventions
No antibiotics or vasoactive medications were newly started during the study period, and no subjects developed acute cardiopulmonary conditions that might cause an increase in AaDO$_2$ and thereby confound the relationship between norfloxacin administration and change in AaDO$_2$ (“Potential Sources of Bias,” Chapter 2).
CHAPTER 4: RARE DISEASE RESEARCH: STRATEGIES FOR IDENTIFICATION AND RECRUITMENT OF RESEARCH SUBJECTS

1. Introduction

About 10% of all diseases are classified as rare, a diverse group of infrequent and unusual disorders which are generally poorly studied, understood and treated. The prevalence at which a disease is classified as rare varies widely (Table 7). Although each individual rare disease is uncommon, over 6000 rare diseases have been described to date, leading to a “paradox of rarity” in which “diseases are rare [but] rare disease patients are many.” Accordingly, the prevalence of any rare disease is about 6-8%, signifying an important public health concern.

Rare disease researchers will face several obstacles, many of which will be common across diseases. In this paper, we summarize difficulties in identifying and recruiting participants and suggest strategies to address these barriers. Where possible, we present the evidence base for the proposed solutions; nevertheless, the study of rare diseases is still in its early stages and many of our proposals are based on anecdote or reasoning. Accordingly, many of our suggestions are also opportunities for future methodological research in this area. We also recognize the existence of a “spectrum of rarity,” and that different strategies may be appropriate for “somewhat rare” versus “ultra rare” diseases; we highlight such concerns throughout.

Table 7: Rare Disease Definitions by Region

<table>
<thead>
<tr>
<th>Region/Authority</th>
<th>Rare Disease definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>World Health Organization</td>
<td>≤ 0.65-1/1000 inhabitants</td>
</tr>
<tr>
<td>USA</td>
<td>&lt; 200 000 cases</td>
</tr>
<tr>
<td>European Union</td>
<td>&lt; 1:2000 inhabitants</td>
</tr>
<tr>
<td>Japan</td>
<td>&lt; 1:2500 inhabitants</td>
</tr>
<tr>
<td>Australia</td>
<td>&lt; 2000 cases</td>
</tr>
</tbody>
</table>

89, 92, 93
Table 8: Examples of Rare Diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>US Prevalence (cases)¹¹³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatopulmonary Syndrome (HPS)</td>
<td>40 000 – 96 000</td>
</tr>
<tr>
<td>Cystic Fibrosis (CF)</td>
<td>30 000</td>
</tr>
<tr>
<td>Hereditary Hemorrhagic Telangiectasia (HHT)</td>
<td>60 000</td>
</tr>
<tr>
<td>Angelman’s Syndrome</td>
<td>25 000</td>
</tr>
<tr>
<td>Achalasia</td>
<td>3000</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>230*</td>
</tr>
<tr>
<td>Wilms Tumour</td>
<td>460*</td>
</tr>
<tr>
<td>Human Brucellosis</td>
<td>100-300*</td>
</tr>
<tr>
<td>Murine Typhus</td>
<td>&lt;100*</td>
</tr>
</tbody>
</table>

¹¹³ # = based on an estimated US population of 300 million persons

* = annual incidence

2. Issues in the Identification and Recruitment of Research Participants (Tables 12, 13)

i. Subject Identification

a) Disease Detection

Rare diseases are poorly recognized by both the general public and by most health care professionals. Approximately 30% of rare disease sufferers had to wait up to five years for a diagnosis¹⁰⁴ and about 40% of patients received an erroneous initial diagnosis¹⁰⁵. Rare diseases may be particularly difficult to detect in primary care settings with high patient volumes and rapid turnover, where they may be understandably diagnosed as a common disease presenting with rare manifestations¹⁰⁶. Thus, the proportion of rare disease sufferers who are undiagnosed is likely to be significant.

b) Disease Definitions

Rare disease research is sometimes hampered by the lack of a consensus disease definition. As a result of both limited and fragmented previous research, existing literature may often be inconsistent with respect to diagnostic test thresholds or syndromic criteria. For example, the definition of the “abnormal gas-exchange” criterion in HPS has varied widely, leading to commensurate variability in prevalence estimates among patients with chronic liver disease (Table 9), and concerns about the generalisability of research results across centers.
Table 9: Varying Definitions of “Abnormal Gas-Exchange” with Varying Prevalence of Hepatopulmonary Syndrome (HPS) in Chronic Liver Disease

<table>
<thead>
<tr>
<th>Definition of “Abnormal Gas-Exchange”</th>
<th>HPS Prevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO₂ &lt; 70</td>
<td>5.3%</td>
<td>107</td>
</tr>
<tr>
<td>AaDO₂ ≥15 AND PaO₂ ≤ 70</td>
<td>10%</td>
<td>11</td>
</tr>
<tr>
<td>AaDO₂ &gt; age-corrected value, defined as:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 +0.43 (age in years - 20)</td>
<td>10.4%</td>
<td>108</td>
</tr>
<tr>
<td>PaO₂ ≤ 70 OR AaDO₂ &gt; 20</td>
<td>16%</td>
<td>3</td>
</tr>
<tr>
<td>AaDO₂ ≥ 15</td>
<td>17.5%</td>
<td>9</td>
</tr>
<tr>
<td>PaO₂ ≤ 80</td>
<td>18.5%</td>
<td>109</td>
</tr>
<tr>
<td>AaDO₂ &gt; age-corrected value, defined as:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) 109.4-0.26 X age – 0.098 X (weight (kg) X100/Height (cm) – 100) – 14.1 (men)</td>
<td>24%</td>
<td>6</td>
</tr>
<tr>
<td>b) 109.4-0.26 X age – 0.073 X (weight (kg) X100/Height (cm) – 100) – 15.1 (women)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PaO₂ = partial pressure of arterial oxygen (mm Hg)
AaDO₂ = alveolar-arterial oxygen gradient (mm Hg)

Perhaps surprisingly, such considerations are also relevant for genetic disorders, which comprise an estimated 80% of rare diseases\textsuperscript{110}, since unique gene mutations do not necessarily imply a unique and consistent genotype-phenotype correlation. For example, more than 1000 alleles of the cystic fibrosis (CF) gene have been reported\textsuperscript{111}, and different mutations correspond to distinct clinical phenotypes\textsuperscript{112}. Moreover, there are patients with typical CF symptoms who lack a detectable disease-causing mutation in the CF gene, and there have been reports of the CF phenotype having been caused by an identifiable genetic factor other than a CF gene mutation\textsuperscript{113}. As a result of this genetic heterogeneity, a diagnosis of CF is still primarily clinical and biochemical rather than genetic\textsuperscript{112,113}. Even single-point mutation genetic diseases may require case definitions.

ii. Subject Recruitment

To recruit rare disease sufferers, researchers must often look across geographic, cultural, and/or institutional boundaries\textsuperscript{114}. Such efforts require added time and cost. For example, achieving comparable operating characteristics for diagnostic tests across institutions may be particularly challenging. As an additional example, rare diseases often require multi-center studies to recruit sufficient numbers, but lack the infrastructure
and resources to deal with multiple sites that may be available to typical large multi-center randomized controlled trials of common diseases.

Rare genetic illnesses may be clustered in specific subpopulations or geographic areas, however subjects may still be difficult to access. For example, the rare genetic vascular disease hereditary hemorrhagic telangiectasia (HHT) has a prevalence of only 1/16 500 in Vermont 115, but in Curacao and Bonaire, two small Dutch islands in the Caribbean, the prevalence is more than 10 times the North American average due to a founder effect 116. However, because clinical research in HHT is conducted predominantly by North American and European centers, recruitment from these islands is limited by social, cultural and geographic barriers.

3. Strategies for Identification and Recruitment of Research Participants (Tables 12, 13)
   i. Subject Identification
      a) Disease Detection
      The main strategy that rare disease organizations such as the National Organization for Rare Disorders (NORD) and the European Organization for Rare Disorders (EURORDIS) 90 have promoted to increase detection of rare diseases is to make information more accessible and to coordinate specialized care centers and networks of rare disease professionals 105, 117. While this approach generally focuses on connecting isolated patients with specialized centers after a diagnosis has been made, rare disease research should focus on reducing initial diagnostic delays 117.

   I. Detection in Primary Care
   Because primary care physicians will often encounter rare diseases first, efforts to improve early identification should be focused in their offices and clinics. However, since primary care physicians will never encounter most rare diseases, it would be unrealistic and inefficient to expect them to diagnose each condition that they encounter. The goal of increased detection in primary care should not, therefore, be diagnosis of specific diseases, but rather early recognition that a rare disease might be present and an appropriate threshold for referral to specialized services 91, 106, 118. One strategy for
identifying such patients focuses on recognizing uncommon clinical patterns that suggest a rare disease. Such patterns include atypical symptom clusters, unexpected responses to therapy, delayed or abnormal infant development \(^{118}\), and unusual family histories \(^{104,110}\). While this approach has face validity, no empiric testing of any of these strategies has been undertaken.

Another strategy for identifying rare diseases in primary care is the use of electronic diagnostic aids. While computer-assisted diagnostic systems have limited utility for common conditions \(^{119,120}\), they may prove to be a valuable tool for recognizing rare diseases, with algorithms including specific background information and symptom clusters. For example, a computer-based decision support system to identify rare infectious and parasitic diseases shortened the time from the initial patient visit to the time of correct diagnosis from 17.9 to 4.5 days for human brucellosis (p<0.01) and from 11.5 to 8.6 days for murine typhus (p<0.01), compared to historical controls \(^{121}\) (Table 8). The average physician-computer interaction time was approximately 3 minutes \(^{121}\). Further work is needed to develop more comprehensive applications and to assess the specificity of such systems in high volume settings.

There is also a need to develop innovative and effective educational interventions to increase rare disease identification in primary care. For example, a free and widely-distributed national physician association journal might be used as an instrument for targeted physician education. Also, incentive programs may be effective, such as rewarding physicians with continuing medical education (CME) credits for completing on-line modules on “how not to miss” rare diseases. The internet presents a convenient avenue for knowledge dissemination, and a qualitative assessment of primary care physicians’ attitudes toward rare disease education has suggested that an online resource with easy-to-obtain, optimized criteria for referrals would be desirable \(^{122}\).

Such interventions may also be helpful for other health care professionals, including non-physicians, specialty physicians, physicians-in-training, and even patients. Medical educational interventions could include teaching the presenting features of rare diseases, reinforcing the importance of family history, using video or computer-based simulations
when case examples are not available, and teaching health professionals how to recognize errors and biases in diagnostic reasoning. Patients with undiagnosed conditions can be educated through specific patient-oriented websites and web-based tools providing differential diagnoses for entered symptom clusters, then conveying these suggestions to their primary care physicians directly. Such interventions to improve patient participation in the consultation process are promising, but require further study \(^{123}\).

An important example of a unified approach to achieving these goals is the French national strategy for rare diseases, which uses tactics such as distinct rare disease telephone information services for patients and physicians and an online French rare disease information database called Orphanet \(^{124}\). Proposed innovations include a directory of services to guide patients without a precise diagnosis through the healthcare system based on their symptoms, the introduction of a rare diseases theme in national qualifying exams, teaching modules in medical school, residency training, and CME curricula, and education for health and social sector professionals such as nurses, therapists (occupational therapists, speech therapists, psychomotor therapists), social workers, and psychologists. A planned evaluative component to these measures will be instructive in designing future strategies to improve rare disease detection \(^{117,125}\).

II. Screening Programs

In certain diseases, recruitment may be facilitated by specific rare disease screening programs. However, several factors influence the broad applicability of a screening program, including the nature of the required testing, associated costs and availability of tests, and whether screening is clinically justifiable (e.g. in a high-risk population) or should only be done in a research context. For example, in testing protocols for genetic rare diseases, adoption of clinical testing requires verification of test accuracy, demonstration of clinical utility, and adoption of strict laboratory standards \(^{126}\). In such cases, centralized laboratories often act as common testing centers, and screening programs simply coordinate tissue collection.
Newborn screening is an excellent example of a highly successful rare disease screening initiative. In the late 1990s, a new technology called tandem mass spectrometry (MS/MS) transformed newborn screening abruptly by giving clinicians the ability to detect over 40 rare disorders with a single test \cite{1}. Such screening programs have gained wide acceptance in Europe and the United States. Importantly, the majority of these disorders are treatable if diagnosed early, and in many cases, early detection and treatment have averted morbidity or death \cite{2-5}.

b) Disease Definitions
The lack of a uniform disease definition for some rare diseases may be addressed by establishing consensus among world experts in the area. An ideal consensus statement is evidence-based, achieved through an accepted mechanism (such as voting or a Delphi process), endorsed by an appropriate specialty society, inclusive of patients in the process, evaluated against an external standard such as the AGREE instrument \cite{6}, externally reviewed, and widely published and disseminated.

