# Decreasing Leptospirosis Emergence through Prognosis and Treatment Optimization (DeLEPTO) Project 1: Preventive Strategies for Early and Late Complications of Leptospirosis

## Study Protocol and Statistical Analysis Plan

## Version 6

## 2025-03-13

**Component Project Details**

**Program Title**: Decreasing Leptospirosis Emergence through Prognosis and Treatment Optimization (DeLEPTO)

**Proposal Title:** Preventive strategies for early and late complications of leptospirosis

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**UP Manila site primary investigator:** Jose B. Nevado Jr., MD, PhD

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**Target Start Date:**April 1, 2023 **Target End Date:** March 2026

**Implementing Agency:** National Kidney and Transplant Institute

**Co-implementing Agency(ies):** San Lazaro Hospital (SLH)

**Cooperating Agencies:** Institute of Human Genetics, NIH UP Manila

**Type of Research:** Applied Science

**R&D Priority Area and Program (HNRDA):** Health: National Unified Health Research Agenda

**Select HNRDA Item:** Diagnostics Program

**Sustainable Development Goal:** Good Health and Well-Being

**Line-Item Budget**

| Source of Fund | PS | MOOE | EO/CO | Total |
|---|---|---|---|---|
| DOST-GIA | 9,613,137.60 | 46,168,765.60 | 4,570,000.00 | 60,351,903.20 |
| National Kidney and Transplant Institute | | 15,480,000.00 | | 15,480,000.00 |
| UP National Institutes of Health-Institute of Human Genetics | 798,310.08 | | | 798,310.08 |
| Total Direct Cost (incl counterpart) | 8,829,118.08 | 57,867,400.00 | 4,500,000.00 | 71,196,518.08 |
| Total Indirect Cost | 1,582,329.60 | 3,781,365.60 | 70,000.00 | 5,433,695.20 |
| **Overall** | **10,411,447.68** | **61,648,765.60** | **4,570,000.00** | **76,630,213.28** |

1. **Executive Summary (not to exceed 300 words):** *an abstract of the project including details on the requested budget and the targeted 6 P's*

Leptospirosis is a bacterial disease of severe public health concern in the Philippines. Aware of the increasing number of individuals dying from this disease and its impact on society, the National Kidney and Transplant Institute (NKTI), San Lazaro Hospital (SLH) and the Institute of Human Genetics, National Institutes of Health-University of the Philippines Manila (IHG, NIH-UPM) teamed up to investigate ways to improve the clinical outcomes of leptospirosis patients. This collaboration intends to explore using a serum protein recently identified as a potential prognostic biomarker for

organ complications in leptospirosis and as a predictor of a patient's response to different preventive strategies. Unfortunately, no conclusive evidence yet supports a prognosticator aided intervention. It is hoped that valuable insights into the progression of severe leptospirosis could be generated through this study. Furthermore, these insights will expand on the currently limited information on host responses against leptospirosis and help inform patient management and clinical guidelines. Prognostic biomarker-based assays and early interventions will be developed, which could lead to shorter hospital stays of patients, reduced healthcare costs for both the patient and the hospital, lesser facility and human resources stress, and decreased mortality and morbidity.

A budget of at least 76M Php (including 23% counterpart funding) spread out over three years is proposed to implement a clinical study with a high confidence level from which impactful and biologically meaningful data can be generated. With those funds, NKTI will be able to explore the real potential of prognosticator-guided preventative approaches for severe leptospirosis. In addition, because we are targeting the host immune responses during infection, findings from this study may be translated into other fatal infectious diseases such as sepsis, dengue, and malaria.

2. **Project Description (not to exceed 300 words):** *a formally written declaration of the project and its idea and context to explain the goals and objectives to be reached and other relevant information that explains the need for the project startup and aims to describe the amount of work planned for implementation; refers to a simple explanation or depiction of the project that can be used as Press Material.*

   This study aims to determine the clinical utility of complement factor I (CFI) as a prognosticator in patients with complicated leptospirosis without severe pulmonary complications and to determine if its guidance to preemptive measures can lead to a reduction in adverse clinical outcomes, specifically the occurrence of pulmonary bleeding and acute respiratory distress syndrome (ARDS), and mortality. Hence, the results of the study may lead to novel treatment approaches that can be readily applied in clinical practice. The decision to provide preemptive non-invasive therapies or early intensive care admission could lead to significant breakthroughs in managing the disease.

   Within the DeLEPTO program’s vision of developing tools to increase the survival of leptospirosis patients, this project will explore the avenue of novel tertiary care. Specifically, the program will look into the possibility of CFI repletion using plasma transfusion, cytokine depletion strategies using hemoperfusion (HP), and extracorporeal membrane oxygenation (ECMO). It would be interesting to see how such interventions could work individually or in a pipeline with other proposed interventions. In addition to plasma therapy, ECMO was observed to improve outcomes in severe leptospirosis (1). As a secondary endpoint, it would also be interesting to know if CFI can prognosticate who will benefit the most from such interventions.

3. **Significance (not to exceed 300 words):** *refers to the alignment to national S&T priorities, strategic relevance to national development, and sensitivity to Philippine political context, culture, tradition, and gender and development.*

   Many leptospirosis patients still develop serious, costly, and potentially fatal complications. Although there are guidelines to manage leptospirosis effectively, these treatment strategies, such as dialysis, ICU care, and hemo-respiratory support, incur a relatively high cost for the patient. For example, patients developing acute kidney injury requiring dialysis or patients with pulmonary failure requiring mechanical ventilation for lung support will incur an additional cost per week amounting to Php 24,000.00 for four sessions of hemodialysis or Php 70,000.00 for daily use of a mechanical ventilator, and a conservative amount of Php 35,000.00 in ICU stay. This still does not include

laboratory tests required daily.

Moreover, despite these interventions, the hospital stay and mortality rate of leptospirosis remain significant. With the need to properly allocate financial, medical, and human resources, clinicians need to have a more efficient and timely way to identify who will develop such complications. There needs to be more understanding of how to determine which leptospirosis patients need treatments the most, especially preemptive care for secondary prevention and treatment of severe cases.

It is essential to underscore that the results of the study can impact the therapeutic and prognostic guidelines of leptospirosis that is prevalent in a tropical country such as the Philippines. Prognosticators can help select persons who will benefit most from limited intensive care; these therapies would instead be made available for other patients requiring these life-saving treatments. The proposed therapy is also a relatively safe and inexpensive plasma transfusion. Plasma is usually among the least-utilized blood products in blood banks and is generally discarded. If found effective, we may repurpose plasma into a life-saving measure.

4. **General Objective:**
   To evaluate the clinical applicability of selected interventions and the use of complement factor I (CFI) gene expression as a prognosticator for developing pulmonary complications in leptospirosis.

5. **Specific Objectives:**
   1) To determine the effect of plasma component transfusion in moderate leptospirosis on the following outcomes:
      a) Mortality
      b) Significant pulmonary involvement
      c) Refractory hypotension
      d) Days to renal recovery
      e) Hospital stay
   2) To determine the effect of hemoperfusion in moderate to severe leptospirosis on the following outcomes:
      a) Mortality
      b) Significant pulmonary involvement
      c) Refractory hypotension
      d) Days to renal recovery
      e) Hospital stay
   3) To verify technologies for measuring CFI gene expression levels in the blood
      a) PBMC mRNA expression
      b) Blood protein levels
   4) To correlate CFI gene expression with the development of clinical complications
      a) Concerning the eventual occurrence of the aforementioned complications (controls only)
      b) Concerning response to treatment

6. **Review of Literature:** *discuss here the following: (a) related research that has been conducted, state-of-the-art or current technologies from which the project will take off; (b) scientific/technical merit; (c) results of related research conducted by the same Project Leader, if any; (d) Prior Art Search; and (e) other relevant materials*.

   **Leptospirosis as a public health concern**
   Leptospirosis is a prevalent zoonotic disease typically affecting tropical and subtropical countries. The most affected countries include those in Southeast Asia, including the Philippines (2). It is

mainly contracted from water contaminated with pathogenic leptospires that enter through an interruption in the skin or mucosa of the host. It is typically seasonal, with increased incidence during times of flooding. However, it could be sporadic, especially among occupations associated with immersion in contaminated water, such as sewage management or farming (3).

Though most cases manifest with flu-like illness, about 5-10% will have a more severe form called Weil's syndrome. In a systematic review published in 2015, it was estimated that there were about 1.03 million cases and 58,900 deaths due to leptospirosis worldwide (2). According to the PH Department of Health, in 2019, there were 3,011 cases, whereas in 2018, there were 5,116. A higher mortality rate is expected in the tertiary hospital setting (4). Most fatalities are males aged 20–49 years (2). In addition to hepatic, renal, and central nervous system (CNS) involvement, which are classic complications of leptospirosis, the disease may also be complicated by acute respiratory distress syndrome (ARDS) and pulmonary hemorrhage (3,5). In the Philippines, the National Kidney Transplant Institute of the Philippines reports that during the 1st three quarters of 2019, there were a total of 226 leptospirosis-associated admissions in their hospital alone, with almost 78.3% requiring dialysis due to renal complications.

**The presentations and complications of leptospirosis**

Due to its protean manifestations and the range of spectrum of its severity, leptospirosis is classified presently as mild, moderate, or severe. The following description of the severity of leptospirosis is provided in detail by Haake and Levett (5), Barthi et al. (3), and the clinical practice guideline of The Leptospirosis Task Force (6). Mild leptospirosis does not have jaundice, has stable vital signs, and is without renal failure and pulmonary complications. Nonspecific symptoms, such as fever, headache, myalgia, nonproductive cough, and a maculopapular rash are present. More than 90% of mild leptospirosis does not become more severe and resolves in a few days. Conversely, those with severe leptospirosis experience severe complications, such as renal failure, episodes of hypotension, and pulmonary complications. In between, moderate leptospirosis patients have jaundice, with altered but not gross organ failure, such as altered mental status, lessening of urine output, and unstable vital signs. The occurrence of gross pulmonary hemorrhage or congestion can happen in moderate to severe leptospirosis, herein defined as pulmonary complications. To be more specific, such complication could be described as pulmonary injury as indicated by severe dyspnea and/or needing mechanical ventilator support plus one of the following in radiographs: patchy alveolar infiltrates in at least 1/3 of the lung area, presence of alveolar infiltrates in the chest radiograph, or gross hemoptysis/hemorrhage. However, in a clinical setting, pulmonary complications are challenging to evaluate and can be missed early on (7). Notably, the reports on the prevalence of pulmonary hemorrhage vary but can go as high as 39% (8).

**Leptospirosis as a detrimental host response**

Weil's syndrome can be considered as a host response mechanism to leptospiral pathogens. It is generally characterized by hyperinflammatory reactions in one or more of the host's organs, such as the kidneys, liver, joints, meninges, and other organs (3,5). Molecularly, such responses are manifested as increases in blood levels of tumor necrosis factor- alpha (TNF-alpha) or interleukin 6 (IL6) (5,9). Such a storm could be due to the loss of modulatory molecules, the brute force of the degree of activity of inflammatory pathways, or both. If both, such phenomena could reinforce each other. On the one hand, the hyperactivation of pathways, such as the alpha-interferon pathway and the Toll-receptor- mediated pathways, is strongly suspected (5). On the other hand, hyperactivity of the alternative complement pathway, through the lack of CFI inhibition, is also strongly suspected (Nevado et al. 2019, unpublished). These speculated mechanisms will be the central basis for this proposal's depletion and repletion approaches. Since Weil's syndrome is a host response, intuitively, one considers the human host the most practical source of an indicator biomarker. This is pertinent

to recommendations on the diagnosis and therapy of leptospirosis that advocate the importance of early diagnosis and treatment.