The potential impact of a uniform diagnostic guideline is well illustrated in HHT. Historically, several HHT experts each used slightly different diagnostic criteria, resulting in a fragmented literature with uncertain inter-study comparability. In Table 10, we summarize five different examples of HHT diagnostic criteria used in studies prior to 2000. In 2000, a panel of international clinical and research experts developed consensus diagnostic criteria for HHT, called “The Curacao Criteria” (Table 11) \cite{7}, which were endorsed by the HHT Foundation International. These criteria are now widely adopted and used worldwide for both clinical management and research. To illustrate the reach of these criteria, of 527 HHT-publications published between 2000 and August 2007, 183 (35%) referenced the original publication of the Curacao Criteria, the most cited HHT publication during this period.
Table 10. Various Definitions of HHT Prior to Publication of The Curacao Criteria

<table>
<thead>
<tr>
<th>HHT Definition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Presence of multiple (&gt; 15) telangiectatic lesions</td>
<td></td>
</tr>
<tr>
<td>2) Either a family history of bleeding or recurrent episodes of bleeding;</td>
<td>134</td>
</tr>
<tr>
<td>a) Criterion of heredity: presence of telangiectatic lesions in at least one</td>
<td></td>
</tr>
<tr>
<td>first-degree relative</td>
<td></td>
</tr>
<tr>
<td>b) Criterion of recurrent bleeding: telangiectatic lesions at the bleeding</td>
<td></td>
</tr>
<tr>
<td>site</td>
<td></td>
</tr>
<tr>
<td>1) Recurrent epistaxis</td>
<td></td>
</tr>
<tr>
<td>2) Multiple telangiectases</td>
<td></td>
</tr>
<tr>
<td>3) Mendelian dominant transmission</td>
<td>135</td>
</tr>
<tr>
<td>1) Presence of recurrent epistaxis</td>
<td></td>
</tr>
<tr>
<td>2) Presence of telangiectases elsewhere than in the nasal mucosa</td>
<td>136</td>
</tr>
<tr>
<td>3) Family history of the disorder</td>
<td></td>
</tr>
<tr>
<td>Presence of two of the three following criteria:</td>
<td></td>
</tr>
<tr>
<td>1) Nosebleeds one to four times per month</td>
<td>137</td>
</tr>
<tr>
<td>2) Telangiectases on the skin</td>
<td></td>
</tr>
<tr>
<td>3) A mother or father who has been diagnosed with HHT, or one or the</td>
<td></td>
</tr>
<tr>
<td>other who has frequent nosebleeds</td>
<td></td>
</tr>
<tr>
<td>Presence of two of the three following criteria:</td>
<td></td>
</tr>
<tr>
<td>1) Nosebleeds at least four times a month</td>
<td>138</td>
</tr>
<tr>
<td>2) Telangiectasia of the skin</td>
<td></td>
</tr>
<tr>
<td>3) A mother or father with HHT</td>
<td></td>
</tr>
</tbody>
</table>

Table 11. The Curacao Criteria 133

The HHT diagnosis is
Definite - if 3 criteria are present,
Possible - or suspected if 2 criteria are present, and
Unlikely - if fewer than 2 criteria are present

Criteria
1. Epistaxis - spontaneous, recurrent nose bleeds
2. Telangiectases - multiple, at characteristic sites:
   - lips
   - oral cavity
   - fingers
   - nose
3. Visceral lesions - such as:
   - Gastrointestinal telangiectasia (with or without bleeding)
   - Pulmonary AVM
   - Hepatic AVM
   - Cerebral AVMs
   - Spinal AVM
4. Family history - a first degree relative with HHT
   according to these criteria
ii. Subject Recruitment

Rare disease cohorts are often based in specialty referral clinics, where clinicians develop expertise over time as a result of centralized clinical care. This unique clinical environment often serves as a fertile ground for hypothesis generation, and these rare disease experts are often in a position to propose and lead the studies that will answer the very questions that they have raised in clinical practice. Since their clinical practice facilitates recruitment through direct access to a cohort of patients, increasing the clinic referral base also increases the number of potentially recruitable subjects for a study.

a) Establishing a Rare Disease Program

Although a specialty program with a dedicated disease-specific clinic may have low initial enrollment and require initial infrastructure support, this approach has several potential recruitment advantages in addition to the obvious advantages in clinical care. First, the associated publicity can help to establish a clinician as the local expert in that rare disease, which not only increases referrals, but also increases the willingness of patients to travel for consultation. Second, in the eyes of colleagues, it legitimizes the disease as an important enough condition to merit a dedicated clinic. Third, knowledge of a clinic might increase awareness of a disease, leading to increased disease detection and referrals.

Given that manifestations and complications of rare diseases may straddle several organ systems, dissemination of a program’s activities should target multiple specialties. Tactics for physician-to-physician outreach include: 1) giving directed talks at hospital rounds, 2) leading symposia or sessions at subspecialty and general medical conferences, and 3) authoring topic reviews in association newsletters or reviews. Conventional marketing suggests that an easily remembered telephone or fax number, a simple email address, targeted mailings, pocket cards with study information, and/or a catchy web address may be of value. Although evidence for each of these interventions in the context of rare diseases is missing, studies have shown that local medical opinion leaders can successfully increase fellow physicians’ adherence to evidence-based practice for common diseases through talks and other interactions 139.
HPS is an example of a rare disease in which the majority of patients initially present with symptoms of liver disease, with the pulmonary complication remaining unrecognized \(^{14}\). As a result, the vast majority of HPS patients are undiagnosed, and followed only by hepatologists. Accordingly, respirologists studying HPS should enlist a hepatology clinical colleague and collaborator, ideally as a study co-investigator who is personally committed, facilitating referrals from other hepatologists and general access to this high-yield audience.

In conclusion, although several potential strategies to augment clinical referrals and study recruitment have been proposed, these recommendations require formal evaluation; this represents an important area for future study by rare disease research methodologists.

b) Recruiting From Multiple Centers
I. Clinical Research Networks
Clinical research networks (CRNs) initially evolved to address the need for large sample sizes and speedy trial completion in studies of cancer therapy. They consist of a network of clinical sites supported by an infrastructure for clinical and statistical coordination. By creating collaborative groups that have access to large patient populations, modern CRNs have had a major impact on the feasibility of clinical studies of rare diseases. In addition to recruitment, CRNs have other advantages, such as allowing for disease-specific diagnostic and outcome measures to be standardized and validated, often through centralized reference laboratories. CRNs can also facilitate identification of target subpopulations by creating comprehensive clinical databases and, by standardizing data collection, can increase data quality. Less measurable outcomes may include enabling cross-disciplinary interactions, providing an environment for mentorship, and raising public awareness \(^{140, 141}\).

Several examples of successful networks exist. Due to the inherent complexity and variability of CF, single center studies in CF have historically lacked adequate statistical power to detect clinically important differences \(^{141}\). To overcome this problem, the Cystic Fibrosis Foundation formed the Cystic Fibrosis Therapeutics Development
Network (CF TDN) in 1988, a network of up to 50 international clinical centers. After its first 3.5 years of existence, the network had conducted 18 clinical trials involving 900 patients, with progressively higher productivity in each year. A further example is provided by the National Wilms Tumor Study Group, which enabled the development of novel therapies for this rare childhood cancer (Table 8) through cross-disciplinary interactions between surgeons and oncologists. Research conducted through the Group has led to a survival rate of 96% for favorable cancer stages from a historical low of 20%. Similarly, rhabdomyosarcoma is a fast-growing, highly malignant tumor seen in children (Table 8); the Intergroup Rhabdomyosarcoma Study Group led five generations of successive clinical protocols involving a total of 4292 patients, tripling overall survival.

II. Rare Disease Clinical Research Network
An important resource for multi-center rare disease recruitment efforts was established by the National Institutes of Health (NIH) in 2003. The Rare Disease Clinical Research Network (RDCRN), a conglomerate of conventional rare disease CRNs who share access to geographically dispersed populations through a centralized data and technology coordinating center, consists of 10 research consortia grouped by clinical area, each directing several studies in a variety of rare diseases. Over 20 rare disease studies have begun recruiting at 50 sites in the United States and other countries, and a request for applications for new CRNs has recently been announced. Recruitment to CRN studies has been facilitated by including a coalition of Patient Advocacy Groups in the Network’s steering committee and by creating a comprehensive website that lists diseases under study along with actively recruiting trials with easy on-line registration for patients.

III. Community-Based Centers
Many CRNs have developed “satellite” research sites in small, community-based centers, which serves to both broaden the population base for recruitment, and to provide community physicians with education in specific rare disease detection along with resources not normally available to their patients. However, research infrastructure
may be lacking in research inexperienced community-based clinics and hospitals, a situation which may be challenging since privacy legislation in many jurisdictions requires community organizations to identify potential subjects and to acquire some level of consent or assent to be contacted by study staff. Previously identified barriers to recruitment from such “research novice agencies” include: 1) no prior experience in program development, 2) skepticism about the potential success of the program, and 3) lack of personnel with available time to take on a new initiative. Thus, research teams must provide maximal support for program and protocol development, education for agency personnel, adequate financial support and training, and - where possible - simplified recruitment procedures and protocols (such as flow sheets and checklists) tailored to each agency’s processes, to minimize negative impacts. Further measures could include finder’s fees, subject to approval by institutional review boards, reminder items such as pens and coffee mugs with project logos, and periodic feedback sessions about recruitment targets which are scheduled in advance.

An example of a successful community hospital recruitment program is the 1976 Eastern Cooperative Oncology Group (ECOG) initiative to involve community hospitals in multi-institutional clinical studies of cancer therapies. Over the next five years, 4506 patients were recruited to 97 different randomized controlled trials from over 100 community hospitals. Comparisons between community hospitals and member institutions showed no deleterious effects on quality of data or therapeutic outcomes, with similar ineligibility rates, protocol compliance, data submission, and objective outcome measures.

c) Direct Patient Recruitment: The Emerging Role of the Internet

Patient recruitment by direct contact allows researchers to circumvent the institutional “gatekeepers” that normally control access to potentially recruitable patients in medical clinics. These techniques are used frequently in common disease research, but may be more challenging in rare diseases. Certain direct recruitment methods, such as direct mailings or phone calls to known subjects from a previous study or to members of a rare disease patient organization are likely to be highly effective in rare diseases. While most population-based recruitment methods, such as random digit dialing, mass mailing,
and general print and broadcast media advertisements are likely of low yield, the internet has promise as a powerful recruitment tool for rare diseases. More than 75% of Americans now have access to the internet, and over 111 million Americans accessed the internet in 2004 in search of health and medical information.

I. Recruitment through Disease-Specific Internet Groups

Electronic peer-to-peer community venues, such as disease-specific internet mailing lists, news-groups, web-based discussion forums, and live chat rooms, have been used by individuals with rare diseases to share experiences and resources. These groups represent a concentration of individuals with a particular rare disease, presenting a unique and convenient opportunity for efficient recruitment. Practically, recruitment usually requires the researcher to post a study summary and contact information in cooperation with the internet site moderator, though some disease sites have dedicated research bulletin boards on which study information can be posted.

Bedgood, et.al. used the internet to identify a cross-section of patients with achalasia, a rare gastrointestinal disease (Table 8), for a survey-based study. Using common search engines, they identified six active internet-based achalasia support groups. From the largest, with 298 members, the investigators recruited 83 respondents in only one month, a sample size comparable to that in the most frequently cited existing studies. Furthermore, they achieved global outreach, with 25% of respondents from a total of eight foreign countries, and 75% from the US.

II. Recruitment through Regional Rare Disease Organizations

In the US, the National Organization for Rare Disorders (NORD) is a non-profit federation of approximately 124 voluntary health organizations and 5000 patients, healthcare providers and individuals dedicated to helping rare disease sufferers. The NORD website is an excellent resource, with an online database with group leader contact information for 2000 different rare-disease patient organizations. Furthermore, the website offers patients and physicians networking assistance and educational materials, including articles on how to start and fund-raise for a new rare disease patient.
organization. The site also offers secure online health and wellness support networks\textsuperscript{156}. Finally, the NORD website posts information about active studies along with researcher contact information for patients and their physicians, at no cost\textsuperscript{157}.

In Europe, the leading rare disease coalition is The European Organization for Rare Disorders (EURORDIS)\textsuperscript{90}. EURORDIS represents more than 260 rare disease organizations in more than 30 European countries. The EURORDIS website is another potent recruitment tool for researchers, offering information about existing online communities and/or mailing lists for rare diseases, as well as a search function which provides links to EURORDIS member patient groups, national rare disease group alliances, and a list of international support groups. Furthermore, the website offers a service to facilitate the formation of new, disease-specific online communities for European rare disease sufferers, similar to that offered by NORD\textsuperscript{158}.

Orphanet is a high-quality database of rare diseases and orphan drugs established in 1997 by the French Ministry of Health, and now run by a consortium of European partners. The Orphanet website is another valuable resource for researchers in search of a particular rare disease population, offering a unique search function for rare disease-specific clinics and experts in both public and private institutions, as well as ongoing rare disease studies and active patient support groups, all searchable by specific disease and geographical region. Furthermore, the site supports an on-line registry which allows patients to register for current and/or future studies directly\textsuperscript{124}.

An example of successful study recruitment through rare disease organizations using web-based techniques, is a study of Angelman’s Syndrome, a rare neurogenetic disorder (Table 8)\textsuperscript{159}. Researchers recruited subjects by sending study information to national Angelman’s Syndrome organizations in Canada, Australia, the United Kingdom, and the US. Measuring outcomes with a web-based or paper questionnaire, they received 75 electronic and 13 handwritten responses from participants in six different countries, with the majority arriving within the first week. Given the rarity of this syndrome, this recruitment approach proved highly successful and efficient\textsuperscript{159}.  

III. Use of a Study-Specific Website

A study information website with a dedicated patient recruitment area may act as a vehicle for “passive” recruitment. Attracting appropriate “hits” to this website depends on the use of suitable keywords (META-tags) so that the site will be appropriately recognized by internet search engines (e.g. Google, Yahoo, etc.). If designated strategically, these keywords can attract a very specific disease population to a study website at no incremental cost to the researcher. A complementary internet recruitment strategy is to have links to the study site on other related websites. Examples include specific disease foundation websites, support-group websites as discussed earlier, general rare disease websites (NORD, EURORDIS, Orphanet sites), and therapeutic information websites hosted by pharmaceutical companies.

IV. Use of Paid Advertisements on Popular Search Engines

Paid advertisements for a study website can be placed on popular search engines. For example, Google AdWords service can offer text ads with a link to a study website which appears in a “paid search results” section when Google users search for relevant terms. Because researchers are only charged when users click on their text ad, this type of a strategy has been found to be highly cost-effective. Furthermore, “snowball” approaches can be used to compliment this strategy, whereby ad respondents can be asked to send the study link to other patients.

Although internet recruitment is promising, certain disadvantages are noteworthy. First, the evidence for the efficacy of any of these interventions is lacking. Second, although internet users have the same sex and ethnicity as the general population, they tend to have higher education and income, and a younger age. This “digital divide” raises concerns about sampling bias, though conventional clinical recruitment for rare disease studies may also include a biased population of subjects who were able to both identify an expert center and had the financial means to travel there. Third, researchers may be crowded out of the internet by 2-3 billion existing web pages, and a barrage of spam e-mails facing potential subjects. As a result, rare disease researchers will have to be increasingly sophisticated in their web-based recruitment tactics, and may benefit from
the services of web-technology companies in designing strategies for internet-based recruitment. Lastly, ethical considerations in web-based recruitment and consent are poorly defined, and there are concerns about potential for erroneous data entry by subjects in studies limited to web-based data collection \cite{154, 160, 162, 163}.

4. Summary and Conclusions
Rare diseases are an important public health concern, and rare disease clinical researchers face numerous obstacles in identifying and recruiting research subjects. We have proposed several strategies to overcome these barriers, but evidence for their effectiveness is limited. In order to facilitate rare disease research, it is imperative that researchers work to develop and improve these methodologies through objective testing, seeking inter-disciplinary and inter-disease collaborations.

Earlier identification of rare diseases is particularly important for both clinical care and the advancement of research. Educational tools and aids should be designed for primary care physicians, however the uptake and testing of these interventions will be challenging given existing onerous CME demands. Primary care oriented electronic diagnostic aids have shown potential, and might be studied in RCTs using cluster randomization of a large number of primary care practices. Empowering patients to prompt physicians about rare diseases through direct internet education is also promising, and effects could be compared to a historical control (prior to website availability). Important outcomes would include time to rare disease diagnosis and resulting patient morbidity, mortality and quality of life. These outcomes could be measured through patient surveys; however, establishing rare disease-specific International Classification of Diseases (ICD) codes would greatly facilitate these measurements through health services databases, and should be considered.

Next, recruitment from multiple centers is crucial in rare diseases, and clinical research networks including community-based centers are among the few approaches with evidence of effect. Though the establishment of such networks is time and labour
intensive, their importance has been recognized by funding agencies such as the NIH RDCRN, and researchers should pursue these resources aggressively.

Finally, direct subject recruitment through the internet is an emerging modality. Currently, few examples are available, and rare disease researchers across disciplines should apply this strategy and compare its effectiveness with traditional recruitment techniques used in prior studies. Eventually, these experiences could be summarized in a systematic review in order to determine effect size, optimal methods, and the nature of any recruitment bias.

Also, rare disease researchers that have successfully recruited subjects to a clinical study should seek to maximize the data available from their clinical cohort. For example, since the natural history of many rare diseases is poorly understood, researchers involved in multi-center RCTs of new therapeutics can concurrently collect descriptive data on placebo subjects, and continue to follow the cohort after study completion. Furthermore, creative research designs such as crossover designs, internal pilot studies, Bayesian methods, and ranking and selection designs can maximize the yield of research involving small numbers.

Though individual studies focus on specific rare disease questions, research methodologies are often adaptable from one disease to another, and rare disease researchers must seek out and learn from these common features. Fellow researchers must facilitate this by collecting and reporting qualitative data regarding the research process along with conventional outcomes of their studies. Also, researchers must not only apply the tools presented in this paper, but continue to seek out creative new solutions that will contribute to this growing body of literature. With these collaborative efforts, the quality and impact of clinical research in rare diseases should continue to improve.
## Table 12: Rare Disease Subject Identification: Summary of Common Obstacles and Strategies

### i. Disease Detection

<table>
<thead>
<tr>
<th>Suggested Recommendations and Strategies</th>
<th>Relevant Examples/Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Detection in Primary Care</td>
<td>- Computer-assisted diagnostic systems are useful in rare infectious disease detection, may be adapted for broad use in rare diseases[^121]</td>
</tr>
<tr>
<td>- Education and diagnostic aids directed at primary care physicians</td>
<td>- Qualitative research suggests that an online resource with optimized criteria for referrals is desirable for primary care physicians[^122]</td>
</tr>
<tr>
<td>- Rare disease education for allied health staff and at all levels of medical training</td>
<td>- Success of newborn screening programs in detecting rare diseases[^28-31]</td>
</tr>
<tr>
<td>- Physician-prompting by patients through web-based patient-oriented rare disease diagnostic resources</td>
<td></td>
</tr>
<tr>
<td>- Instituting Screening Programs</td>
<td></td>
</tr>
</tbody>
</table>

### ii. Disease Definitions

<table>
<thead>
<tr>
<th>Suggested Recommendations and Strategies</th>
<th>Relevant Examples/Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Development and dissemination of a consensus document</td>
<td>- Broad acceptance and application of HHT Curacao Criteria[^133]</td>
</tr>
</tbody>
</table>
Table 13: Rare Disease Subject Recruitment: Summary of Strategies

<table>
<thead>
<tr>
<th>Suggested Recommendations and Strategies</th>
<th>Relevant Examples/Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Increasing the referral base</td>
<td>- Evidence for ability of “opinion leaders” to change clinical behaviour 139</td>
</tr>
<tr>
<td>- Specialty program with a devoted disease-specific clinic</td>
<td></td>
</tr>
<tr>
<td>- Physician-to-physician outreach</td>
<td>- Research successes of CF TDN 141</td>
</tr>
<tr>
<td>- Directed talks, symposia, topic reviews in regional newsletters and reviews</td>
<td>- Pediatric cancer cooperative groups’ impact on outcomes in pediatric cancers 142, 143</td>
</tr>
<tr>
<td>- Recruitment of cross-disciplinary collaborators</td>
<td>- ECOG’s success in recruiting from community centers 151</td>
</tr>
<tr>
<td>- Specialty-specific mailings</td>
<td></td>
</tr>
<tr>
<td>- Recruiting From Multiple Centers</td>
<td>- Successful internet recruitment of achalasia subjects through internet support groups 102</td>
</tr>
<tr>
<td>- Developing clinical research networks</td>
<td></td>
</tr>
<tr>
<td>- Using the Rare Disease Clinical Research Network</td>
<td>- Successful recruitment of Angelman’s Syndrome subjects from rare disease organizations 159</td>
</tr>
<tr>
<td>- Recruiting from community centers</td>
<td></td>
</tr>
<tr>
<td>- Direct Patient Recruitment</td>
<td></td>
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<tr>
<td>- Disease-specific internet groups</td>
<td></td>
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<tr>
<td>- Regional rare disease organizations</td>
<td></td>
</tr>
<tr>
<td>- Study website</td>
<td></td>
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<tr>
<td>- Paid advertisements on popular search engines</td>
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</table>
CHAPTER 5: DISCUSSION

We performed a pilot randomized, controlled, crossover study in order to guide planning for a future full-scale randomized controlled trial (RCT) of norfloxacin therapy for HPS. HPS is a rare disease with a poor prognosis and no known effective medical therapy. Previous studies in cirrhotic subjects have established the relationship between gut bacterial translocation with systemic endotoxemia, increased vascular NO levels, and systemic vasodilatation. Furthermore, elevated exhaled NO levels have been demonstrated in humans with HPS, and a rat model of HPS supports the role of endotoxemia in pulmonary NO overproduction and subsequent intrapulmonary vascular dilatation.

Though this pilot study was not designed to measure statistically significant changes in outcomes, observed trends provide numerous insights into future study design, and are discussed below.

1. Primary Outcome: AaDO$_2$

   Although sample size was too small to provide an accurate estimate of the actual treatment effect, there were no significant changes in AaDO$_2$ or PaO$_2$. However, observed mean changes in both AaDO$_2$ and PaO$_2$ were in the opposite direction to that which was hypothesized (AaDO$_2$: 1.1 +/- 4.7 mm Hg on norfloxacin and -6.0 +/- 12.9 mm Hg on placebo, PaO$_2$: 0.3 +/- 4.6 mm Hg on norfloxacin and 5.3 +/- 12 mm Hg on placebo). It is worth considering the influence of several key factors on these results.

   i. Population Studied

   Due to slow recruitment, inclusion criteria were amended to allow inclusion of pre-HPS subjects with elevated exhaled NO levels. Given their normal baseline AaDO$_2$ (≤ 20 mm Hg), we did not expect these subjects to have a decrease in AaDO$_2$ with norfloxacin therapy (the study focused instead on changes in exhaled NO in this subgroup). Prior to study initiation, only two of seven subjects had pre-HPS, five had HPS. However, two of these five had an AaDO$_2$ ≤ 20 mm Hg on the first study visit, leaving three subjects with
HPS; two of these three had at least one AaDO2 value ≤ 20 mm Hg during the course of the study, leaving only one subject with unequivocal HPS. Given this population, it is not surprising that the expected trend toward decreased AaDO2 with norfloxacin therapy was not observed.