Moreover, in leptospirosis, neutrophil extracellular traps are innate mechanisms of the body against infection by extruding chromatin from neutrophils to sequester circulating pathogens through a process called netosis. Netosis was observed to be active during leptospirosis (10) and may be involved in the detrimental effects of the infection. Gasdermin D (GSDMD) is a marker for netosis. Its circulating levels increase with netosis, as GSDMD appears to be a central molecule to induce netosis (11).

**Limitations of the current management of leptospirosis**

The present management of leptospirosis is mainly clinical and supportive (5). The cornerstone of the current management of leptospirosis heavily relies on antibiotics, supportive treatment, and alleviation of symptoms. At the start of suspicion of the conditions, antibiotics such as beta-lactams (penicillin G, ampicillin, and ceftriaxone), doxycycline, and azithromycin are typically administered. These are accompanied by supportive interventions, such as intravenous fluids, electrolyte correction, inotropic support, ventilatory and renal replacement support if applicable, and nutritional support. Medications to mitigate manifestations that include fever, headache, and bodily pain, as well as for bleeding manifestations. In general, the approaches are reactive and still need further refinement, as reflected by the persistently significant mortality in complicated conditions.

**Importance and challenges of early intervention in leptospirosis**

Management at the early phase of the disease seems to lower the risk of complications and fatality (12), and early diagnosis and treatment remain the most practical public health strategy to deal with leptospirosis outbreaks. This is particularly important in pulmonary complications because of the associated increased mortality, consistently observed to be typically more than 50% in cases of acute lung injury and more than 80% in the presence of pulmonary hemorrhage (8,13,14,15,16,17). Early diagnosis and prognosis of leptospirosis present a significant challenge even in tertiary hospitals because both remain heavily dependent on clinical parameters, such as the presence of jaundice or low urine output, which are mainly concurrent with the severe state of patients.

Contrary to expectations, clinical and laboratory parameters for leptospirosis do not help predict the development of complications. Instead, laboratory testing of biomolecules, such as high creatinine levels and blood electrolyte levels to indicate renal failure, low partial thromboplastin time, aspartate aminotransferase or alanine aminotransferase to indicate liver failure, and the derangement of blood gases to indicate lung failure, are used to detect the presence of end-organ complications (16,18,19,20). Other generic predictors include blood cell counts and gender (21). However, all the parameters mentioned above occur during the phase wherein end-organ damage is apparent and may have less impact on preventing complications.

**Current practices in prognosticating leptospirosis**

Clinical parameters are currently the most supported means of prognosticating leptospirosis. Though there are clinical prognosticators, such as dyspnea, oliguria, presence of pulmonary rales, hyperkalemia, abnormal serum creatinine, leukocytosis, thrombocytopenia, elevated bilirubin, hypotension, arrhythmia, acute respiratory distress syndrome, pulmonary hemorrhage, and altered mental status, these are likely already late manifestations and could be less reliable in the crucial clinical decision-making. This is not surprising as most evidence was obtained from clinical studies with minimal molecular data. For example, a study on patients with evident acute lung injury concluded that hemodynamic disturbance, high serum creatinine level, and elevated serum potassium level were determinants of mortality (16,18). Moreover, such a panel of clinical

determinants typically differs widely across various studies (18,19,23,24). Even the determination of the presence of anti- leptospiral immunoglobulins, more commonly detected through IgM microagglutination tests and IgG, cannot predict the eventual development of complications in leptospirosis, probably because of the nonspecific immune responses of the host at this point of the disease history.

**The concept of host molecular markers**

Because molecular host changes may occur early during the pathogenesis of diseases, they could be better alternatives for the prognosis and diagnosis of diseased conditions. Molecular biomarkers are molecules that are taken from an organism to indicate a particular condition. Traditionally, diagnostics for infectious diseases mainly rely on biomarkers that originate from the corresponding pathogen. While these biomarkers confirm the presence of the pathogen, they do not provide physicians with a way to predict whether the patient will develop Weil's disease. Meanwhile, antibody-detecting diagnostics are only capable of diagnosing leptospirosis but not differentiating between milder forms and complicated forms.

This underscores the importance of prognostication by host response. Host inflammatory processes are supposedly evolution-derived reactions designed to protect an organism from injury. However, through various phenomena, such as the co-evolution of the pathogens to evade or the persistence of genetic derangements resulting in acute-onset hyperinflammatory responses, inflammation by itself has become a cause of disorders that can adversely affect the human host. More likely, the detrimental reactions occur as a result of active biomolecular and hyperinflammatory reactions that perturb host homeostasis, resulting in disease or even death. In other words, in the case of severe leptospirosis, the presence of a pathogen may indicate infection. However, the issue of developing complications can be better assessed by the host response.

It is also important to underscore that, in general, molecular markers on infectious diseases traditionally rely on the features of the pathogens, such as the amplification of the flagellin B gene (flB) in pathogenic Leptospira. However, such markers may only appear in the acute phase and may not mean that the patient will go into Weil's syndrome. Investigations on the use of host biomarkers are relatively novel, considering that high throughput technology has become available to countries that are greatly affected by infectious diseases. This technology is able to screen a large number of candidate markers that can have an impact on the diagnosis and prognosis of diseases. In addition, candidate pathways/genesets can be inferred, which in turn can serve as markers themselves and may also suggest potential mechanisms of injury. In summary, by considering the host response mechanism as a potential source of markers for patients with leptospirosis, the present discovery can help in the early diagnosis and in the prediction of complications, as well as in the triage of patients who are at risk for the complications of leptospirosis. However, as in the case of uncomplicated leptospirosis, a biomarker that can sufficiently predict complicated leptospirosis from the early onset of symptoms has yet to be found.

**Complement factor I (CFI) and its possible role in the pathogenesis of leptospirosis**

The gene complement factor I (CFI) codes for an important regulator of the complement system, particularly the alternative pathway. It inactivates C4b and C3b and prevents the formation of C3 and C5 convertase enzymes (24). This leads to the inactivation of the complement system, including the formation of the membrane attack complex. Loss-of-function mutations in the gene resulting in loss-of-function per se, lack of secretion to the extracellular space, or the lack of expression of the protein has been associated with susceptibility to organ damage via atypical hemolytic uremic syndrome (aHUS), and severe recurrent bacterial infections (25,26). The encoded protein is a heterodimer composed of a light (L) and a heavy (H) chain from a single peptide

processed in most cells, including the PBMCs. The H chain is an allosteric inhibitor, and the L chain is the catalytic component. Inhibition of complement targets occurs upon binding to its target substrate that complexes with an appropriate cofactor that can disrupt the binding of the H chain. The exact mechanism of organ injury remains unclear. However, complement activation is increased, and C3 levels were found to be decreased in those with decreased CFI activity. A possibility of a self-imposed injury by complement overactivity is speculated but needs to be substantiated. On the other hand, the resulting lessening of activity could result in dire consequences due to the complex interaction of the system with other immunologic processes. In summary, it is surmised that the increased complement system may directly contribute to organ injury, either as a direct mechanism or a mediator of other immunological processes that result in complications.

Concerning leptospirosis, it was observed that pathogenic leptospira are resistant to complement attack and may contribute to sustained hyperactivity of the complement system (27,28). The bacteria activate the complement system mainly through the alternative pathway, consistent with the CFI hypothesis above. In addition to activating the complement system, it was also observed that it prevents the modulated inhibition of the complement system by binding and inhibiting factor H, a cofactor for CFI. Interestingly, a recent report considers the possibility of severe leptospirosis being a variant of HUS (29). Hence, this raises the consideration that Weil's syndrome is a hemolytic uremic syndrome variant due to complement activity.

**CFI as a marker for pulmonary complications in leptospirosis**

A study from Nevado and colleagues in 2019, sponsored by the Department of Science and Technology (DOST Philippines) and the Philippine Council for Health Research and Development (PCHRD), showed a verified association of CFI levels with the eventual development of pulmonary complications in leptospirosis. Using PBMC samples from the first 24 hours after admission from patients with leptospirosis who have no evident severe organ damage, transcriptional profiling is then performed. Microarray values of CFI mRNA levels were lower in cases who eventually developed clinically serious pulmonary complications compared with those who did not (4.10 [95%CI: 3.89-4.30] versus 4.60 [95%CI: 4.50-4.70; unadjusted $p<0.01$). ROC area is 0.89. The levels were only significantly different (which is lower) for those with complications and were even lower when compared with healthy participants (4.52 95%CI: 4.45-4.60]; unadjusted $p<0.01$) and with febrile controls (4.44 [95%CI: 4.36-4.53]; unadjusted $p<0.05$).

Subsequent verification in a separate and independent sample of patients using quantitative polymerase chain reaction (qPCR) showed a generally similar and significant trend. Using deltaCq values, the mean for those who eventually developed pulmonary complications is significantly higher (lower CFI expression compared with those without (15.68 [95%CI: 14.54-16.81] versus 11.65 [95%CI: 10.72-12.59; unadjusted $p<0.01$). ROC area is 0.92, with optimum specificity of 85.42% on 100% sensitivity.

Curious as to the occurrence of most deaths in complicated leptospirosis due to pulmonary complications, especially pulmonary hemorrhage, we correlated CFI levels with mortality. Overall, 50% (3/6) of those with pulmonary complications died. Four deaths occurred, with 3 (75%) having pulmonary complications. For all deaths with pulmonary complications, the adverse respiratory condition was assessed two days after the determination of the low CFI mRNA levels. In contrast, the lone mortality had minimal pulmonary complications and had died two weeks after admission. Noticeably, the CFI level is higher than the other deaths.

Hence, levels of CFI can be used to indicate the eventual occurrence of complicated leptospirosis,

especially pulmonary complications. The possibility that this molecule is causative suggests that it is a potential therapeutic target in the alleviation and prevention of complications.

**Proposed interventions in the treatment of complications in leptospirosis**

*A. Prophylactic plasma transfusion (PPT) for less complicated leptospirosis*

Studies on plasma transfusion in leptospirosis indicate potential benefits in terms of reduction in mortality due to complications from leptospirosis. In particular, most studies focused on the stages when complications are already present; hence, termed therapeutic plasma exchange (TPE) is also an interesting approach as it could be a cost-effective means to replete CFI and remove toxins from the body. Fresh frozen plasma has been found to be stable during blood collection. Because most people have normal CFI activity (data unpublished), such could explain a potential therapeutic application of TPE. Several studies have shown potential promise for the intervention (Table 1). In particular, the mid-sized study by Herath et al. (15) and Trivedi et al. (8) showed a more than 60% reduction in death in severe leptospirosis using therapeutic plasma exchange despite the occurrence of pulmonary hemorrhage.

In the present study, such repletion therapy as a preemptive treatment for less severe leptospirosis appears promising in the context of CFI repletion. As pathway analyses of CFI gene expression demonstrate the downregulation of the alpha interferon signaling and the upregulation of the alternative complement pathway due to the relative lack of intrinsic inhibition that is predictive of the development of serious complications (Nevado et al. 2019, unpublished), the study entertains the possibility of repletion therapy as a prophylactic means to prevent deteriorations in leptospirosis. Thus, to differentiate from prior studies, the present study will look into the benefit of prophylactic plasma transfusion (PPT) if administered before the onset of complications.