Furthermore, all of our subjects were male. Prior studies have demonstrated an increased proportion of men amongst subjects with HPS and/or cirrhosis, likely due to a higher prevalence of hepatitis risk factors among men. Estrogen is a vasodilator that contributes to systemic capillary vasodilatation in cirrhotic subjects of both sexes and has been studied as a possible mediator of intrapulmonary vascular dilatation in HPS. Yol, et.al. used a common bile duct-ligated rat model of HPS and demonstrated that female rats with simultaneous common bile duct ligation and bilateral oophorectomy had significantly lower serum estrogen, vascular NO and extent of intrapulmonary vascular dilatation, and higher PaO2 than common bile duct-ligated female rats. They hypothesized that estradiol may dilate pulmonary vasculature by activation of nitric oxide synthase (NOS) or by calcium-channel blockade, and progesterone may have a similar effect through 3’, 5’ cyclic AMP. In a human study, Aller, et.al. demonstrated elevated progesterone and estradiol levels in a sex balanced cohort of 19 HPS subjects, compared to other cirrhotics. These data suggest a possible role for sex hormones in the pathogenesis of HPS, which may imply between-sex differences in disease pathogenesis and progression. Given that any such differences might also mitigate the effect of norfloxacin on NO production and intrapulmonary vascular dilatation formation, the lack of female subjects in this study is a potential weakness.

ii. Differences Compared to the Rat Model of HPS

The effects of norfloxacin on HPS have only previously been studied in a rat model with important differences from human HPS. Firstly, common bile duct ligation with resulting biliary cirrhosis is the only insult that causes HPS in rats, with chemical cirrhosis (e.g. thioacetamide-induced) or portal hypertension alone (e.g. portal vein ligation) being ineffective. In contrast, human HPS can be caused by biliary cirrhosis, alcoholic cirrhosis, or portal hypertension in the absence of cirrhosis. Given that
none of the subjects in this study had biliary cirrhosis, HPS pathophysiology may have been different than that studied in the rat model. Furthermore, norfloxacin has been shown to mitigate the development of HPS in rats immediately after common bile duct ligation, whereas our study evaluated the effect of norfloxacin on established HPS; it is possible that certain preventable changes during disease development may not be reversible once disease is established.

2. Variables Supporting the Pathophysiologic Model
Several measures addressed the validity of the proposed pathophysiologic model.

i. Exhaled NO
There was no substantial difference in change in exhaled NO levels while subjects were on norfloxacin as compared to placebo, and a large variability in levels was observed (-0.5 +/- 5.5 versus -0.8 +/- 2.7 mm Hg, respectively). The inter-subject coefficient of variation (CoV) of 30.0% in our study was larger than the 13.9% reported in a stable cohort of 26 cirrhotic subjects (20 male) using the same technique (expiratory flow rate 12L/min) 40. However, exhaled NO output demonstrated an inter-subject CoV of 22.1% in nine HPS subjects 42, and exhaled NO concentration at an expiratory flow rate between 5-15 L/min demonstrated an inter-subject CoV of 30.0% in five HPS subjects 43, suggesting that HPS subjects may have more variable results. Also, mean intra-subject CoV over the three-month study period was 16.7%. In subjects with elevated pulmonary NO and resulting regional intrapulmonary vascular dilatations 175, measured exhaled NO levels are influenced by the degree of ventilation and perfusion of lung units with intrapulmonary vascular dilatations, which may vary with minor changes in patient position and intra-abdominal pressure. This high degree of both inter- and intra-subject variability indicates that much larger numbers would be required to show statistically significant treatment effects on this surrogate outcome.

Pre-HPS subjects in the study were required to have an elevated exhaled NO level, defined by NO >12.6 ppb (one SD above the mean value determined in a cohort of 11 cirrhotic subjects without intrapulmonary vascular dilatations tested at SMH, unpublished
data). However, given that the outcome of interest in HPS subjects was AaDO₂, inclusion criteria did not require HPS subjects to have an elevated exhaled NO level, and only three of seven total subjects had an exhaled NO >12.6 ppb at the time of the first study visit. This is an important pilot RCT finding, but also an important study weakness. These low baseline NO levels challenge the biological mechanism of HPS which we proposed to modify with norfloxacin administration (i.e. if NO levels were not elevated, there is no support for bacterial translocation-mediated NO production and vasodilatation, and antibiotics may not have attenuated pulmonary vasodilatation).

Though reports from other centers ⁴¹-⁴³ as well as our own prior data ¹⁷⁶ have demonstrated significantly higher exhaled NO levels in HPS subjects than in other cirrhotics, it is possible that mechanisms other than NO-mediated intrapulmonary vascular dilatation dominate in the subset of HPS subjects with normal exhaled NO levels. Several NO-independent candidate mediators of intrapulmonary vascular dilatation formation have previously been proposed, including adrenomedullin ¹⁷⁷, glucagon ¹⁷⁸, calcitonin-gene related peptide ¹⁷⁸, atrial natriuretic factor ²⁸, ¹⁷⁸, platelet activating factor ²⁸, ¹⁷⁸, arachidonic acid metabolites ¹⁷⁸, and interleukins-1 and 6 (IL-1, IL-6) ¹⁷⁸.

In a future full-scale RCT, an additional inclusion criterion requiring an elevated exhaled NO level for all HPS subjects may select for the population that is most likely to respond to norfloxacin. However, this desire for a more selected patient population must be balanced against limited availability of exhaled NO testing and more restrictive inclusion criteria, which may limit enrollment and impair generalisability of findings to all patients with clinical HPS.

ii. Endotoxin

Endotoxin levels decreased with norfloxacin compared to placebo (-0.022 +/- 1.5 versus 0.009 +/- 0.11 EA units). Endotoxin has been shown to be elevated in subjects with cirrhosis ³⁰, ³¹ and was included as a surrogate marker for the effectiveness of selective intestinal decontamination. However, at baseline, mean endotoxin level across all subjects was 0.278 +/- 0.14 EA units (normal < 0.4 EA units) and only two of seven
subjects had an elevated baseline level (≥ 0.4 EA units). These results were unexpected, and given that the hypothesized drop in endotoxin with selective intestinal decontamination depended on elevated baseline endotoxin levels, it is not possible to accurately assess the success of norfloxacin in achieving selective intestinal decontamination.

Prior studies used the quantitative chromogenic Limulus lysate assay to measure endotoxin concentration, whereas our study used a newly developed assay that relies on complement-mediated responses to measure endotoxin activity (EA Units). Due to the significant inter-individual variability in response to endotoxin, this assay is considered to be only semi-quantitative, and inter-subject comparisons are limited. Furthermore, this assay has never before been applied in a cirrhotic population, and differences in protein synthesis and/or immune function in this population may have impacted its accuracy, precision, and/or expected normal values.

Though the chromogenic Limulus lysate assay would thus be the preferred test in a future full-scale RCT, the availability of this test is limited. However, because endotoxemia mediates pulmonary intravascular macrophage sequestration and inducible nitric oxide synthase induction through tumor necrosis factor alpha (TNF-α) 47, 50, 179, TNF-α may be another marker of the effectiveness of intestinal decontamination. Accordingly, an Enzyme-Linked ImmunoSorbent Assay (ELISA) will be used to measure TNF-α levels in batched future analyses of frozen plasma collected from study patients at baseline, four, eight and twelve weeks. This measure has yet to be validated in humans with cirrhosis, and these test results will help to determine its potential role as a more widely available measure of selective intestinal decontamination than endotoxin, for use in a future full-scale RCT of norfloxacin.

3. **Other Variables**
   i. **Hemodynamics**
   
   Cardiac output (CO) decreased with norfloxacin compared to placebo, by both echocardiography (-0.4 +/- 1.7 L/min versus 0.6 +/- 0 1.4 L/min) and arterial tonometry
(-0.6 +/- 0.6 L/min versus -0.1 +/- 0.5 L/min). Total peripheral resistance (TPR) decreased with both norfloxacin and placebo by echocardiography, but increased with norfloxacin compared to placebo, by both echocardiography (-16.3 +/- 278.4 versus -104 +/- 400.4 dynes-sec-cm\(^{-5}\)) and arterial tonometry (77.3 +/- 179.7 versus 70.7 +/- 158.9 dynes-sec-cm\(^{-5}\)). These hemodynamic parameters were included as a measure of the effectiveness of norfloxacin therapy \(^{180}\) in improving the hyperdynamic circulatory state of cirrhosis. Findings confirmed those of a prior placebo-controlled crossover study of norfloxacin in 14 male subjects with alcoholic cirrhosis over a four-week treatment course in which authors used invasive thermodilution in order to maximize the accuracy of CO and TPR measurements \(^{37}\).

Agreement between thermodilutional and tonometric CO measurements are fair \(^{181}\), whereas thermodilutional and echocardiographic CO measurements have poor to fair correlation \(^{182}\). Also, echocardiography may underestimate stroke volume \(^{182}\) and CO, and is more variable \(^{183}\). If hemodynamics are to be measured in a future full-scale RCT, these test characteristics, as well as the higher costs of echocardiography will have to be balanced against the limited availability of tonometry, in choosing the ideal test.

ii. Diffusion Capacity

Diffusion capacity increased slightly with norfloxacin compared to placebo, though there was large variability (3.4 +/- 7.3 versus 2.6 +/- 7.8 percent predicted). Diffusion capacity has been shown to be decreased in all cirrhotic subjects, and further decreased in HPS subjects \(^{3}\). Potential explanations include an increased membrane component of resistance to diffusion due to intrapulmonary vascular dilatations \(^{184}\) or frank structural changes such as increased collagen deposition in alveolar capillary basement membranes \(^{27}\). The latter theory is supported by reports showing persistently decreased diffusion capacity in HPS subjects after liver transplantation, despite an improvement in oxygenation \(^{26,27}\). A significant improvement on norfloxacin in a full-scale study would support the role of intrapulmonary vascular dilatations in the etiology of low diffusion capacity, leading to an improved understanding of gas-exchange abnormality in HPS.
4. **Functional Measures**

i. **Six-Minute Walk Distance**

Six-minute walk distance (6MWD) improved in four of six subjects, with a mean difference in change of 23 m between norfloxacin and placebo; the minimal clinically important difference for the six-minute walk distance is 54 m (95% CI: 37-71 m)\(^{185}\). Only one subject was unable to perform this test, due to hip arthritis. Other subjects did not report any extra-pulmonary factors limiting their ability to walk, suggesting that this was a useful measure of respiratory capacity. Though variability was high, this is an important functional measure that should be considered for inclusion in a future full-scale RCT.

ii. **Questionnaires**

Baseline dyspnea index (BDI) scores were similar in norfloxacin and placebo (5.3 +/- 2.7 versus 5.4 +/- 2.6), suggesting good intra-subject reproducibility, as previously demonstrated in chronic obstructive pulmonary disease (COPD)\(^{186}\). Transitional dyspnea index (TDI) was positive (indicating improvement) in both norfloxacin and placebo; mean improvement was larger with placebo, but responses were highly variable (0.4 +/- 0.8 versus 0.6 +/- 1.0, respectively). We used the BDI and TDI to measure changes in dyspnea-related functional capacity (Appendix 9). The minimal clinically important difference for TDI is 1 unit\(^{186-188}\); two subjects improved by > 1 unit, two subjects declined by > 1 unit, and three subjects had no change on norfloxacin compared to placebo. In a future full-scale RCT, correlations between BDI and baseline values of 6MWD and AaDO\(_2\), and between TDI and changes in these parameters could be measured to further assess the construct validity of these tools in HPS\(^{189}\).

Mean changes in Chronic Respiratory Disease Questionnaire (CRQ) domain scores ranged from 0–0.6 with either norfloxacin or placebo, with differences in change favoring norfloxacin. The minimal clinically important difference for each domain score is 0.5\(^{72}\) and Dyspnea was the only domain with a clinically important change, improving 0.6 units with norfloxacin, consistent with previous studies demonstrating that Dyspnea is the
most treatment-responsive domain in various chronic lung diseases \(^7\). This instrument is promising and may be further explored in a full-scale study.

5. **Duration of Therapy**
   The choice of a four-week treatment period in this study was based on two previous studies. Rasaratnam, et.al. demonstrated significant changes in hemodynamic parameters with a four-week course of norfloxacin \(^3\) and Rabiller, et.al. demonstrated mitigation of the severity of HPS with norfloxacin therapy over five-weeks in common bile duct-ligated rats \(^4\). However, the former study was limited by a failure to report the calculated difference in change between norfloxacin and placebo treatments, and the second study by differences between human disease and the rat model.

Post-liver transplantation (LT) results may provide an index of the expected time course of improvement in gas-exchange in HPS subjects. Arguedas, et.al. reported a pre-operative PaO\(_2\) < 75 mm Hg in 16 of 17 HPS subjects, improving to > 75 mm Hg in 12 of 17 subjects by six months post-LT, and in 17 of 17 subjects by 12 months post-LT \(^6\) and Schiffer, et.al. reported normalization of AaDO\(_2\) six months post-LT in six of six surviving subjects \(^1\). In a report of 19 subjects by Taille et.al., an improvement in PaO\(_2\) by > 5 mm Hg was seen in 3 subjects at 1 month, 11 at 3 months, 15 at 6 months, and 18 at 12 months post-LT \(^2\).

Assuming that the proposed pathophysiologic model is valid, given that gut bacterial translocation likely stops very soon after liver transplantation, it is unlikely that the effect of norfloxacin on gas-exchange abnormalities would be seen any sooner than that of liver transplantation. Furthermore, certain authors have suggested that NO-mediated vascular dilatation in HPS may lead to long-term vascular remodeling which would require months to reverse \(^9\), \(^9\).

In our study, subjects treated with norfloxacin first (five subjects) had a mean decrease in AaDO\(_2\) of 3.6 +/- 5.2 mm Hg after 8 weeks, 5.3 +/- 10.4 mm Hg after 10 weeks, and 5.8 +/- 9.3 mm Hg after 12 weeks, suggesting a possible delayed effect. Though longer
treatment was considered infeasible in this crossover design, these various findings suggest that a future full-scale parallel group trial of norfloxacin should test a six-month treatment course.

6. Recruitment
i. Discrepancies Between Planned and Actual Initial Recruitment Algorithms (Figure 3, Chapter 2 and Figure 6, Chapter 3)

Poor recruitment was a major problem in this study, with only five of the planned 20 HPS subjects successfully recruited and studied (two pre-HPS subjects were studied, but pre-HPS patients were not initially to be included). Several sources of lost recruitment were identified. Firstly, a total of 187 of 310 (60%) pre-liver transplantation listed patients were from outside of Toronto. Due to the frequency of visits in our crossover design, we initially believed that it would be impractical to recruit these patients, and added this as an exclusion criterion at the time of screening. Eventually, we did amend the study to include subjects from other centers, however this applied only to subjects who had diagnosed HPS and met eligibility criteria. In contrast, routinely screened cirrhotic patients had a very low likelihood of having HPS (see later), and for this reason, these patients from outside of Toronto were not pursued. In order to minimize loss due to subjects residing outside of major centers, a future full-scale RCT of norfloxacin therapy must: 1) employ more specific screening criteria (to justify evaluation and recruitment of outside subjects) (“Study Implications of a New Definition for Abnormal Gas-Exchange,” see later) and 2) decrease the frequency of study testing (this may be easier in a parallel-group study) and/or limit outcomes to those that can be tested locally in referring centers, in order to alleviate the travel burden.

Next, seven of 43 (16%) local subjects with AaDO2 > 20 mm Hg and no other identifiable cause for hypoxemia either died or had liver transplantation before they could be seen in clinic, and among eligible HPS subjects, two of five died before they could be enrolled in the study (Figure 6, Chapter 3). These lost subjects reflect the overall severity of illness in the cirrhotic population. Another unforeseen consequence of this severity of
illness was that six of 43 (14%) subjects were considered by their treating physicians to be too ill to endure the frequent travel and extensive testing required in the study, adding an additional limit to recruitment. Similarly, two eligible pre-HPS subjects that refused to participate cited objections to frequent travel, with a reference to frail health. A future study design must account for the impact of underlying cirrhosis-related fatigue and deconditioning on recruitability, and must also attempt to minimize these losses by limiting 1) the number of subject visits, 2) subject travel time, and 3) subject testing time. One interesting avenue would be to develop methods and resources for measuring simple outcomes at home in this small group of subjects.

Furthermore, resource limitation in our SMH HPS clinical program acted as a bottleneck for research recruitment. Both limited clerical resources and limited availability of testing caused delays between subject identification by screening and subject assessment at the SMH HPS Clinic. In response, we improved both clinical and research efficiencies by prioritizing bookings for the study, increasing clinician availabilities, and limiting testing to HPS diagnostic tests only. In a future trial, research resources must be allocated to complement routine clinical care protocols, which may often be limited in a rare disease model. In HPS, this is particularly important in order to ensure prompt assessment of potentially eligible subjects at the time of study initiation, to minimize lost recruitment due to death or liver transplantation before assessment.

Finally, among the 17 subjects with AAaDO2 > 20 mm Hg on TGH ABG that were evaluated at the SMH HPS Clinic, nine of 17 (53%) in fact had AAaDO2 ≤ 20 mm Hg by the time they were assessed at SMH, excluding HPS by definition. In response to this, the cutoff for gas-exchange abnormality triggering referral from TGH was increased from AAaDO2 > 20 mm Hg to > 22 mm Hg at the time of the third screening. This inter-center variability was an important cause of discrepancy in recruitment (“Intra-Subject Variability of AAaDO2,” see later).
ii. Role of Research Ethics Board Approval Time in Lost Recruitment
Research ethics board delays in the overall study approval and specific amendment approvals also contributed to a significant loss of recruitable subjects. A total of three eligible subjects died and four had liver transplantation during waiting periods for approvals (Table 2, Chapter 3). If approvals were received sooner, four of seven would have been able to complete the three-month study before death or liver transplantation. A future RCT will require a multi-national, multi-center approach, and lost recruitment due to research ethics board-related delays at multiple institutions must be considered in feasibility calculations.

7. Intra-Subject Variability of AaDO$_2$
As noted, AaDO$_2$ was highly variable both within screened subjects between centers and within recruited subjects between study time-points. These effects were closely examined in larger clinical cohorts followed at our center.

i. Inter-Center Variability
Inter-center variability was determined in 16 cirrhotic subjects who had an ABG and clinical assessment at TGH and SMH within one year, with no identifiable intercurrent change in cardiopulmonary status (unpublished data). Among these subjects, 11 of 16 (69%) had a drop in AaDO$_2$ at SMH compared to TGH; mean drop was 8.4 +/- 12.7 mm Hg. Seven of 15 subjects (47%) who had AaDO$_2$ > 20 at TGH had AaDO$_2$ $\leq$ 20 at SMH, by definition excluding HPS. The most important cause for this discrepancy was likely the difference in ABG protocols between centers. At SMH, all ABGs are done after 20 minutes at rest in order to eliminate any possible effect of exertional desaturation, while at TGH, there is no mandated rest period. Furthermore, SMH ABGs are performed in the standing position, while TGH ABGs are performed in the seated position. Though HPS subjects have increased intrapulmonary shunting with a resulting higher AaDO$_2$ in the standing position compared to the supine position $^{175}$, differences between standing and seated positions are unknown. Furthermore, non-HPS subjects might have a higher AaDO$_2$ in the seated position due to basilar lung compression - an effect which is likely exaggerated by abdominal obesity and/or ascites $^{192,193}$. Another possible contributor
could be a systematic measurement bias between ABG analyzers at each center, though measurement characteristics have not been compared. A future study must account for any differences in ABG protocol and analyzers between referring centers and study centers, and technique must be harmonized in order to improve reproducibility.

ii. Intra-Center Variability

As a result of intra-center (SMH) intra-subject variability, two subjects with a diagnosis of HPS based on an SMH ABG prior to the study were re-classified as pre-HPS on study visit one, and two other HPS subjects had at least one AaDO2 \( \leq 20 \text{ mm Hg} \) during the course of the study, leaving only one unequivocal HPS subject in the study.

Intra-center variability was assessed in a cohort of 29 cirrhotic subjects at the SMH HPS Clinic having two or more ABGs within a one-year period with simultaneous clinical assessment revealing no change in cardiopulmonary status (unpublished data; abstract submitted to the American Thoracic Society International Conference 2008, Appendix 10). There were a total of 93 ABGs (mean three ABGs/subject), with a mean intra-subject difference between maximum and minimum AaDO2 of 13.1 +/- 9.3 mm Hg (range 1.1 - 36.7 mm Hg). Over one year, eight of 29 (28%) subjects fluctuated either above or below the threshold AaDO2 value of 20 mm Hg. The overall mean of intra-subject CoV for AaDO2 was 29.8% +/- 30.0%. This compares to a CoV of 8.0% +/- 5.4% for PaO2. Since the CoV is defined as the SD/mean and the majority of subjects had a higher mean PaO2 than AaDO2, it is not surprising that the CoV was larger for AaDO2. However, mean intra-subject SD’s were also slightly higher for AaDO2 than for PaO2 (6.8 mm Hg and 6.0 mm Hg, respectively).

Biological variation including regional changes in ventilation and perfusion matching due to transient atelectasis or variable flow through intrapulmonary vascular dilatations likely contribute to intra-subject variability in AaDO2, however additional artifactual variation may be produced by assumptions in the ideal alveolar gas equation.
AaDO₂ is defined as:
\[ \text{PAO}_2 - \text{PaO}_2 \]
where \( \text{PAO}_2 \) is the partial pressure of alveolar oxygen estimated by the ideal alveolar gas equation, and \( \text{PaO}_2 \) is the measured (ABG) partial pressure of arterial oxygen.

The ideal alveolar gas equation is as follows:
\[ \text{PAO}_2 = \text{FIO}_2 \cdot (\text{Pb-Ph}_2\text{O}) - \frac{\text{PACO}_2}{\text{RER}} \]
where \( \text{FIO}_2 \) is the fractional concentration of oxygen in inspired air, \( \text{Pb} \) is the barometric pressure, \( \text{Ph}_2\text{O} \) is pressure exerted by water vapour, \( \text{PACO}_2 \) is the partial pressure of alveolar CO₂ (approximated by partial pressure of arterial CO₂ measured in ABG), and RER, the respiratory exchange ratio, is the ratio of CO₂ exiting the lungs to O₂ entering pulmonary capillaries from the alveolar space at the time of ABG acquisition, assigned an estimated value of 0.8.

Using \( \text{PaCO}_2 \) as an index of ventilation, this equation adjusts \( \text{PAO}_2 \) with changes in ventilation to eliminate the effect of ventilation on the calculated AaDO₂. Accordingly, while changes in \( \text{PaO}_2 \) reflect changes in both ventilation and gas-exchange, changes in AaDO₂ reflect changes in gas-exchange exclusively, suggesting that it might be both a less variable and a more appropriate measure of gas-exchange than \( \text{PaO}_2 \). However, RER in this equation adds variability, and conventional use of an RER value of 0.8 may introduce error. A previous cross-sectional analysis of ABG results in liver transplant-listed cirrhotic subjects at TGH revealed that 33 of 248 (13%) subjects had a physiologically impossible “negative” calculated value for AaDO₂, suggesting that the conventionally applied RER value of 0.8 was incorrect in at least a subset of patients. Subsequent RER measurements during ABG acquisition in six cirrhotic subjects at SMH confirmed a wide intra-subject variability in RER (largest intra-subject range 0.85-1.06 over a one hour period) (unpublished data). It is likely that a similar or greater intra-subject variability exists across serial ABGs over time, and that the erroneous use of a fixed RER contributed to the observed intra-subject variability in AaDO₂. Furthermore, these data demonstrated a wide inter-subject range in RER (0.7 – 1.06) at the time of
arterial puncture, suggesting that the use of a standard value leads to errors in calculation, and may be inappropriate for inter-subject comparisons as well.