*B. Hemoperfusion (HP) and cytokine depletion for complicated leptospirosis*

The hypothesis that certain molecules which are able to drive the cytokine storm may be present is plausible. The presence of traditional molecules like TNF-alpha and IL6 (9) could result in self-inflicted tissue damage. Also, unique to leptospirosis, the low CFI expression may be a result of the presence of certain inhibitors, such as the leptospiral immunoglobulin-like proteins (30). Thus, the means to remove injuring molecules in the blood is a possible therapeutic approach.

One feasible approach that is available at the National Kidney and Transplant Institute (NKTI) is hemoperfusion (HP). The procedure involves passing the blood of patients ex-vivo to activated carbon or resin cartridges to remove potentially detrimental molecules either from the pathogen or the host that contribute to the development of tissue injury (31). Mainly utilized in sepsis, some report potential benefits on mortality, with a relative risk reduction of 0.88 (1,33). However, conclusive evidence, particularly in leptospirosis, is needed. More importantly, there is no known evidence for its use as a preemptive strategy. One report suggested the potential use of hemosorption (33).

Although there was scarce evidence on the use of hemoperfusion in leptospirosis, its utility in hyperinflammatory conditions can be approximated by evidence in sepsis and other severe infections. The most similar to our study would be those that will use Jafron HA330. Interestingly, the device was found to improve organ function at day seven significantly, and decreased both ICU admissions (24% vs. 57% in control; p=0.02) and 28-day mortality (28% vs. 67% in control; p=0.009) in the hemoperfusion group compared to controls in patients with nonpulmonary sepsis with significant lung injury (34). There is also a substantial lowering in mechanical ventilatory time.

**Table 1.** List of reports on the use of TPE as an intervention for pulmonary complications in severe leptospirosis

| Authors | Year published | Description of pulmonary complication | Description of TPE | Survival | Other remarks |
|---|---|---|---|---|---|
| *Herath et al.*[15] | 2019 | Severe pulmonary hemorrhagic syndrome (SPHS) | Within 48 hours after SPHS | 35/59 (74.5%) | Without TPE, 10.5% (2/19) survived |
| *Ekinci et al.*[35] | 2018 | Diffuse bilateral pulmonary infiltrates | 5 courses of plasma exchange | | Turkey; patient is 8-years old |
| *Taylor and Karamadoukis*[36] | 2013 | Pulmonary hemorrhage | Two 4-litre exchanges using a combination of fresh frozen plasma and human albumin solution | 1/1 | United Kingdom |
| *Cerdas-Quesada*[37] | 2011 | Diffuse bilateral pulmonary infiltrates | 5 courses of plasma exchange | 1/1 | Argentina |
| *Bourquin et al.*[38] | 2011 | ARDS | 5 sessions of plasma exchange | 1/1 | Switzerland |
| *Trivedi et al.*[8] | 2010 | Pulmonary hemorrhage | 2 cycles of plasma exchange, 24 h apart, 25 ml/kg per cycle | 70/114 (61%) | India; Without TPE, 17% (5/30) survived |
| *Chen et al.*[39] | 2007 | Pulmonary hemorrhage | 8 courses of plasma exchange | 1/1 | Taiwan |
| *Landini et al.*[40] | 1981 | Severe leptospirosis with hemorrhagic manifestations | Average 2 - 5-3 liters per session; frequency not indicated | 6 patients recovered | Italy |

Aside from resin absorption, a more specific approach is to deplete endotoxin levels using polymyxin B or to target hypothetically toxic innate molecules (41). A systematic review of controlled studies using the endotoxin-specific polymyxin B-immobilized hemoperfusion showed a significant reduction in mortality (0.68. 95% CI 0.51–0.91; P=0.01) with significant differences in the presence of sepsis or with an Acute Physiology and Chronic Health Evaluation II (APACHE II) score <25 is considered (42). Again, similar to the Huang study, an interesting trend emerged that suggests early intervention using hemoperfusion may be more beneficial than late treatment in the preemptive management of inflammatory conditions, thus supporting and justifying a preemptive role rather than a reactive one.

Besides the HA series, for the cytokine-targeted approach, recent trials on hyperinflammatory conditions also commonly used CytosorbTM cartridges. However, most studies showed no conclusive benefit if used in patients with more severe conditions. In a meta-analysis of patients with inflammatory conditions requiring intensive care, hemoperfusion using CytosorbTM was reported to have a significant increase in mortality in the CytosorbTM group compared with control (29% versus 24%; RR = 1.24 [95% CI, 1.04–1.49; p=0.02) (43). Data on earlier interventions is lacking.

*C. Extracorporeal membrane oxygenation (ECMO) for severe respiratory compromised leptospirosis as a rescue treatment*

ECMO involves the use of a pump that circulates the blood out of the human body to an artificial lung that oxygenates the blood and pumps the blood back to the body. Considered a lung by-pass means, it was proven to be useful in cases when severe respiratory decompensation occurs. Several studies found a promising trend in the use of ECMO in leptospirosis. Several cases of using ECMO in leptospirosis showed an encouraging trend towards a decrease in mortality (Table 2; 44). In the Philippines, a case report on the use of ECMO was documented in a leptospirosis-afflicted male with severe ARDS and pulmonary hemorrhage (1), resulting in the resolution of complications.

At the same time, Vandroux et al. (51) reported a 75% survival rate among patients with moderate to severe acute respiratory distress syndrome (ARDS) (6 out of 8).

To assess the potential benefit of ECMO in severe pulmonary cases of leptospirosis, an indirect approach is warranted. This is because the use of ECMO in leptospirosis was mostly reported in small case series with minimal controls.

For the sake of evaluating benefits, the nearest evidence that used controlled comparators had been acute respiratory distress syndrome (ARDS) and severe pneumonia. In these studies, the potential clinical benefits, such as decreased mortality and hospital stay, are significant. For this purpose, a combined analysis of 2 large RCTs, namely, the Conventional Ventilator Support vs Extracorporeal Membrane Oxygenation for Severe Acute Respiratory Failure (CESAR) trial and the ECMO to Rescue Lung Injury in Severe ARDS (EOLIA) trial comprising 214 ECMO cases and 215 control was performed (45). Results showed a significant reduction in death (36% vs 48%, p=0.013) after a terminal 90-day period. This is despite rescue ECMO administered in 17% of the control group. Thus, this finding may serve as a satisfactory justification for the extrapolation of ECMO benefits to pulmonary complications of leptospirosis.

**Safety concerns on the proposed study interventions**

*A. Prophylactic plasma transfusion (PPT)*

Although the rates are generally low, adverse events associated with plasma transfusion do exist and can be life-threatening. In general, the more common serious events can be grouped into 3: (1) transfusion-associated circulatory overload (TACO), (2) transfusion-related acute lung injury (TRALI), and (3) anaphylactic reactions (46,47,48). According to the US-based National Healthcare Safety Network Hemovigilance Module, from Jan 1, 2013, to 2018, there were a reported 8.34 million transfusions of any blood component, with 1.25 million transfusions being of plasma. Of these, there were about 23,083 adverse reactions, with just 1,340 being serious. Serious TACO, TRALI, and anaphylaxis occurred in 353, 44, and 75 cases, respectively, making their cumulative prevalence well less than 0.04% (49). Such rarity of serious events is supported by a 7-year (2006-2012) review of the International Surveillance of Transfusion- Associated Reactions and Events (ISTARE) database, with each individualized adverse event having less than 100 per 100,000 transfusions, with TACO and TRALI reported in <10 per 100,000 transfusions (50). Interestingly, whole blood-derived plasma was noted to be less associated with allergic and hypotensive reactions compared with apheresis-derived plasma, as the former will be applied in the present study.

**Table 2.** List of sample reports on the use of ECMO as an intervention for pulmonary complications in severe leptospirosis

| Authors | Year published | Description of presenting pulmonary complication | Description of ECMO | Survival | Other remarks |
|---|---|---|---|---|---|
| *Chavez et al.*[1] | 2019 | Pulmonary hemorrhage | | 1/1 | Philippines |
| *Vandroux et al.*[51] | 2019 | Moderate or severe ARDS | Typically, on the day of ARDS, for 5-13 days | 6/8 (75%) | France |
| *Cantwell et al.*[52] | 2017 | Pulmonary hemorrhage | On the day of admission, for eight days | 1/1 | Chile used a double setup |
| *Umei & Ichiba*[53] | 2017 | ARDS with endobronchial bleeding | On the day of referral, five days after onset of ARDS for 11 days | 1/1 | Japan |

| | | | | | |
|---|---|---|---|---|---|
| *Pardinas et al.*[54] | 2017 | ARDS with hemoptysis on day 2 | On day 2 for 14 days | 1/1 | USA |
| *Liao et al.*[55] | 2015 | Pulmonary hemorrhage | On the day of hemoptysis, for six days | 1/1 | Taiwan |
| *Kahn et al.*[56] | 2006 | ARDS with endobronchial bleeding | On the day of admission (3- 4 days after documented bleed), for 60 hours | 1/1 | Austria |
| *Arokianathan et al.*[57] | 2005 | Pulmonary hemorrhage | Provided post-hemoptysis for 183 hours | 1/1 | United Kingdom |

Other important but less serious events include hemolytic, anaphylactic, septic, febrile, delayed hemolytic, and delayed serologic reactions. Fortunately, they all have been observed to occur in less than 1% (46,49).

*Measures to prevent and manage the occurrence of adverse events in plasma transfusion*
The latest definition of TACO was adopted in 2018 from the collaborative works of the International Society of Blood Transfusion (ISBT), International Haemovigilance Network (IHN), and the former American Association of Blood Banks (AABB) as an onset of respiratory symptoms within 12 hours of blood product transfusion. This may be determined by clinical physical examination, radiographic chest imaging, other noninvasive assessments of cardiac function, or a combination. Signs include tachycardia, a widened pulse, and rales heard bilaterally. The chest radiographs may show signs of congestive heart failure: bilateral infiltrates and an enlarged cardiac silhouette. An elevated pulmonary capillary wedge pressure (PCWP) and central venous pressure (CVP) can be observed (58). Hence, patients at risk include those with heart failure, renal failure, and those >70 years old. However, the most important risk factors are the rate of infusion and the amount of infusion. High rates (>300 ml/hr) or high volume (> eight units per day) significantly increase the risk for TACO (59,60). According to the 2011 annual prospective United Kingdom-based Serious Hazards of Transfusion Report (SHOT), an optimum rate with significantly fewer incidents is <120 ml/hour for whole blood (58). Subsequent recommendations to minimize TACO place the infusion at <120 ml/hour for low-risk and <85 ml/hour for those with risk (age >60, history of congestive heart failure, myocardial infarction, chronic renal failure, or chronic lung disease). In addition, furosemide 40 mg is recommended to be injected every 6 hours for those aged >60 years old (58). Aside from the use of loop diuretics, placing the patient in a seated position, providing supplemental oxygen, and prompt consultation with cardiology are also recommended (58,60).