8. New Proposed Definition for Abnormal Gas-Exchange

Given the above noted threats to the validity of AaDO₂ as a measure of gas-exchange, the marked intra-subject variability of this measure, and the fact that it has not been associated with any clinical outcomes, it is a poorly suited variable by which to define the “abnormal gas-exchange” criterion for HPS, for both clinical and research purposes. In contrast, PaO₂ is measured directly, was less variable in our population, and has been shown to be clinically relevant. Among cirrhotic subjects with a positive contrast echocardiogram, 1) those with a PaO₂ < 70 mm Hg exhibit dyspnea more commonly, 2) long-term mortality is elevated in those with a PaO₂ ≤ 60 mm Hg without liver transplantation, 3) a PaO₂ ≤ 60 mm Hg is associated with increased peri-liver transplantation mortality, and 4) there is a trend toward increased long-term post-liver transplantation mortality in those with a PaO₂ ≤ 60. Thus, PaO₂, and particularly a PaO₂ ≤ 60 mm Hg, has been associated with important clinical outcomes. In defining a “clinically relevant” diagnostic PaO₂ cutoff, given that PaO₂ declines at an estimated rate of 6.3 mm Hg/year and > 70% of subjects await liver transplantation for ≥ 1 year, we propose to include a “buffer” above this value in order to ensure early disease recognition, appropriate monitoring, and timely liver transplantation. Accordingly, we propose a PaO₂ diagnostic cutoff value of < 70 mm Hg for HPS, which is already employed practically by experts to identify “clinically significant” HPS. An additional criterion of AaDO₂ > 20 mm Hg should also be included, to rule out rare cases of hypoventilation with normal gas-exchange. Finally, because the moderate intra-subject variability of PaO₂ still renders disease definition susceptible to ambiguity due to fluctuation above and below this threshold in “borderline” cases, we propose an additional requirement that at least two separate ABGs performed on different days demonstrate the defined PaO₂ and AaDO₂ abnormalities (Table 14).
Table 14: New Proposed Definition for “Abnormal Gas-Exchange” in the Syndromic Definition of HPS

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<tbody>
<tr>
<td>1.</td>
<td>PaO$_2$ &lt; 70 mm Hg</td>
</tr>
<tr>
<td>2.</td>
<td>AaDO$_2$ &gt; 20 mm Hg (partial pressure of alveolar oxygen calculated with the ideal alveolar gas equation)</td>
</tr>
<tr>
<td>3.</td>
<td>Criteria (1) and (2) fulfilled on 2 separate ABGs performed on different days</td>
</tr>
</tbody>
</table>

Schenk et al. 7 performed ABGs and CEs in 98 cirrhotic subjects and compared the population defined by an AaDO$_2$ > 20 mm Hg with that defined by a PaO$_2$ < 70 mm Hg. As demonstrated in Table 15, 30 of 80 (38%) subjects with AaDO$_2$ > 20 mm Hg had a positive contrast echocardiogram (CE) and 15 of 18 subjects (83%) with AaDO$_2$ ≤ 20 mm Hg had a negative CE. Thus, a total of 45 of 98 (46%) subjects had concordant results, in which both diagnostic criteria were consistent with either a diagnosis of HPS or no diagnosis of HPS, and 53 of 98 (54%) had discordant results, in which one diagnostic criterion suggested HPS, but the other did not. In contrast, Table 16 demonstrates that 14 of 15 (93%) subjects with PaO$_2$ < 70 mm Hg had a positive CE and 64 of 83 subjects (77%) with PaO$_2$ ≥ 70 mm Hg had a negative CE. Thus, 78 of 98 subjects (80%) had concordant results and only 20 of 98 (20%) had discordant results. Though there is no gold standard test for HPS, our diagnostic certainty is much higher in cases of concordance, and a PaO$_2$ < 70 mm Hg cutoff clearly yields more concordant cases. This suggests that if a gold standard test for HPS was available, a PaO$_2$ < 70 mm Hg cutoff would yield a higher specificity than an AaDO$_2$ > 20 mm Hg cutoff. Though sensitivity might be lower with a PaO$_2$ < 70 mm Hg cutoff, given that we have identified this as the clinically relevant threshold, we do not anticipate many deleterious consequences of “missing” potential HPS cases with PaO$_2$ ≥ 70 mm Hg. Also, among subjects with discordant results, the number of subjects with a negative “abnormal gas-exchange” criterion and a positive CE was higher using the PaO$_2$ < 70 mm Hg cutoff (Table 16) than the AaDO$_2$ > 20 mm Hg cutoff (Table 15). However, it is unlikely that it is clinically important to detect isolated positive CEs in cirrhotic subjects (pre-HPS), given that no deleterious consequences of intrapulmonary vascular dilatations (IPVDs) in
the absence of gas-exchange abnormalities have been described (IPVDs are seen in 34-47% of cirrhotics)\textsuperscript{3,6-11}.

Table 15: Concordance of AaDO\textsubscript{2} > 20 mm Hg Cutoff for Positive CE\textsuperscript{7}

<table>
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<tr>
<th>Contrast Echocardiogram</th>
<th>+</th>
<th>-</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>AaDO\textsubscript{2} &gt; 20 mm Hg</td>
<td>30</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>AaDO\textsubscript{2} ≤ 20 mm Hg</td>
<td>3</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>65</td>
<td>98</td>
</tr>
</tbody>
</table>

Table 16: Concordance of PaO\textsubscript{2} < 70 mm Hg Cutoff for Positive CE\textsuperscript{7}

<table>
<thead>
<tr>
<th>Contrast Echocardiogram</th>
<th>+</th>
<th>-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO\textsubscript{2} &lt; 70 mm Hg</td>
<td>14</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>PaO\textsubscript{2} ≥ 70 mm Hg</td>
<td>19</td>
<td>64</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>65</td>
<td>98</td>
</tr>
</tbody>
</table>

Overall, this proposed cutoff defines a more homogeneous population with demonstrated poor outcomes, providing better ethical justification for investigation of new therapies such as norfloxacin. A universal acceptance of this definition of HPS would harmonize research efforts, enabling comparisons between findings at different centers and facilitating a future multi-center study. In order to achieve this, a consensus must be reached among world experts in HPS and effectively disseminated to fellow researchers and clinicians (“Strategies for Identification and Recruitment of Research Participants,” Chapter 4).

9. Study Implications of a New Definition for Abnormal Gas-Exchange

Use of the proposed new definition for abnormal gas-exchange in the clinical definition of HPS would have several important implications for a future parallel-group RCT of norfloxacin therapy for HPS.
i. Feasibility

In our study, only one of seven recruited subjects had a PaO$_2$ < 70 mm Hg (Table 3, Chapter 3), and use of this cutoff would decrease the proportion of cirrhotic patients diagnosed with HPS on the TGH pre-liver transplantation list from 11% to 4.5%. Similar prevalences were reported by Krowka, et.al. (5.3%)$^{107}$ and Schiffer, et.al. (10%)$^{111}$ using this cutoff. However, the likely improved specificity of the proposed criteria might balance these losses by 1) justifying evaluation and recruitment of subjects residing outside of study centers, and 2) diminishing the number of unnecessary clinical evaluations, thereby alleviating possible bottlenecks in clinical assessment and reducing recruitment losses due to death or liver transplantation before assessment. Conversely, loss to follow-up would be a greater concern with these new criteria, since a higher proportion of subjects would have a PaO$_2$ < 60 mm Hg, at which threshold an increased liver transplantation priority is awarded, and time to transplantation becomes shorter$^{17}$. If a longer therapeutic period of six months was tested, this problem would be further compounded. Overall, this suggests that a future study using the new proposed criteria for abnormal gas-exchange would require a multi-center, multi-national effort and use of novel recruitment techniques in order to be feasible.

ii. Analysis and Sample Size

The new proposed definition has important implications for analysis and corresponding sample size required in a future RCT. Given its clinical relevance, PaO$_2$ would be the preferred primary outcome variable in a future study. In terms of planned analysis, given that baseline and end-of-treatment PaO$_2$ values were found to be highly correlated within subjects (Table 4, Chapter 3), power would be maximized by adjusting end-of-study PaO$_2$ values for baseline values, by either 1) comparing the difference in change in PaO$_2$ between groups directly, or 2) using an analysis of covariance (ANCOVA), in which a single regression equation solving for end-of-study values would include baseline values as a covariate$^{196}$. Sample sizes for RCTs measuring continuous variables have often been calculated using the standard deviation (SD) of the outcome measure itself, and comparing end-of-study results only, assuming similar mean baseline scores between groups$^{196}$. However, in cases where pre- and post- treatment values are highly correlated
(such as with PaO₂), these sample size calculations yield erroneously large sample sizes, adding unnecessary cost and time to the planned study. This is particularly problematic in a rare disease such as HPS, in which recruitment is the main threat to study feasibility. Given this, the difference in change in PaO₂ between groups should be used as the outcome measure in a sample size calculation for a future study, and this will require an estimate of the standard deviation of the expected change in PaO₂ with treatment.

10. Rare Disease Research Strategies in the Study of HPS

i. Strategies Employed

We undertook several of the rare disease research strategies discussed in Chapter 4 before and during this study (Tables 17, 18). In terms of subject identification, we launched evidence-based finger-oximetry screening programs at a three different sites prior to study initiation, with variable results (Table 17). In addition to logistic difficulties, many physicians remained unconvinced of the utility of screening, and extensive staff education and frequent reminders were not always effective. Several other hepatologists were approached to set up similar screening programs, however these physicians were unwilling to perform screening themselves and allied health care workers were not available for this task. These examples underscore the difficulty of establishing new screening programs for rare diseases. The new proposed definition for HPS would guide new oximetric screening criteria and might facilitate a culture change by adding clinical relevance to identified cases.

In terms of subject recruitment, we increased our referral base by establishing a specialty program with a dedicated HPS clinic at SMH, and undertook physician-to-physician outreach initiatives such as rounds and presentations aimed at general, respirology and hepatology audiences, and a topic review in a regional newsletter distributed to respirologists and allied respirology health care staff in Ontario (Table 18). We also set up screening programs and recruitment channels at other centers and established a dedicated HPS Clinic at the University of Montreal.
ii. Strategies to Consider in a Future Study

Given the requirements for a multi-center future RCT, a clinical research network (CRN) for HPS research should be established, linking multiple centers through a central infrastructure. Our attempts to create a Canadian CRN have been limited by a lack of dedicated collaborators. However, efforts will now focus on collaborating with US centers in order to establish HPS in one of the Rare Disease Clinical Research Network (RDCRN) consortia. Future efforts must also seek to involve community gastroenterologists, who may enable access to a large population of cirrhotic subjects.

Finally, direct patient recruitment strategies have not yet been developed. Currently, no HPS-specific internet support group exists, and regional rare disease organizations have not focused on this disease. Also, direct web-based maneuvers such as a study website or paid advertisements have not been attempted. Two separate HPS patients in the US learned of this study on the open-access clinical trial registration site “ClinicalTrials.gov,” and recently contacted us regarding eligibility. This underlines the potential of web-based direct recruitment strategies in this disease, and a future multi-center study should include specific plans to use this avenue.

Table 17: Strategies for HPS Subject Identification

<table>
<thead>
<tr>
<th>Strategies</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Screening Programs</td>
<td></td>
</tr>
<tr>
<td>- Evidence-based finger-oximetry screening programs 198, 199</td>
<td>- 0 referrals received; low screening yield, screening abandoned by staff within 2 months</td>
</tr>
<tr>
<td>- SMH Hepatitis C-HIV co-infection clinic (August 2006) 200, 201</td>
<td></td>
</tr>
<tr>
<td>- TGH pre-liver transplantation clinic (September 2006)</td>
<td>- 5 referrals received; screening performed routinely but criteria for referral often ignored, likely only severe abnormalities being referred</td>
</tr>
<tr>
<td>- General Hepatology clinic (October 2006)</td>
<td>- 2 early referrals received; screening no longer performed routinely as hepatologist unconvinced of utility and concerned about risks of ABG</td>
</tr>
</tbody>
</table>
Table 18: Strategies for HPS Subject Recruitment

<table>
<thead>
<tr>
<th>Strategies</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increasing the referral base</strong></td>
<td></td>
</tr>
<tr>
<td>- Specialty program/dedicated SMH HPS Clinic (2004)</td>
<td>- 3 referrals received from inpatient medical wards (SMH)</td>
</tr>
<tr>
<td>- Physician-to-physician outreach:</td>
<td>- Increased willingness of physicians and allied health team at TGH pre-liver transplantation clinic to participate in screening program</td>
</tr>
<tr>
<td><strong>General:</strong></td>
<td>- Enabled collaboration for research and establishment of screening program at McGill University</td>
</tr>
<tr>
<td>- Grand Rounds presentation (SWCHSC(^1), SMH X 2)</td>
<td></td>
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<tr>
<td>- Multi-organ Transplant Rounds presentation (TGH)</td>
<td></td>
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<tr>
<td><strong>Hepatology:</strong></td>
<td></td>
</tr>
<tr>
<td>- Canadian Association for the Study of Liver Disease Meeting presentation (April, 2006)</td>
<td>- Improved cooperation between TGH and SMH, increased study legitimacy for subjects, facilitating recruitment</td>
</tr>
<tr>
<td>- University of Toronto Hepatology Research Day HPS liver transplantation outcomes research presentation (April, 2007)</td>
<td></td>
</tr>
<tr>
<td>- Mailed letters announcing SMH HPS Clinic (2004) and study (2006) to all hepatologists in the University of Toronto academic hospitals</td>
<td></td>
</tr>
<tr>
<td>- Medical Director of TGH liver transplantation Program recruited as study co-investigator</td>
<td></td>
</tr>
<tr>
<td><strong>Respirology:</strong></td>
<td></td>
</tr>
<tr>
<td>- University of Toronto Respirology Grand Rounds presentation (November 2005)</td>
<td>- 1 referral from a community-based respirologist</td>
</tr>
<tr>
<td>- Ontario Better Breathing Conference presentation (February 2007)</td>
<td>- In press</td>
</tr>
<tr>
<td>- Ontario Thoracic Reviews (newsletter) lead article HPS review</td>
<td></td>
</tr>
<tr>
<td><strong>Recruiting From Multiple Centers</strong></td>
<td></td>
</tr>
<tr>
<td>- University of Montreal Respirology Rounds Presentation</td>
<td>- Led to establishment of a local screening program</td>
</tr>
<tr>
<td>- University of Montreal and McGill University screening protocols for HPS</td>
<td></td>
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<tr>
<td>- HPS clinic at the University of Montreal (monthly, as of January 2007)</td>
<td>- 9 referrals received</td>
</tr>
</tbody>
</table>

\(^1\) = Sunnybrook and Women’s College Health Services Center
11. Conclusions

This pilot study was designed to address several issues relevant to the planning of a future full-scale RCT.

Firstly, we evaluated the hypothesized pathophysiologic model for HPS linking systemic endotoxemia with increased pulmonary vascular NO production and abnormal gas-exchange. Though observed trends in systemic circulatory changes supported findings of a previous study demonstrating improvements in hyperdynamic circulation with norfloxacin\(^{37}\), findings did not support parallel decreases in pulmonary nitric oxide levels and improvements in gas-exchange. However, this pilot study did not have the power to detect these changes. Furthermore, the endotoxin measurement technique used in the study was likely inaccurate in the given population, and exhaled NO was found to be a highly variable measure. Given that these are crucial variables in the proposed pathophysiologic model, no conclusions can be drawn regarding validity of the model. Use of a chromogenic Limulus lysate endotoxin assay and inclusion of a larger number of subjects (given the variability of exhaled NO) will be required to address this question, and these measures may be incorporated in a full-scale study of the effects of norfloxacin on gas-exchange in HPS.

This pilot study also offers important design insights into both study duration and choice of outcomes. First, observed trends along with liver transplant outcome data suggest that a future study should test a six-month rather than a one-month treatment period. Next, AaDO\(_2\) has been used widely in the syndromic definition of HPS, however this outcome has no known clinical significance, and we found it to be both highly variable and of questionable validity due to assumptions required in its calculation. Given these factors, we suggest 1) using PaO\(_2\) as a primary outcome in a future study, and 2) using a PaO\(_2\) cutoff as part of a new definition of “abnormal gas-exchange” in HPS (Table 14), for both clinical and research purposes. Finally, novel parameters such as six-minute walk distance, baseline and transitional dyspnea indices, and the Chronic Respiratory Disease Questionnaire appear to be useful in this population as well, and may be further explored in a future study.
Next, in terms of feasibility, no significant adverse events or compliance problems were noted in the study, however we identified recruitment as a major obstacle. We determined that limited mobility and severity of illness of the study population were important contributors to lost recruitment; study procedures must be simplified and limited in order to encourage participation, and home assessment of outcomes should be considered. Furthermore, study design must address possible resource limitations in clinical HPS programs which may limit assessment for study eligibility and enrollment. Overall, a future full-scale study will likely require recruitment through a multi-center clinical research network for HPS and use of innovative direct patient recruitment strategies.

Finally, this pilot study aimed to measure the magnitude and standard deviation of the change in the outcome measure in order to guide sample size calculations for a full-scale study. However, these data are now of limited value given two important differences between the proposed future study and the current pilot study - 1) a longer treatment duration (six months), and 2) a different population (according to the new proposed diagnostic criteria). One option to address this issue would be to conduct a separate pilot study in order to determine these values under the newly proposed study conditions. However, rather than devoting additional resources to this isolated question in a second pilot study, we would recommend designing a full-scale study in accordance with the numerous lessons already learned in this pilot study, and using an internal pilot study to address sample size calculations. This way, the pilot population would be highly representative of the final study population, pilot subjects would not be rendered ineligible for the larger study, and extra costs and delays due to the pilot testing phase would be eliminated.

The study of HPS is characterized by many of the difficulties that are often encountered in other rare disease research, and this pilot study suggests multiple solutions that will be required in the design and conduct of a future randomized controlled trial of norfloxacin therapy for HPS.
REFERENCES


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154. Hamilton RJ, Bowers BJ. Internet recruitment and e-mail interviews in qualitative studies. Qualitative Health Research 2006;16:821-35.

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APPENDIX 1

Telephone Follow-Up Log
**Phone/ Follow Up Log**

Participant Name:
Phone Number:
  Home:
  Work cell:
  Pt. preferred number and time to call:

<table>
<thead>
<tr>
<th>Week</th>
<th>Date</th>
<th>*Note any side – effects/complications/new medications</th>
<th>Comment/Action Taken</th>
<th>Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
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<td>3</td>
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<tr>
<td>11</td>
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</tbody>
</table>

Specifically Ask:
  A - new rash
  B - throat or tongue swelling C - rupture (a complete tear) of any tendon
  D - any new infection (e.g. pneumonia, as diagnosed by another physician)
  E - any new diarrhea
  CM - New Medication (list in Con/Med sheet)
  O - Other:
APPENDIX 2

Clinic Safety Log
**Clinic Safety Log**

Participant Name: 

**Complete at WEEK 2, 4, 8, 10 and 12 (and every week in-between by phone)**

<table>
<thead>
<tr>
<th>Week</th>
<th>Date</th>
<th>*Note any side – effects/complications/new medications</th>
<th>Comment/Action Taken</th>
<th>Initials</th>
</tr>
</thead>
</table>

Specifically Ask:

A - new rash  
B - throat or tongue swelling  
C - rupture (a complete tear) of any tendon  
D - any new infection (e.g. pneumonia, as diagnosed by another physician)  
E - any new diarrhea  
CM - New Medication (list in Con/Med sheet)  
O - Other:
APPENDIX 3

Adverse Event and Cointervention Log
ADVERSE EVENT and COINTERVENTION LOG

REB # 06-120
P.I: Dr. Faughnan

NEW MEDICAL EVENT OR COMPLICATION:

NEW MEDICATIONS OR OTHER COINTERVENTIONS:

Start Date: End Date:

Recovery:
  o Recovered with sequelae:
  o Recovered without sequelae:

Patient seen by: Please attach any doctor’s notes.
  o Family doctor ___________________________
  o Specialist: ___________________________
  o Emergency Room:_______________________

Was the Patient hospitalized: □ yes □ no
If yes: Date of Admission: ______________
       Date of discharge: ______________

Study Medications: Patient’s Baseline date:

<table>
<thead>
<tr>
<th>Name</th>
<th>Start Date</th>
<th>Stop date</th>
<th>route</th>
<th>dosage</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Relationship to intervention:
  o Related
  o Likely
  o Unlikely
  o Not related

Comment: Report Summary. Include unblinding if unblinding was necessary
(Please sign and date at end)
NOTE:

Contact study coordinator for the following events (these events result in an immediate appointment for the patient (if assessed by phone), unblinding of the study physician, and immediate study withdrawal):

- hypersensitivity reaction to norfloxacin
- any tendon rupture
- new hemolytic anemia, leukopenia, neutropenia and thrombocytopenia 
  prolongation of the QT interval (QTc)
- pregnancy
- infection with a quinolone-resistant organism
- Clostridium difficile diarrhea
APPENDIX 4

Patient Information Form
Patient Information Form

“A pilot Study of Norfloxacin For Hepatopulmonary Syndrome (HPS)”

Because this is a “blinded” study, neither you, nor your treating physician will know whether you are taking the actual medication (norfloxacin), or the placebo (a pill with no active ingredients), during any given phase of the study.

Though norfloxacin has been used safely for many years in other conditions (e.g urinary tract infections), there are certain side-effects or complications that we would like to be aware of, and we will therefore be contacting you weekly by phone (on the weeks that you will not be seen in clinic as part of the study protocol). Also, there are certain symptoms for which we would like you to contact us **immediately**, and these may result in us asking you to stop taking the drug:

- new rash
- throat or tongue swelling (please go to the closest emergency room if this occurs)
- rupture (a complete tear) of any tendon
- any new infection (e.g. pneumonia, as diagnosed by another physician)
- any new diarrhea

Should any of these side effects occur, please call 416-864-5431, and ask for the HPS study physician to be paged. You will be able to speak to a study physician 24 hours/day, and he/she will schedule an appointment with you for the same or next day, if necessary.

Also, this drug may cause birth defects, and should not be taken by pregnant patients; please alert us immediately if you may be pregnant
APPENDIX 5

Norfloxacin Safety Considerations
Norfloxacin belongs to the quinolone group of antibiotics, which have extensive activity against enterobacteriaceae, without affecting anaerobic colonic flora – an effect referred to as selective intestinal decontamination. For these properties, quinolones have emerged as the antibiotics of choice for the prophylaxis of spontaneous bacterial peritonitis (SBP), and as noted in Chapter 2, norfloxacin has been studied extensively in cirrhotic patients, for both primary and secondary prophylaxis of SBP, and for prophylaxis in the context of variceal hemorrhage. Given that these subject populations are very similar to our own, they provide considerable insight into the possible complications and safety concerns relating to norfloxacin administration; accordingly, results in these groups have been reviewed.

Firstly, several studies have examined the prevalence of quinolone-resistant fecal flora in subjects receiving norfloxacin prophylaxis (400 mg po qd). In a prospective study, Aparicio, et.al. demonstrated that 13/30 (43.3%) consecutively hospitalized subjects who had received norfloxacin prophylaxis had quinolone-resistant (QR) E.coli in stools. Furthermore, 6/14 (42.8%) subjects who were newly started on norfloxacin prophylaxis developed QR E.coli in a mean time of 18.5 +/- 9.8 days. Similar findings were reported by Dupeyron, et.al., also in a hospitalized cohort. Conversely, a study by Gines, et.al. examined 40 outpatients receiving norfloxacin for secondary prophylaxis of SBP, and demonstrated no sustained QR gram-negative strains in the stool, and no infections due to QR bacteria during a mean follow-up of seven months. Other studies have reported that the prevalence of QR bacteria in stool samples from the general healthy population is as high as 24%, and the proportion of infections caused by QR bacteria among cirrhotics not taking norfloxacin prophylaxis is as high as 29%.

The actual incidence of infections caused by QR organisms among subjects taking prophylaxis has been well evaluated. In the longest prospective study of norfloxacin prophylaxis, Novella, et.al. compared 56 subjects receiving regular norfloxacin prophylaxis to 53 subjects receiving prophylaxis only during hospitalization, for a mean period of 43 +/- 3 weeks. Though 9/10 E.coli strains isolated from subjects on regular prophylaxis were found to be QR (vs 4/11 in the other group, p<0.05), the overall
incidence of infections caused by QR bacteria was not significantly different between groups (19.6% and 15%, respectively)\textsuperscript{85}. Next, a large retrospective series by Llovet, et.al. compared SBP in subjects with and without norfloxacin prophylaxis. This study showed a shift in the microbiology of SBP infections in the norfloxacin-treated group, toward gram-positive organisms. However, the clinical course and outcome of SBP was similar in both groups, and none of the isolates in subjects taking norfloxacin prophylaxis showed in vitro resistance to quinolones\textsuperscript{84}. Conversely, in a prospective study of bacterial infections in cirrhotic subjects by Fernandez, et.al., 50% of culture-positive SBP episodes in subjects taking norfloxacin prophylaxis were caused by quinolone-resistant gram-negative bacilli, compared to 16% of episodes in those not taking prophylaxis. However, disease evolution and clinical outcomes were again similar in subjects with QR versus quinolone-sensitive infections, and in subjects on norfloxacin prophylaxis versus those on no prophylaxis\textsuperscript{80}.

In conclusion, though results have been conflicting, more recent data suggests that the emergence of QR stool flora is a significant phenomenon among prophylactic norfloxacin users. However, reports of the incidence of infections due to QR organisms have been inconsistent, and studies have not shown any differences in the clinical outcomes of subjects on norfloxacin prophylaxis compared to those on no prophylaxis.