TRALI is another potentially serious side effect. By consensus, the 2019 TRALI definition states that it is the presence of an acute onset of hypoxemic respiratory distress during or within 6 hours of a blood product transfusion. Specifically, the hypoxemic state is defined as PaO2/FiO2 <300 mm Hg or O2 saturation <90% on room air (46,48). Currently, the main notion is that the injury is caused by neutrophil-mediated damage to the lungs due to interaction with infused antibodies from the transfused blood (47). In 20% of TRALI cases, instead of antibodies, the culprit is suspected to be biologically active lipids (BALs). The main risk predictor is the blood being from multiparous women who had developed antibodies from previous pregnancy tissues (48). Despite a 5-10% mortality, the incidence of such events is very low (61). Due to the rarity of the event, it is decided that patients be preferentially provided with plasma exclusively from men or, if from women, who are uni- or nulliparous. Monitoring of signs and symptoms and vital signs can be done hourly until 8 hours after the end of transfusion. Management will be mechanical ventilation, if indicated, applying restrictive tidal volume and possibly diuretics, fluid restriction, and, in extreme cases, extracorporeal

membrane oxygenation (46).

Note that both TACO and TRALI are usually observed in the setting of plasma transfusion with high volume indications (~8 units or more a day) (62). Thus, as a precaution, a volume of just four units per day is then prescribed in this study.

For the present study, FFP will be infused using units two times a day, with each unit infused for about 3 hours. This assumes a low-volume (12-15 ml/kg) volume at <85 ml/hour. This range of plasma dose is within the recommended range in coagulation factor repletion therapy, as observed by Chowdary and colleagues (59). Despite previous studies on TPE in leptospirosis-afflicted patients done in critical patients having used high volume exchanges at about 25-30 ml/kg per day in exchange procedures (8,16), the volume to be used in the present study is deemed to suffice due to the recipients having less serious conditions. The important assumption is that the indicated volumes and rates are within the limits for precaution for TACO and TRALI.

The third common serious reaction is the cumulated group of anaphylactic and hemolytic reactions. Usually occurring as reactions to blood additives, the clinical manifestations include hypotension, tachycardia, nausea, vomiting, abdominal pain, severe dyspnea, pulmonary or laryngeal edema, and bronchospasm, usually within 4 hours after transfusion (46). Related to this, hemolytic reactions are mainly due to blood ABO/Rh mismatching resulting in increased temperature, increased heart rate, chills, dyspnea, chest or back pain, abnormal bleeding or shock, hemoglobinuria, epistasis, oliguria or anuria, disseminated intravascular coagulation, and pain or oozing at the intravenous site (46). Presently, with routine use of typed matched donors and more advanced blood processing, the events are considered rare and, if occurring, rarely fatal, more so in cell-depleted plasma. The events are less than 1%, and if these occur, supportive care is generally sufficient, which in extreme cases may include respiratory support and/or the use of antihistamines and inotropic drugs (46,47). In the present study, to prevent this from occurring, cross-matched plasma will be used as the basis for transfusion.

Less serious events may also occur, such as febrile nonhemolytic transfusion reactions, graft versus host type reactions, delayed hemolytic reactions, and post-transfusion purpura that usually manifests with mild and temporary symptoms. However, these are usually less than 1% in incidence and are given supportive treatment (47,48).

To minimize the occurrence of these adverse events, hemovigilance guidelines were made and published, more notably by the United States Biovigilance Network and International Haemovigilance Network (IHVN) (48). Several procedures were suggested to lessen the occurrence of the events. Clinically, blood transfusion cessation is recommended in the presence of clinical signs and symptoms that include fever, chills, respiratory distress, hypo/hypertension, skin manifestations (redness, edema, urticaria), hemoglobinuria, nausea/vomiting, oliguria/anuria, or anaphylaxis that is suspiciously associated with transfusion. Recommended standard laboratory workup after an event includes clerical checks (bag, label, patient sample), repeat ABO testing on posttransfusion samples, visual checks of the physical product unit, and direct antiglobulin test.

Locally, the monitoring of patients during transfusion can be guided using the guidelines set by the Lung Center of the Philippines (63). Here, the following symptoms will require stoppage of the transfusion (as adapted from the proposed guidelines of the Lung Center of the Philippines): (1) hives, pruritus, flushing, swollen lips, tongue, or uvula; (2) new or worsening dyspnea, wheezing, stridor, hypoxemia; (3) hypotension, a systolic or diastolic drop of >30% from baseline; (4) tachycardia or bradycardia; (5) syncope; confusion or decrease in sensorium; and (6) other signs or

symptoms for which the attending physician determines warrants the discontinuation of infusion (such as gastrointestinal symptoms).

*Risk-benefit justification for research investigation on plasma transfusion in leptospirosis*
Because of the precautions mentioned above (blood screening, cross-matching, <6 units/day, at <80 ml/hour), the estimated risk for adverse events should be at most <5%, with the serious events to be <1%. If the estimated benefit is placed conservatively at a 30% reduction in death (which is objectively much lower than the >60% reduction predicted by both Herath et al. (15) and Trivedi (8), the projected benefits still greatly outweigh the risks involved; thus, justifying the implementation of the study.

*B. Hemoperfusion (HP)*
Hemoperfusion has been used as a depletion procedure in order to lessen the concentration of detrimental molecules that can lead to adverse outcomes. In particular, this treatment has been attempted in severe conditions, such as sepsis, kidney and liver failure, drug overdose, and poisoning (41).

As part of procedures, the following adverse events may result. Complications may occur due to the vascular accesses used during hemoperfusion, such as bleeding, pain, and infections. This is more likely due to the use of anticoagulation that prevents blood from clotting, especially while it is outside the body. Although optional, anticoagulation may be recommended to protect the patient from developing clots that may lead to thrombosis (42).

The most important consideration in increasing the material-related safety concern of hemoperfusion is the improvement of material biocompatibility. From the initial use about a century ago of aluminosilicates (zeolites) and charcoal, then to organic polymer ion exchange resin, and presently to synthetic porous polymers (e.g., styrene or acrylic acid-based), biopolymers have greatly improved in terms of safety, resulting in designs with generally minimal risk (42). Previous concerns related to biocompatibility include cytotoxicity due to released matter from the sorbents, the abnormal and possibly detrimental induction of intrinsic mechanisms, such as the coagulation or complement pathways, the destruction of cells resulting in leukopenia, thrombocytopenia or similar effects, or even the depletion of unwanted molecules resulting to loss of homeostasis (64).

Another important feature of present-day designs is that they minimize the direct contact of sorbent material with the cells of the blood and/or the use of various sorbent coatings to minimize such inductive interactions (42). In general, present innovations in hemoperfusion (material, specific/limited affinity, different pore sizes, membrane choices, biocompatibility) have resulted in more selective depletion compared with plasma exchanges and plain hemofiltration. The latter has concerns about the removal of potentially beneficial molecules, such as clotting factors, albumin, antibiotics, and protective antibodies. In sum, because of newer materials and filter designs, the incidence of long-term or serious reduction in blood cell levels is considered minimal.

As the safety of hemoperfusion depends largely on materials used in consumables and in those associated with the procedures themselves, we will limit the adverse events unique to Jafron HA330, which is an inorganic resin-based polystyrene sorbent-containing hemoperfusion used in the present study.

Most reports on the use of Jafron HA330 had reported only minor adverse events, with minimal serious events that were no more than comparable to either a historical reference or a parallel control. In fact, several studies on a total of hundreds of patients showed no serious adverse events,

except for occasional fever and rashes that cannot be differentiated from controls (64). Bleeding and thrombocytopenia, if noted, were transient and did not result in emergency intervention. In addition, in 1 case series, using a related Jafron HA280 that differs only in resin loading capacity with HA330, 5 cases in 41 had adverse effects after hemoperfusion: 2 with nausea and vomiting, 2 with hypotension, and 1 with skin bruising after the perfusion (65).

Reiterating, the present study will use a resin-based Jafron HA330 ultrafiltration/absorbent system. In brief, the sorbent part has hydrophobic properties and, therefore, molecules in contact with it. Previous versions of this approach used charcoal. However, years of research have led to newer and presumably safer materials, such as the present non-ionic macroporous resin. The resins are similar to charcoals as they are made of micro-sphere agglomerates, which adsorb toxins on their surface. Presently, most nonspecific resin-based hemoperfusion, such as Jafron HA330 which makes use of styrene-divinylbenzene-based copolymers, are generally used in clinical practice (64). The Jafron HA330 (resin loading capacity = 330 mL) is indicated for the depletion of cytokines, complements, free hemoglobin, and so on, as it has a pore size range from 500 Da to 60 kDa. It is sterilized using irradiation.

*Measures to prevent the occurrence of adverse events in hemoperfusion*
To prevent the occurrence of adverse events during hemoperfusion, several measures can be implemented. Patients with ongoing bleeding or with a platelet count below 80,000 will not undergo hemoperfusion. Due to assumed hepatic dysfunction, heparinization will be deferred unless the attending physician orders. The patients will be monitored at least every hour while on the procedure using the following parameters until 2 hours after hemoperfusion: vital signs, fever, rashes, and bleeding (bruising, hemoptysis, GI bleeding, etc.) prior to hemoperfusion.

*Measures to address adverse events if they happen after hemoperfusion*
In the occurrence of common problems, mitigation measures may help in the management. For all suspicious cases, hemoperfusion should be stopped. For mild cases, supportive therapy with assurance is indicated. Paracetamol can be prescribed for fever and antihistamine for rashes. For more serious cases, specialized treatment should be provided, such as the transfusion of fresh frozen plasma and/or packed red blood cells for significant bleeding.

*Risk-benefit justification for research investigation on hemoperfusion in leptospirosis*
Overall, the hemoperfusion procedure has risks compared with the standard of care but with substantial possible benefits in hyperinflammatory conditions. Objectively, in preemptive designs, we can assume that a conservative estimate of relative risk reduction of 30% (which is less than the actual preemptive estimate of >60% in the study of Huang et al. (34)), the projected benefit can be justified to outweigh the risk, which is projected to be minimal with <1% reported rate for a serious adverse event.

*C. <u>Extracorporeal membrane oxygenation (ECMO)</u>*
Considering that the present study will use the veno-venous ECMO (VV-ECMO) because of the primarily pulmonary nature of leptospirosis-associated lung complications and because such procedure will be used in the study, we will limit the discussion of ECMO adverse events to such, while giving proper regards to adverse events common with veno-arterial ECMO (VA-ECMO).

Although VV-ECMO has been considered an important life-saving procedure in patients who need pulmonary support, it is still an extraordinary measure with expected adverse effects, some of which are potentially debilitating or even fatal. Nonetheless, with proper expertise and equipment, the overall prevalence is generally low. In a review of the post-marketing surveillance data from the

Food and Drug Administration (FDA) Manufacturer and User Facility Device Experience (MAUDE) database from January 2009 to March 2019, Khalid and his colleagues (66) reported the most common adverse events. The most common patient-related adverse events were hemodynamic decompensation of patients, death, atrial perforation, and bleeding. Other less common complications noted were infection and air embolism. The most commonly reported mechanical failure modes were in the following circuit components: mechanical pump membrane oxygenator and access cannulae. However, it is important to note their inferences. First, the total reported adverse event is just 82 out of the estimated >100,000 ECMOs being monitored. Although this may imply a low rate of complications, post-marketing registries have been noted to be notorious for underestimating the true rate of complications. Despite this, it is fair to assume that this extraordinary procedure is relatively safe but needs to be done under the strictest of indications. It is also important to underscore that due to the critical or serious condition of the patients, it is difficult to discern if death or hemodynamic instability is due primarily to ECMO.

In a pooled analysis of 20 studies encompassing 1,866 patients with cardiogenic indications, VA-ECMO has been observed to be associated with renal replacement therapy in 6.0% and major or significant bleeding in 40.8%. Other reported adverse events include lower extremity ischemia, fasciotomy for compartment syndrome, lower extremity amputation, stroke, neurologic complications, acute kidney injury, thoracotomy for bleeding or tamponade in postcardiotomy patients, and significant infection (67). However, if such adverse events were due mainly to the arterial access/complication or due to the primary disease, it is not clearly indicated.

Due to the foresight that ECMOs are complicated with the need for specialized and yet diverse expertise, the Extracorporeal Life Support Organization (ELSO) has recommended the establishment of a multidisciplinary team to handle cases (68). Such an approach has resulted in significant reductions in mortality, hospital stay, and adverse events in pulmonary cases undergoing VV- ECMO (69).

*Measures to prevent and manage the occurrence of adverse events in ECMO*
For VV-ECMO, the most anticipated serious complication is bleeding. As intrinsic coagulation derangements and the use of anticoagulation is mostly required, such adverse reactions are expected. In a study by Popugaev et al. (70), the coagulation derangements are multifactorial and may need to be assessed before, during, and even after the procedure. Coagulation testing should be done and corrected to acceptable levels prior to interventions and during the management of bleeding. The key is to maintain hemostasis and cardiorespiratory stability during the ECMO process.

Another preventable complication is infection. As in any invasive procedure, standard care requires the use of aseptic procedures and proper wound care, as well as the judicious use of antibiotics and antiseptics. Infections should be treated aggressively and preferably guided by culture and sensitivity. Historically, the rate of infection at the wound site has been minimal in VV-ECMO (66).

Lastly, as for hemodynamic instability, it is important to point out that both ECMO and the patient's condition, or both, may contribute to its occurrence. However, in most cases, such etiology is hard to establish, especially the contribution of ECMO. With prudence, perhaps the justification of likely benefit compared to harm will justify only the application of ECMO to those who are deemed to require such to save their lives.

*Risk-benefit justification for research investigation on ECMO in leptospirosis*
Using only the strictest inclusion criteria of severe pulmonary hemorrhage, the use of ECMO can be

justified in leptospirosis. In the presence of severe pulmonary hemorrhage, an expected mortality of 50-80% is observed. ECMO has been observed to reduce mortality by 25% in ARDS (68). Thus, with this potential clinical benefit with an expected rate of serious adverse events at <10%, the present study can argue that investigating ECMO is, therefore, justifiable in ARDS-like leptospirosis-associated pulmonary complications.

7. **Methodology:** *Discuss here the following: (a) variables or parameters to be measured and evaluated or analyzed; (b) treatments to be used and their layout; (c) experimental procedures and design; (d) statistical analysis; (e) evaluation method and observations to be made, strategies for implementation. (Conceptual/Analytical framework).*

**Study design, participants, and comparators**

The present study is the first of the four-project Decreasing Leptospirosis Emergence through Prognosis and Treatment Optimization (DeLEPTO) Program. It will focus on clinical studies on possible clinical applications that can be used to prognosticate and treat leptospirosis. Project 2 will involve the construction of cell culture models, including epithelial endothelial cells and immune cells. This will serve as test models for leptospira supernatant-activated and patient-derived plasma. With these test models, plasma derangement based on pathologic (hyperinflammatory) findings from the plasma of patients with leptospirosis and corresponding interventions for plasma calibration will also be evaluated. Project 3 will involve in vitro (using modified human RBC and endothelial cell culture) and in vivo (using golden Syrian hamsters) testing of candidate interventions or therapeutic strategies using blood plasma. Findings from these studies will also be tested in cell culture models developed in Project 2. Lastly, Project 4 will be done in parallel with Project 1, which will aim to develop a fast point-of-care device that will assess CFI gene expression in patients. Microfluidics and digital droplet PCR (ddPCR) technologies will be utilized to create this device. Initial patient samples and subsequent pilot scale testing will be done with the assistance of NKTI in Project 1.

Of note, Project 1 will not be dependent on the samples and the results of the other projects in the program but will provide them with vital clinical data and biological samples. In turn, the other projects, among their numerous works, will investigate how CFI is involved in the development of complications to leptospirosis and rationalize the treatment mechanism (e.g., plasma transfusion). The results of the other projects will be used to investigate the possible mechanisms of CFI. Further, conditions will be optimized for the digital droplet PCR (ddPCR) under Project 4 (LIGTAS), a technology developed by Dr. Pobre and his team at the DLSU. The ddPCR platform has many advantages compared with the usual qPCR, such as portability and accessibility, shorter processing time, reduced patient sample and reagent volume requirements, and may be more cost-effective in the long run.

The project is composed of 4 objectives, with the third and fourth having sub-objectives, hereon referred to as Objectives 1, 2, 3a, 3b, 4a, and 4b (see the section on Objectives).

All subjects will be recruited from the emergency rooms, wards, and clinics of the National Kidney and Transplant Institute (NKTI) and San Lazaro Hospital (SLH), as these hospitals serve as tertiary care centers for Weil's disease patients in Metro Manila. Patient recruitment will be conducted upon approval of the Single-Joint Research Ethics Board (SJREB). Participants will be recruited only after signing an informed consent form. In the event that the patient is not physically or mentally capable of giving consent, a legally authorized representative or guardian may provide consent on their behalf. Only trained research personnel can and will interact with patients or their legal representatives to be included in the study. The screened patients will be respectfully asked to join the study by explaining carefully the details of the study, especially the responsibilities and rights of the participants, as well as answering their concerns. Patients will be given adequate time to decide

within the study constraints.

For Objectives 1 and 2, the design would be open-label controlled trials. Objective 1 participants would be moderate and severe leptospirosis without hypotension (or those indicated for vasopressor support) AND pulmonary complications (Table 3). These participants will be recruited from both the National Kidney and Transplant Institute and San Lazaro Hospital as part of the multi-center collaboration. They will be randomized to receive either prophylactic plasma component therapy with conventional treatment (PPTTRT) or conventional treatment alone (PPTCONV). For Objective 1 (plasma transfusion), the decision to exclude those with hypotensive episodes is to allocate the patients for Objective 2 (hemoperfusion), where initial evidence had shown significant benefit for those with hypotension (Danguilan et al. 2022, unpublished). According to that study, a calculated 0% (0/18) mortality in HP compared with 36.84% (7/19) in the standard of care was observed. Moreover, Objective 1 may have overt acute kidney failure. As CFI level is a marker for the occurrence of respiratory failure, not renal failure, evidence suggests that plasma intervention works on severe leptospirosis (even those with overt renal failure) in preventing pulmonary hemorrhage. Thus, the study will hereon include patients with overt renal failure as long as there is no pulmonary hemorrhage.

Objective 2 will involve patients with moderate to severe leptospirosis (Table 3) and will be randomized to receive either hemoperfusion treatment with conventional treatment (HPTRT) or conventional treatment alone (HPCONV). In addition to clinical characterization, the baseline blood levels of TNF- alpha, IL6, Gasdermin D, hsCRP, procalcitonin, and CFI (for Objectives 3 and 4) will be measured. Outcomes to be observed will be mortality, end-organ complications, need for emergency procedures, and hospital stay.

Objective 3 will obtain data pertinent to the blood CFI levels of all participants, either in the form of PBMC RNA levels (Objective 3a) or whole blood protein levels (Objective 3b). The assays would be optimized quantitative PCR (qPCR, also known as real-time PCR or RT-PCR). The two sets will be compared for head-to-head correlation and will be optimized as needed depending on observed problems. The process should produce normalized values for the tests with optimized conditions. Although the qPCR has been optimized to measure CFI expression levels, there is a need to verify our conditions in a different laboratory (NKTI setting) and validate these findings in the clinical setting.

Objective 4 aims to correlate CFI gene expression with the eventual occurrence of clinical complications and outcomes. The purpose of this objective involving CFI is to validate if CFI can be used to predict the occurrence of complications. Although such applications may identify who might benefit from interventions such as therapeutic plasma exchange or hemoperfusion, at present, the main aim of the study is to establish the correlation of CFI levels; it will not be used to determine the type of intervention the patients will receive. It will be divided into two sub-objectives. To assess the potential of CFI as a prognosticator, Objective 4a will compare the CFI values of the patient who will eventually have clinical outcomes (death, refractory hypotension, significant organ damage, and prolonged hospital stay) to those who did not upon hospital stay. The patients who belong to the controls (PPTCONV or HPCONV) will be included in the analyses. On the other hand, Objective 4b will compare the CFI values of patients who either responded to PPT or HP. The CFI values will be divided into high (within mean + 2SD of normative value) (CFIHIGH) or low (CFILOW). Subgroup analyses of the effects of the treatment will be compared in the two subsets. Convalescent CFI gene expression will also be determined to see whether the patients were genetically predetermined to develop pulmonary complications (if CFI expression remains low) or whether the reduction in CFI levels was lepto-induced (return to normal levels of CFI).

An overview of the study design is provided in the schema in Figure 1. When a participant is recruited in the study as having moderate leptospirosis without significant pulmonary or renal complications, he/she will be assigned to either Objective 1 (Prophylactic Plasma Transfusion) or Objective 2 (Hemoperfusion) then to be randomized into the treatment group (PPT TRT or HP TRT) or control group (PPT CONV or HP CONV) and will receive their assigned intervention. Patients can deteriorate from the particular objective they are assigned to and the attending physician may employ any rescue treatment, such as Extracorporeal Membrane Oxygenation (ECMO), that he/she may deem necessary.