Next, the reported incidence of norfloxacin-related side-effects in the context of long-term prophylactic therapy has been consistently low, and specific side-effects have varied, as follows: 3/40 (severe diarrhea, granulocytopenia, urticaria)\textsuperscript{79}, 2/53 (hypersomnia and nausea)\textsuperscript{78}, 0/48\textsuperscript{77}, 1/56 (oral candidiasis)\textsuperscript{85}. In addition, hypersensitivity reactions have occasionally been reported with norfloxacin use (for this and other indications), including a case report of necrotizing granulomatous hepatitis with eosinophilia in a subject with no history of prior liver disease\textsuperscript{87}. In this latter report, when norfloxacin sales figures up to 1990 were cross-referenced with a registry of adverse reactions to this medication, authors estimated that the incidence of norfloxacin-related liver damage is one case per five million daily doses of 800 mg\textsuperscript{87}. Finally, rare potential adverse events include tendon rupture, Clostridium difficile diarrhea (a risk with
any antibiotic therapy), hemolytic anemia (in subjects with glucose-6 phosphate dehydrogenase deficiency), and arrhythmia (in subjects with a prolonged QTc) (“Study Sample,” Chapter 2).
APPENDIX 6

Eligibility Checklist
A pilot Study of Norfloxacin For the Hepatopulmonary Syndrome
Inclusion/Exclusion CRF

Subject ID:

Inclusion criteria:

1a. AaDO2 ____ (must be > 20) mm Hg

Date of ABG:

2. + Contrast Echo

Date:

3. Portal Hypertension
   Identified on:
   EGD
   CT or U/S
   Isolated splenomegaly
   Ascites (SAAG > 1.1 mg/dL)
   hepatic vein wedge pressure > 12 mm Hg

Exclusion criteria:
TICK BOX IF PATIENT DOES NOT HAVE:
- FEV1 < 70% pred
- FVC < 70% pred
- FEV1/FVC < 0.7
- inability to perform PFTs
- ECHO RVSP ≥ 50 mm Hg/RHC MPAP > 25 mm Hg
- inadequate ECHO window
- antibiotic use within the last 1 month
- norfloxacin intolerance:
- allergy or intolerance to norfloxacin or other fluoroquinolones
- history of tendon rupture associated with fluoroquinolones
- G6PD deficiency
- QT > 50% of R-R interval
- QT prolonging drugs
- uncorrected hypokalemia
- known bradyarrhythmias
- acute myocardial ischemia
- pregnancy

- age < 18 or > 70
- expected death/transplantation within 3 mo
- lactose intolerance
- smoking within the last 1 month
- current use of exogenous nitrates (may increase exhaled NO levels)

**CYP1A2 substrates:** Norfloxacin may increase the levels/effects of CYP1A2 substrates (strong inhibitor).

**CYP1A2 Substrates:** Acenocoumarol; Alosetron; Aminophylline; Betaxolol; ClomipRAMINE; Clozapine; Cyclobenzaprine; Dacarbazine; Doxepin; Duloxetine; Flutamide; Fluvoxamine; Guanabenz; Mexiletine; Mirtazapine; Olanzapine; Pimozide; Propranolol; Ramelteon; Ropinirole; Ropivacaine; Tacrine; Theophylline; Thiothixene; Tizanidine; Trifluoperazine

**CYP3A4 substrates:** Norfloxacin may increase the levels/effects of CYP3A4 substrates (moderate inhibitor).

Example **CYP3A4** substrates include cyclosporine, benzodiazepines, calcium channel blockers, mirtazapine, nateglinide, nefazodone, sildenafil (and other PDE-5 inhibitors), tacrolimus, and venlafaxine. Selected benzodiazepines (midazolam and triazolam), cisapride, ergot alkaloids, selected HMG-CoA reductase inhibitors (lovastatin and simvastatin), and pimozide are generally contraindicated with strong CYP3A4 inhibitors (norflox is a moderate only – so OK).
The following is a list of QT prolonging drugs:

Abarelix; Amiodarone; Amitriptyline; Apomorphine; Arsenic Trioxide; Bretylium; ChlorproMAZINE; Cisapride; Clarithromycin; Disopyramide; Dofetilide; Dolasetron; Domperidone; Droperidol; Erythromycin; Flecainide; Fluoxetine; Flupenthixol; Foscarnet; Gatifloxacin; Halofantrine; Haloperidol; Ibutilide; Imipramine; Indapamide; Isradipine; Levofloxacin; Loxapine; Mesoridazine; Moxifloxacin; Octreotide; Pentamidine; Pimozide; Probucol; Procainamide; Propafenone; Quetiapine; Quinidine; Ranolazine; Sotalol; Sparfloxacin; Telithromycin; Thioridazine; Thiothixene; Voriconazole; Ziprasidone; Zuclopenthixol
APPENDIX 7

Enrollment Log
**Enrollment Log**

*A Pilot Study of Norfloxacin For the Hepatopulmonary Syndrome*

<table>
<thead>
<tr>
<th>Name</th>
<th>ID NUMBER</th>
<th>IC Date</th>
<th>Enrollment date</th>
<th>Nonparticipation Reason</th>
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APPENDIX 8

Exhaled Nitric Oxide in Hepatopulmonary Syndrome, Pre-Hepatopulmonary Syndrome and Cirrhosis

S. Gupta, MD and M.E. Faughnan, MD, MSc, St. Michael’s Hospital, University of Toronto, Canada

Introduction

• Hepatopulmonary Syndrome (HPS) is defined by a triad of:
  1) Liver dysfunction or portal hypertension
  2) Intrapulmonary vascular dilations
  3) Alveolar-arterial gradient (AaDO₂) > 20 mm Hg

• Nitric Oxide (eNO) is thought to be an important mediator of the intrapulmonary vascular dilations (IPVDs) that characterize HPS

• Exhaled NO (eNO) levels have been shown to be elevated in HPS subjects compared to other cirrhotics and normal controls

• However, eNO levels have not been reported in cirrhotic patients with IPVDs but an AaDO₂ < 20 mm Hg - a condition we have termed "pre-hepatopulmonary syndrome" (pre-HPS)

Methods

• Consecutive patients referred to a specialized clinic for possible HPS were prospectively recruited

• The following were studied:
  • 14 HPS subjects
  • 9 pre-HPS subjects
  • 15 cirrhotic subjects with no IPVDs

• All patients had contrast echocardiography (CE) for detection of IPVDs, arterial blood gas (ABG) for determination of AaDO₂, DLCO, and eNO measurement at a constant expiratory flow of 200 ml/s (FE,No200)

• Subjects were compared to 47 healthy, non-smoking, non-asthmatic volunteers assessed with FE,No200

• Inter-group differences were tested using two sample T-tests

• Pearson’s correlation coefficient was calculated for the correlation between AaDO₂ and DLCO in HPS and pre-HPS subjects

• Significance level was set at 0.05 for all tests

Results

Figure 1: Distribution of Child’s-Pugh Classes Within Groups

Table 1: Demographics and Results in Each Group

<table>
<thead>
<tr>
<th>Variable</th>
<th>HPS (n=14)</th>
<th>Pre-HPS (n=9)</th>
<th>Cirrhosis with no IPVDs (n=15)</th>
<th>Controls (n=47)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>8/6</td>
<td>8/1</td>
<td>9/6</td>
<td>20/27</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>66.4 +/- 9.9</td>
<td>56.3 +/- 7.1</td>
<td>59.5 +/- 9.0</td>
<td>39.4 +/- 16.3</td>
</tr>
<tr>
<td>FE,No200 (ppb)</td>
<td>14.9 +/- 4.36</td>
<td>13.4 +/- 2.50</td>
<td>10.1 +/- 2.78</td>
<td>10.2 +/- 2.76</td>
</tr>
<tr>
<td>DLCO (% pred)</td>
<td>62.8 +/- 10.1</td>
<td>79.5 +/- 9.74</td>
<td>77.0 +/- 13.0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Figure 2: FE,No200 Between Groups

• No significant between group differences in distribution of Child’s-Pugh classes

• DLCO significantly lower in HPS subjects (62.8 +/- 10.1% pred) vs pre-HPS subjects (79.5 +/- 9.74% pred, p<0.01), and cirrhotic subjects with no IPVDs (77.0 +/- 13.0% pred, p<0.01)

• Among HPS and pre-HPS subjects, strong negative correlation between AaDO₂ and DLCO (r = -0.75; p<0.01)

Discussion

• Largest series of eNO measurements in HPS subjects

• First series to report eNO levels in pre-HPS subjects separately

• Used a constant expiratory flow rate of 200 ml/s for eNO measurement — more representative of the alveolar fraction of eNO

• Finding of elevated alveolar eNO in HPS subjects compared to cirrhotic subjects without IPVDs supports the theorized role of NO in the formation of IPVDs

• In addition, finding that eNO levels in pre-HPS are similar to those in HPS suggests that NO may mediate IPVD formation even before significant gas-exchange abnormalities develop

Conclusions and Future Directions

• HPS and pre-HPS subjects have an elevated alveolar fraction of exhaled NO (eNO)

• As a possible marker of intrapulmonary vascular dilations (IPVDs), progressive elevations in eNO may prove a useful predictor of evolving pre-HPS and HPS among cirrhotic subjects

• A future study with greater numbers and long-term serial eNO measurements is necessary to further evaluate this, and to evaluate the natural history of pre-HPS

Acknowledgements

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APPENDIX 9

Questionnaire Characteristics
<table>
<thead>
<tr>
<th>Questionnaire</th>
<th>Domains</th>
<th>Individual Domain Scoring</th>
<th>Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDI</td>
<td>1) Functional impairment (impact of breathlessness on activities)</td>
<td>Range: 0 (very severe impairment) to 4 (no impairment)</td>
<td>Sum of 3 domain scores, range: 0 to 12</td>
</tr>
<tr>
<td></td>
<td>2) Magnitude of task (type of task that causes breathlessness)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3) Magnitude of effort (level of effort that causes breathlessness)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDI</td>
<td>1) Functional impairment (impact of breathlessness on activities)</td>
<td>Range: -3 (major deterioration) to 3 (major improvement)</td>
<td>Sum of 3 domain scores, range: -9 to 9</td>
</tr>
<tr>
<td></td>
<td>2) Magnitude of task (type of task that causes breathlessness)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3) Magnitude of effort (level of effort that causes breathlessness)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRQ</td>
<td>1) Dyspnea</td>
<td>Range: 1 (maximum impairment) to 7 (minimum impairment)</td>
<td>No summary score</td>
</tr>
<tr>
<td></td>
<td>2) Fatigue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3) Emotional function</td>
<td></td>
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<td></td>
<td>4) Mastery</td>
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</tbody>
</table>
APPENDIX 10

Title: Large Variability in Alveolar-Arterial Gradient (AaDO2) in Cirrhotic Subjects: Implications for The Hepatopulmonary Syndrome (HPS)

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Introduction
Arterial blood gas (ABG) is performed in liver transplant (LT) candidates to screen for HPS, defined by 1) liver disease, 2) intrapulmonary vascular dilatations (IPVDs), and 3) AaDO2 > 20 mm Hg. Accordingly, several LT candidates with AaDO2 > 20 mm Hg were referred to our HPS clinic for evaluation. We noted that with serial ABGs over time, several subjects fluctuated above and below the threshold AaDO2 value, making a definitive diagnosis of HPS difficult. We sought to characterize the intra-subject variability of this measure over 1 year, in order to propose a more stable definition.

Methods
Retrospective review of ABGs in a cohort of 29 liver disease subjects with 2 or more ABGs within a 1-year period at our dedicated HPS clinic, with corresponding clinical assessment revealing no cause for a change in gas-exchange.

Results
Twenty-seven/29 subjects had evidence of IPVDs by contrast ECHO. A total of 93 ABGs were done (mean 3.2 ABGs/subject), with mean PaO2 75.3 +/- 5.9 mm Hg, PCO2 31.6 +/- 1.8 mm Hg, and AaDO2 35.0 +/- 6.8 mm Hg. Mean intra-subject difference between maximum and minimum AaDO2 was 13.1 +/- 9.3 mm Hg (range 1.125 - 36.725 mm Hg). Over a period of 1 year, 8/29 (28%) subjects fluctuated randomly above or below the threshold AaDO2 value of 20 mm Hg. The mean intra-subject CoV was 29.8% +/- 30.0% for AaDO2 and 8.0% +/- 5.4% for PaO2. Ten/29 subjects had 2 consecutive PaO2 < 70 mm Hg, and 10/10 remained < 70 mm Hg on subsequent ABGs.

Conclusions
Given that AaDO2 is highly variable in cirrhotic subjects with IPVDs and has not been associated with any clinical outcomes, it is a poorly suited variable by which to define HPS. Given that PaO2 has been associated with mortality, we propose a cutoff of AaDO2 > 20 mm Hg and PaO2 < 70 mm Hg on 2 separate ABGs performed on different days; this represents a more stable and clinically relevant definition for HPS.

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