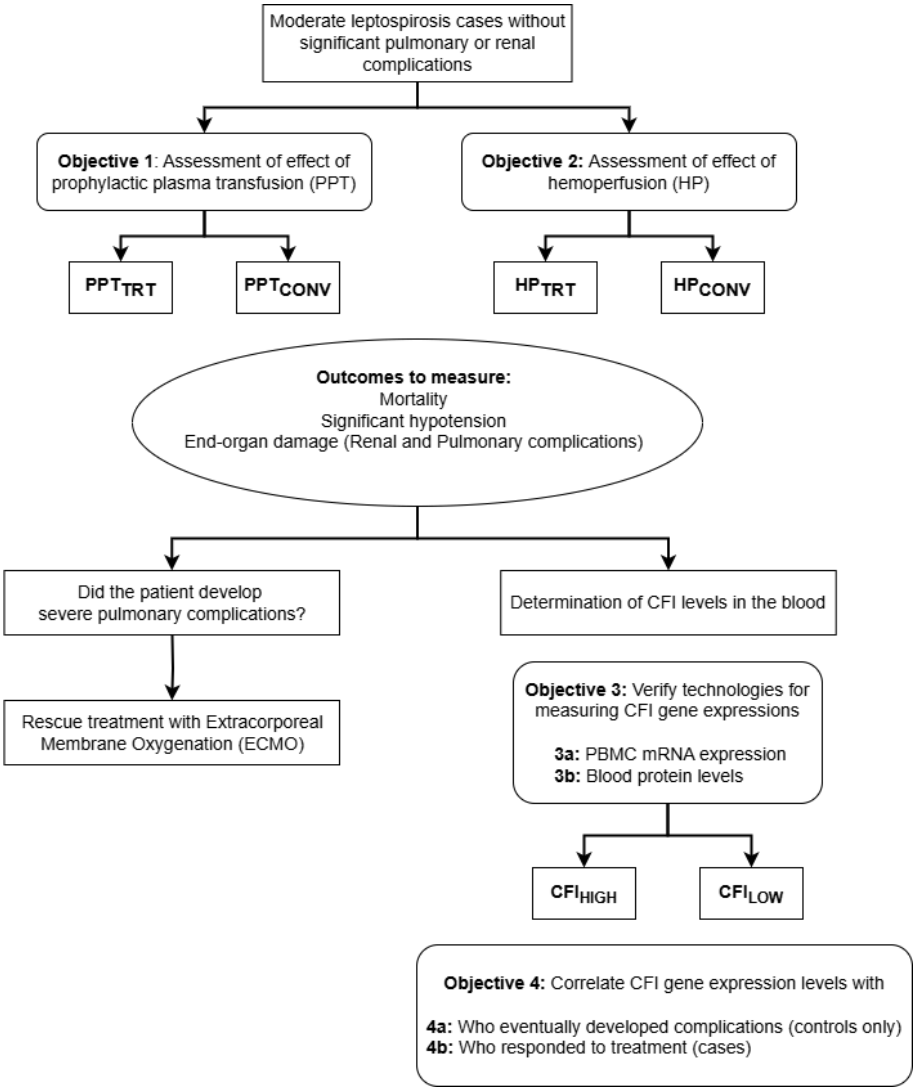



**Figure 1.** Overview of the experimental designs and objectives of the study

**Human subject recruitment**

Subjects will be invited to join the study only after signing an informed consent form (Appendices A and B) duly approved by the ethics review board of the Single-Joint Research Ethics Board (SJREB).

All subjects will be recruited from the emergency rooms, wards, and clinics of the National Kidney and Transplant Institute (NKTI), San Lazaro Hospital (SLH), and other hospitals that admit a

significant number of leptospirosis cases as these hospitals serve as tertiary care centers for Weil's disease patients in Metro Manila. These hospitals have wards, rooms, and intensive care units and have specialized set-ups for tertiary care to address complicated leptospirosis.

**Inclusion and exclusion criteria**

The inclusion and exclusion criteria for the cases are indicated in Table 3. The definitions of the outcomes of interest are summarized in Table 4.

Note that from version 4 onwards, the P/F ratio for inclusion for Objective 1 was increased from 200 to 300. As the study looks into the aspect of plasma transfusion in preventing pulmonary hemorrhage and overt respiratory failure, a higher starting P/F ratio of 300 is proposed rather than 200, which may be clinically late already.

The study will not discriminate against COVID-19 infection. While the effect of COVID-19 infection on CFI levels is not yet known, it would be interesting to note how the co-infection will affect CFI levels through a post-hoc analysis.

**Withdrawal criteria**

A participant will be withdrawn under the following conditions: (1) if s/he withdraws from the study for whatever reason, (2) if the samples are not feasible for testing, and (3) if the study is terminated prematurely. His/her participation in this study is voluntary, and s/he can withdraw anytime, for any reason. This will not affect his/her treatment provided by the participant's doctors. In case s/he withdraws permission, s/he will be withdrawn from the study, his/her record will be deleted from the database, and the sample collected from him/her will be properly disposed of.

**Table 3.** Inclusion and exclusion criteria of participants in the study

| Objectives | Inclusion | Exclusion |
|---|---|---|
| *General* | ● Subjects with acute fever (38ºC for at least two days) and at least one of the following: myalgia, jaundice, headache, meningeal irritation, oliguria, conjunctival suffusion,<br>● Who have a microscopic agglutination test (MAT) that indicates a single serum sample MAT titer ≥1:400,<br>● Or a positive result for the latex agglutination test or a repeat test after seven days,<br>● Or a positive blood culture of leptospira WITHOUT the complication specified in a subgroup of interest,<br>● Should be 18-60 years old | ● Previous diagnosis of chronic kidney disease or on maintenance dialysis<br>● Previous diagnoses of diseases associated with hemoptysis, such as bronchiectasis<br>● Blood dyscrasias, malignancy, severe heart disease, HIV, cavitary PTB, Cirrhosis by ultrasound, severe malnutrition (Wt. < 35kg)<br>● Post cardiac arrest or those with GCS <8 at present. Subject has had chest compressions or CPR.<br>● Pregnancy |
| *Objective 1 (PPT)* | ● As in General<br>● Not requiring ventilator support *(see Case Definitions in Table 4)* | ● As in General<br>● Requiring emergent dialyses *(see Case Definitions in Table 4)*<br>● Significant renal impairment as defined by eGFR<30 |

| | | |
|---|---|---|
| | | ● Significant lung pathology as defined by P/F ratio<300, or obvious respiratory distress<br>● Presence of severe neurological symptoms<br>● Hypotension (or need for vasopressor support)<br>● Ongoing hemodynamic instability |
| *Objective 2 (HP)* | ● As in General, plus:<br>● Dialysis Requiring Acute Kidney Injury. Defined as KDIGO Acute Kidney Injury Stage 3 or requiring renal replacement therapy to correct intractable acidosis, electrolyte abnormality, or over uremic encephalopathy or pericarditis<br>● Vasopressor Requiring – The subject must have received intravenous fluid resuscitation of a minimum of 30ml/kg within 24 hours of eligibility and still with hypotension (blood pressure less than 90/60, MAP < 65) requiring vasopressor support<br>● SOFA SCORE less than 15 | ● As in General |
| **Rescue Treatment** | **Inclusion** | **Exclusion** |
| *ECMO* | ● As in General, plus:<br>● A Murray score of ≥ 2.75 | ● As in General |

**Table 4.** Definitions of clinical outcomes and complications

| Outcomes | Definition |
|---|---|
| **Objective 1 (PPT)** | |
| *Mortality* | Death occurring to be related to the natural course of the present condition of leptospirosis or its complication, but not more than two weeks upon discharge by attending physician after being assessed. as well recovered, or the like. |
| *Leptospirosis with significant pulmonary involvement* | As in leptospirosis (Weil's syndrome) plus evidence of pulmonary injury as indicated by (1) the need for mechanical ventilator support OR (2) P/F ratio <200 OR (3) gross hemoptysis, (4) chest x-ray result consistent with leptospirosis-related pulmonary changes |
| *Refractory hypotension* | Occurrence of systolic blood pressure less than 90 mm Hg, OR mean arterial pressure less than 65 mm Hg, OR a decrease of 40 mm Hg in systolic blood pressure compared to baseline: unresponsive to crystalloid fluid challenge of 20 to 40 mL/kg OR requiring vasopressor support |
| *Leptospirosis with significant renal involvement* | As in leptospirosis (Weil's syndrome), plus evidence of severe acute kidney injury as indicated by the need for emergency dialysis due to intractable acidosis, hyperkalemia, uremic encephalopathy or pericarditis |
| *Hospital stay* | To be computed from the date of admission to the date of discharge. Mortality or discharge against medical advice will be penalized with a maximum stay of at |

| | |
|---|---|
| | least 30 days. |
| **Objective 2 (HP)** | |
| *Mortality* | Death occurring to be related to the natural course of the present condition of leptospirosis or its complication, but not more than two weeks upon discharge by an attending physician after being assessed as well, recovered, or the like. |
| *Need for renal replacement therapy* | Number of days requiring dialysis during hospital stay |
| *Need for inotropic support* | Number of days requiring inotropic support to attain MAP >65<br>Number of days requiring each inotrope |
| *Need for emergent invasive respiratory support* | Inability to maintain Sp02 >92% on maximum non-invasive respiratory support |
| *Need for ECMO* | Attainment of a Murray score ≥ 2.75 or PaO2/FiO2 ratio of less than 200 |
| *Hospital stay* | To be computed from the date of admission to the date of discharge. Mortality or discharge against medical advice will be penalized with a maximum stay of at least 30 days. |
| *ICU days* | Number of days patient is required to stay in ICU (date out of ICU will be date ordered by the attending physician) |

A prospective subject will be screened first based on his/her clinical presentation (Appendix C). If deemed qualified, the prospective subject (or if unable to do so by his/her primary caregiver with legal capacity) will be invited to join the study and, if amenable, will be made to sign an informed consent. The study will recruit subjects who are 18-60 years of age. It will recruit patients with manifestations of leptospirosis, as defined in Table 2, for the specified groups of interest. As much as possible, the blood extraction will be from patients who are receiving initial care from the hospitals. After screening using the clinical criteria, infection with leptospirosis will be confirmed by doing a latex agglutination test (LAT) to be performed in the diagnostics laboratory of NKTI or SLH, and/or the leptospiral microscopic agglutination test (MAT) assays in an accredited institution.

Typically, LAT has the shortest turn-around time, and a positive result becomes the basis for inclusion. For initially LAT negative patients, a repeat LAT will be performed and if positive, the patient will be included in the study. Treatment will be initiated and continued while waiting for the results of the repeat LAT and MAT. If all test results are negative, the patient will then be withdrawn (see withdrawal criteria) from the study, and their data will not be included for analysis. However, their treatment will still continue as their attending physician sees fit.

By definition, the NKTI or SLH Lab services will include procalcitonin and hsCRP assays, while patient tests will consist of routine clinical laboratory tests, like CBC, chemistry, leptospirosis tests, and urinalysis.

Baseline CFI assays will be done on blood collected at most 24 hours after admission. Cytokines will be tested once the inclusion/exclusion criteria are met according to the specific objective. Data, including general information, clinical data, clinical test results, and outcomes, will be collected in a case report form (CRF; Appendix D). In addition to clinical characterization, the baseline blood levels of TNF-alpha, IL-6, gasdermin D, CFI, procalcitonin, and hsCRP (for Objective 2) will be measured on enrollment.

For Objectives 1 and 2, within the first 24 hours of admission, routine laboratory test and imaging data will be expectedly collected as ordered (or else, will be ordered by the project) that include

complete blood count, hemoglobin, hematocrit, creatinine, blood urea nitrogen, serum potassium, chest radiograph, arterial blood gases, protime and partial thromboplastin time, as well as the leptospirosis latex agglutination test and microagglutination tests. Three sets of 4-ml blood samples for CFI assays, pre- and post-transfusion, defined as taken within 24 hours before and after the first and last unit transfused, respectively. Other tests ordered by the attending physician during the hospital stay will be collected.

Additionally, for all procedures, additional imaging and/or tests and monitoring parameters will be at the discretion of the attending physicians. They should be considered as part of the standard of care.

The participants will be randomized for inclusion in Objective 1 or 2 using a preset assignment in which the assigner is blinded.

**Sample size calculations**
The minimum computed sample sizes are provided in Table 5.

For Objective 1, a computed sample size of at least 17 per group (PPTTRT and PPTCONV) was calculated based on the following assumptions: using an independent open-label randomized controlled trial, using hospital stay as a primary outcome, a reduction mean of at least three days in PPTTRT compared with PPTCONV with a standard deviation of 3 days, a one-tailed $\alpha$=0.05; power of 0.80; and a 1:1 case-control ratio. If this sample size takes into consideration adjustments for Objective 4b, in which a minimum sample size should be adequate to assess the effect of low CFI subpopulation (estimated at 12% of the total population), then the estimated sample size would become 142 per group.

For Objective 2, a computed sample size of at least 197 leptospirosis patients for hemoperfusion treatment and 197 patients to undergo standard treatment was calculated based on the latest preliminary data from NKTI showing a 42% risk reduction in mortality among those on hemoperfusion and ECMO. For moderate leptospirosis with an estimated 15% mortality among those on standard care (usual mortality is 5%-15%), the 42% risk reduction translates to 6.3% mortality among those on HP and ECMO. Other assumptions used are alpha of 0.05, power of 0.80, and 1:1 exposed/unexposed.

Taking into account the limited capacity (in terms of equipment and expertise) of NKTI, the number of ECMO procedures as a rescue treatment to be observed will only be 16. This will be the largest case series to describe the potential benefit of ECMO as a life-saving procedure in leptospirosis.

**Table 5.** Estimated sample size of planned clinical groups in the study

| Groups | Description | Estimated Sample Size |
|---|---|---|
| **$PPT_{TRT}$** | Cases for prophylactic plasma transfusion | 142 |
| **$PPT_{CONV}$** | Controls for prophylactic plasma transfusion | 142 |
| **$HP_{TRT}$** | Cases for hemoperfusion | 197 |
| **$HP_{CONV}$** | Controls for hemoperfusion | 197 |
| **TOTAL** | | 678 |
| **ECMO** | Cases that will undergo ECMO as a rescue treatment | 16 |

**Plasma transfusion**

This is for Objective 1 of the project. The assignment of treatment groups will be decided upon recruitment by a random picking of envelopes. Plasma transfusion will commence within 24 hours after inclusion in the study as long as the patient remains eligible. As the study considers the inclusion of overt renal failure as an inclusion in Objective 1 (plasma transfusion), the hemodialysis for the plasma transfusion group will proceed as a preemptive procedure to prevent plasma-induced overload. As for the controls, hemodialysis will depend on the attending physician's decision as part of the standard of care.

Participants in the plasma transfusion group will receive transfusion if the PBMC CFI qPCR deltaCT is found to be at least 25 or more; this was based on the preliminary data gathered by the research team with a mean of 23 using the present set of primers. The previous study of Nevado et al. (unpublished) indicated that all patients with eventual pulmonary complications had a PBMC CFI qPCR deltaCT of a mean of 15.68 with a range from 14.79 to 17.04 and a standard deviation of 0.91. The control has a mean deltaCT of 12.41 with a standard deviation of 0.42.

ABO/Rh-type compatible fresh frozen plasma (FPP) units will be thawed to 37° prior to administration. Plasma transfusion will be administered intravenously, 1 unit for 4 hours every 12 hours for two consecutive days. There will be two consecutive days for the transfusion for a total of 4 units. For practical reasons, the FFP units should be processed and procured through DOH-accredited blood banks specifically from NKTI and SLH Blood Banks, which are properly screened for pathogens, typed, and clinically cleared for use. This will allow fresh frozen plasma readily available for both sites and can have the option to have it transported when needed.

Buffer stocks of FFPs will be stored in -80 freezers designated for the project at any time and will be constantly refilled daily if needed. Different blood type-specific FFPs will be stored to accommodate at least 12 patient-regimen, with 6 being type O and 2 each for types A, B, and AB. Due to a lack of availability, the project will not stock Rh- FFPs.

During the transfusion, parameters will be closely monitored to ensure the safety of the interventions. The following symptoms will require stoppage of the transfusion (as adapted from the proposed guidelines of the Lung Center of the Philippines): (1) hives, pruritus, flushing, swollen lips, tongue or uvula; (2) new or worsening dyspnea, wheezing, stridor, hypoxemia; (3) hypotension, systolic or diastolic drop of >30% from baseline; (4) tachycardia or bradycardia; (5) syncope; confusion or decrease in sensorium; and (6) other signs or symptoms for which the attending physician determines warrants the discontinuation of infusion (such as gastrointestinal symptoms) (63).

In addition to the pre-treatment CFI serum ELISA, post-treatment serum CFI ELISA will be performed for those who were plasma transfused within 3 hours after the last transfusion. Serum CFI ELISA will also be performed on admission and on day 3 or 4 of the control group (PPTCONV).

**Hemoperfusion (HP)**

The HP procedure will follow the standard procedure of NKTI using Jafron HA330 hemoperfusion cartridge. First, an internal jugular catheter is attached to the patient. Alternatively, an arteriovenous fistula or arteriovenous graft may be placed on the patient. The patient will then be hooked to a hemodialysis machine. Blood pump speed will be set to 150-200mL/min, and HP will last for 2 to 2.5 hours. Whole blood will flow through the sorbent HA330 cartridge and back to the patient. Anticoagulation is not necessary due to the short treatment time. Hemoperfusion will be repeated after 12-24 hours for at least three days.

For cytokine assays, ELISA will be performed for serum TNF-alpha, IL-6, and gasdermin D quantification, immunoturbidimetric assay for hsCRP, and chemiluminescent microparticle immunoassay (CMIA) for procalcitonin as serviced by NKTI and SLH Medical Laboratories. Pre-HP assays will be done upon recruitment, and post-HP assays will be done within 3 hours after the 3rd HP session. For the control group (HPCONV), cytokine assays will be done upon recruitment and on day 2 or 3 of hospitalization.

**Extracorporeal membrane oxygenation (ECMO)**
The procedure is adapted from Lee et al. (80). A veno-venous ECMO (VV ECMO) will be applied to the patients by aseptically inserting a venous cannula into the femoral veins. The patients will be hooked to an ECMO machine. For anticoagulation, patients without significant bleeding or vascular intervention will be managed with an activated clotting time set at 140–180 sec by 800–1000 U/h of heparin. Otherwise, the heparin will be titrated to maintain a partial thromboplastin time of 60–80 sec.

ECMO flow will be maintained at a mean blood pressure of >60 mm. SaO2 will be maintained at >90% with a flow of 3.5–4.5 L/min. Hematocrit will be maintained at >35% and platelets >50000–100000/mL. Transfusions will be done when necessary. ECMO will be weaned when arterial blood gas analysis revealed pH 7.35–7.45, PaO2 >80 mm Hg, and PCO2 <45 mm Hg under the following conditions: a gas blender FiO2 of 0.21, sweep gas of 0 L/min at an ECMO flow of 2 L/min, and the ventilator mode (if applicable) set to an FiO2 of 0.6, a tidal volume of 6 mL/kg, a PEEP of 8 cmH2O, and an RR of 12–16/min for VV ECMO or 3 L/min of O2 via nasal prong with awakening ECMO patients.

**Sample collection and total RNA extraction from peripheral blood mononuclear cells**
Blood extractions explicitly for the initial qPCR and ELISA for CFI and cytokine levels will be performed immediately upon recruitment, within 24 hours upon admission. Baseline levels of TNF-alpha, IL6, gasdermin D, procalcitonin, and hsCRP will be taken from participants in Objective 2. The assay will be repeated within 24 hours after initial hemoperfusion. Also, blood CFI protein determination will be repeated within 24 hours after plasma transfusion in participants in Objectives 1. In addition, a random sample of 20 participants in the study will be selected for blood extraction upon discharge to assess CFI protein levels in the convalescent phase of infection in patients with initial low CFI levels. RNA extraction, qPCR, and ELISA procedures will be done at the NKTI, SLH, or the Microarray Core Laboratory of the Institute of Human Genetics, National Institutes of Health-University of the Philippines-Manila (IHG, NIH-UPM). Random sampling of blood for CFI assay upon discharge will demonstrate the effect of convalescence on the level of the CFI upon recovery. This will give us an idea of whether CFI levels are affected by the presence of infection or an inherent deficiency.

For RNA extraction and serum processing, whole blood amounting to about 4 ml (4 ml with anticoagulant for RNA extraction and serum processing) will be collected. Samples for qPCR analysis will be stored in EDTA-containing blood tubes, and the samples for ELISA will be stored in blood tubes without additives. Blood tubes will be stored immediately in ice-cold containers. The other whole blood aliquot collected in the tube without additives will be centrifuged in order to obtain serum. The serum samples will then be stored at -80°C until ELISA is performed. Peripheral blood mononuclear cells (PBMCs) will be isolated from whole blood collected in the EDTA-containing blood tube within 2 hours after extraction through Ficoll density gradient centrifugation using Lymphoprep (Stemcell Technologies, Vancouver, Canada). Isolated PBMCs will be temporarily stored at -80°C until RNA extraction.

RNA extraction of the PBMC will be performed using the QIAmp RNA Blood Minikit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Briefly, the PBMC pellets will be mixed with 600 µl of buffer RLT, which will be placed in a QIAshredder spin column in a 2 ml collection tube that will be spun at maximum speed for 2 min. To the homogenized sample, 600 µl of 70% ethanol will be added and mixed by pipetting. The samples will be placed in a new QIAamp spin column and centrifuged for 15 seconds at >8000 x g. After discarding the eluate, the samples will be added with 700 µl Buffer RW1, centrifuged for 15 s at >8000 x g, and the eluates will be discarded. Two sets of washings with Buffer RPE will be done with successive centrifugations at >8000 x g for 15 sec and at full speed for 3 min. In a new 2-ml tube, the samples will be further centrifuged at full speed for 1 min. The RNAs will be eluted by two successive applications of 40 µl of RNase-free water with centrifugation at >8000 x g for 1 min. RNA quantification and OD260/280 determination will be done using an ND1000 NanoDrop spectrometer. Samples will be kept at -80°C until use.

**Quantitative polymerase chain reaction**

Reverse transcription will be performed using an iScriptTM cDNA synthesis kit (Biorad). Each reaction will contain 1 µl reverse transcriptase, 4 ul reaction buffer, and 200 ng of RNA sample, to be added to nuclease-free distilled water and appropriate primers for a total of 20 µl samples. All preparations will be performed on ice. Primers will be designed to have at least one of a primer pair per segment of interest to bridge across exon-exon borders to lessen the possibility of genomic amplification. Reaction conditions will be 25°C for 5 min, 42°C for 30 min, 85°C for 5 min, and 4°C onwards. Quantitative PCR will use the SsofastTM EvaGreen kit (Biorad). One reaction mix will contain 2 µl cDNA from the previous reverse transcription samples, 2 µl each of 5 mM forward and reverse primers, and nuclease-free water for a total of 20 ul. Quantitative PCR conditions to be used will be as follows: 95°C for 5 min, with 39 cycles consisting of 95°C for 45 sec, chosen annealing temperature for 45 sec, 72°C for 45 sec, read at 78°C, and 95°C for 45 sec. Melting curve determination will range from 72°C to 95°C with 0.2°C increments. The primers that will be used for the CFI qPCR are CFI forward, 5′- ACACAGAAAGCAGATTCTCCA–3', and CFI reverse, 5′-ACAATGTGCAGCAGTCAGAA-3', with an annealing temperature of 58°C. The 18S mRNA will serve as the internal control with the following sequences: 18S forward 5'-GCTTAATTTGACTCAACACGGGA-3' and 18S reverse 5'-AGCTATCAATCTGTCAATCCTGTC-3' (71).

**Enzyme-linked immunosorbent assay**

The assay will utilize an in vitro competitive ELISA for CFI (Abcam, HK, catalog no. ab195460). The 96-well plate is precoated with anti-CFI antibody. Sera will be prepared from blood samples that would be clotted for not more than 3 hours and centrifuged at 3000g for 5 min. Sera will be stored at -80°C until use.

On the day of assay, the sera will be diluted at 20x with PBS as per the instruction of the manufacturer. Next, 25 µL of CFI standard (as supplied with dilution ranging from 0 [blank] to 24 µg/mL) or sample per well was added, together with 25 µL of 1X biotinylated CFI to each well (supplied) and incubated for 2 hours at room temperature. After washing with 200 µL of supplied 1X Wash Buffer, 50 µL of 1X SP Conjugate (supplied) was added to each well and incubated for 30 minutes. After washing, 50 µL of Chromogen Substrate (supplied) was added per well and incubated for 10 minutes. Fifty µL of Stop Solution (supplied) was added to each well. The plate was read at 450 nm.

For cytokine assays, sandwich ELISA with standards will be performed for TNF-alpha, IL-6, and

gasdermin D quantification.

**Outcomes and follow-ups**
For Objectives 1 and 2, 3 sets of 4-ml sera will be taken before and after the transfusion and/or hemodialysis/hemoperfusion to determine blood levels of CFI, and other cytokines that may be deemed relevant for monitoring.

For Objectives 1 and 2, the patients will be followed up during their hospital stay or until the 30th day post-admission. Prolonged hospital stay will be an independent outcome from multi-organ failure, which has a separate definition. However, in the presence of both outcomes, patients with prolonged hospital stays will be interpreted as having multi-organ failures in hierarchical outcome designation in composite analyses (1 outcome per participant). The separate designation for prolonged hospital stay is in recognition that it can be due to other causes that may lead to such outcome, including nosocomial infections, interventional complications, and disease deterioration. A daily monitoring form was incorporated to record the progress of the participants (Case Report Form; Appendix D); it contains the following information: the dose and time of administration of the administered plasma, hemoperfusion, and ECMO intervention; daily monitoring of signs and symptoms associated with the plasma, hemoperfusion, and ECMO intervention; the list of drugs used, their doses and schedule; and the outcomes of interest as stipulated in this protocol (mortality, with significant pulmonary involvement, with significant renal involvement, and hospital stay).

The participant is expected to participate during his/her hospital stay or until the 30th day after inclusion in the study, whatever is shorter.

**Statistical analyses**
For Objectives 1 and 2, we will determine differences in categorical outcomes (mortality, presence of shock) using chi-square tests and continuous variables (number of hospital days, number of days in dialysis) using the Student's T-test. The time-to-event analysis will be done using Kaplan-Meier curves if applicable.

For Objective 2, interim analyses will be done at every quartile (50th, 99th, and 148th sample for each group is completed) of the initial estimated sample size (198 per group), with significance set at $p<0.01$.

According to the latest study on the use of HP on severe leptospirosis by Danguilan et al. (2022, unpublished) as a basis, a calculated 0% (0/18) mortality in HP compared with 36.84% (7/19) in the standard of care was observed. The present study will serve as validation. Hence, in a more conservative scenario of 2% mortality in controls and 30% in cases, the minimum sample size to attain significance at $p<0.01$ at minimum power of 90% is 48 per group. If this is observed, the study group will report to the ethics committee and will request for study pre-termination. Similar assumptions will be applied to Objective 1.

For those that undergo ECMO as a rescue treatment, descriptive analytics will be done, indicating means and ranges of hospital stay, number of mortalities, and complications.

For Objective 3, correlation of values of qPCR results to ELISA data will be performed using Pearson correlation (r2 of 0.80 or higher will be considered highly correlated). Test for concordance using Kendall's W may be done.

For Objective 4, receiver operator characteristic analyses for qPCR and ELISA will be compared for

the eventual development of adverse outcomes using the Wilson-Brown method. Percentage values will be used. An area under the curve of at least 0.80 is considered significant. Data will be analyzed using the deltaCT method. The means and standard deviation will be analyzed using ANOVA or student t-test (for parametric comparison), Kruskal-Wallis test or Wilcoxon test (for nonparametric comparison), or the Chi-square or Fisher exact tests for association with categorical variables depending on the quantitative or qualitative nature, respectively, of the clinical outcomes. A p-value of <0.05 is considered significant. Sensitivity, specificity, and predictive values will be computed for each pair of relevant tests and outcomes, such as mortality, end-organ failure, shock, and hospital stay.

The data of the patients will be encoded and summarized in a Microsoft Excel sheet and analyzed using GraphPad Prism v10.

**Biosafety considerations**

All biological samples from infected subjects of objectives 1 and 2 will be processed under a biosafety cabinet, at least of type 2A, with researchers donning proper PPEs. All disposed samples and containers will undergo decontamination with 10% hypochlorite and autoclaving in a sealed container prior to disposal in a biohazard container under 121°C (250°F) for around 15–20 min prior to disposal as biohazard waste.

**Ethical considerations**

The project leader, co-investigators, and designated clinical research assistants are responsible for the recruitment of human subjects. All research staff will undergo training on Good Clinical Practice (GCP) prior to interviewing patients. Patient recruitment will be conducted upon approval of the ethics review board of the Single-Joint Research Ethics Board (SJREB). Participants from the emergency rooms, wards, and clinics of the NKTI and SLH will be recruited only upon willful signing of an informed consent form. In the event that the patient is not physically or mentally capable of giving consent, a legally authorized representative or guardian may provide consent on their behalf.

The primary investigator or designated clinical research assistant will provide the necessary information about the study and will answer questions from the potential participant. It is important to ensure that the participant will read and understand the contents of the Informed Consent, or if the participant desires, the research assistant will read and explain the contents of the ICF.

Only trained research personnel can and will interact with patients or their legal representatives to be included in the study. The screened patients will be respectfully asked to join the study by explaining carefully the details of the study, especially the responsibilities and rights of the participants, as well as answering their concerns. Patients will be given adequate time to decide within the study constraints.

Medical information, if warranted, will be obtained from medical records, including age, sex, address, case number, laboratory data, and clinical metadata. To gain access to medical records, a request letter will be sent to the medical director of NKTI or SLH, where recruitment will take place. Further, the informed consent will also stipulate permission from the participant to access his/her medical records. Only upon the provision of official permission to access medical records and the signing of the informed consent by the participant will the records be scrutinized and their relevant data abstracted to study forms and databases. Medical records can only be accessed by investigators and research assistants of the study, and data from the records will be treated with utmost confidentiality.

Several measures will protect the data confidentiality and privacy of the participants. All records or personal information related to the study will be kept confidential. These will be coded and kept in secured/locked cabinets and password-protected computers. Any genetic, clinical, or personal information directly traceable to the participant will not be released to others, including family members, without written consent from him/her. All samples will be kept in freezers, with the key kept by authorized personnel only.

The research assistant will also inform the participants that the samples may be stored for at most five years from the end of the study and may be used for future studies upon his/her consent. The informed consent form of the study states that the samples will be stored for at most five years and may be used for future studies upon consent of the participant. Biological samples will be stored in -20°C to -80°C freezers and will be kept at most for five years either at the National Kidney and Transplant Institute (NKTI), San Lazaro Hospital (SLH) or at the Institute of Human Genetics, NIH-UP Manila. The stored samples may be used for future studies only upon the consent of the participant and provided that the identifying data will be removed from the samples. If the participant did not give his/her future use consent, s/he can still participate in the present study as long as informed consent is provided for the present study. In this case, samples will only be used for the present study. The sample collected will be properly disposed of 1 year after this study.

However, the results of the study may be presented at scientific or medical meetings and conferences or published in scientific articles. To maintain privacy, there will be no mention of the name or any information of the participants that can be traced directly to him/her. Aside from project investigators and staff of the current, the study monitor(s), auditor(s), the institutional ethics review board of NKTI, UP Manila, SLH, SJREB and regulatory authorities will be granted direct access to the participants' medical records for purposes only for verification of clinical trial procedures and data. This also applies to future research/es in which the data from the participants are to be used.

The participants have the right to know the full results of this study, and they can request the results of the study. Moreover, though they cannot have direct access to their records, they still maintain their right to know data relevant to them.

The participants or their legal representatives will be informed if there are changes or amendments to the information they supplied in this study.

As the study assists standard of care testing and treatment for all participants, there will be a direct benefit for the patients. However, the results of the study are still of unknown significance, given the current scientific knowledge on leptospirosis. Thus, the participants are also encouraged to communicate anytime with the primary investigator concerning the results of the study and their impact on their health.

All expenses directly related to the use of fresh frozen plasma for the transfusion procedure under Objective 1 will be covered by the project budget. For patients under Objective 2, the study will cover the expenses for a maximum of three hemoperfusion cartridges. Inflammatory marker blood tests, such as Interleukin-6 (IL-6), High-sensitivity C-reactive protein (hsCRP), Procalcitonin, TNF-alpha, and Gasdermin D will also be covered for patients under Objective 2. For patients that will undergo ECMO as a rescue treatment, the study will cover the expenses for the ECMO device, cannulae, and insertion kits used in the procedure. The study will also cover the expenses for the following interventions for all patients, regardless of treatment assignment: Intravenous fluids and medications up to Php 2000.00 and standard-of-care laboratory tests up to Php 2000.00 for participants without other available sources or medical insurance (such as PhilHealth), as

applicable.

The study will still cover the expenses mentioned above for initially LAT negative patients while waiting for the results of the repeat LAT and MAT.

Each participant will receive a Php 1000 monetary remuneration for his/her participation in the study and Php 2000 for intravenous fluids and medicine. If needed, he/she is still expected to spend for transportation on follow-up. Such expenses will not be directly reimbursed, but the study will provide compensation during follow-up to cover food, transportation, and the possible loss of income. Furthermore, s/he will be supported for the following tests, if needed: test for leptospirosis, serum creatinine, blood urea nitrogen, chest X-ray, complete blood count with differential count, and urinalysis. If s/he prefers, health information regarding leptospirosis will be offered. The remaining expenses will be sourced from the participant. The DeLEPTO Project 1 Project Development Officer III is responsible for reviewing, approving, and monitoring the coverage of all expenses. This process is coordinated with the project's clinical team, who will ensure that any intervention aligns with the project objectives and the patient's classification.

There will be risks involved in the conduct of the study. There is minimal risk associated with blood extraction provided that the subjects are hemodynamically stable, without respiratory distress, and have a hemoglobin level of not less than 90 g/li. These include bleeding from the site of injection, pain, local reaction to the tape or cotton, and infection. For patients undergoing plasma transfusion, there may be adverse effects, such as difficulty in breathing, itchiness, rashes, swelling, lowering of blood pressure, and faster or slower beating of the heart. For patients undergoing hemoperfusion, there may be some events such as bleeding in wound sites and other parts of the body, low blood pressure and palpitations, as well as temporary lowering of platelet count that may need monitoring. For patients undergoing ECMO, there may be bleeding in wound sites and other parts of the body, sudden changes in blood pressure and heart rate, and infection in areas where the body is attached to the machines. Moreover, there is also the possibility of the machine having some failure. In most instances, most side effects are mild, and serious occurrences are less than 5%, with the potential benefit projected to be more than the risk of harm for the patients' condition.

Suppose there is a persistence of symptoms (such as rashes and itchiness), sudden difficulty of breathing, new onset of pain, severe bleeding that will not stop with gentle pressure, and worsening fever or weakness. In that case, it is advised that Dr. Romina Danguilan of NKTI and Dr. Rontgene Solante of SLH be consulted. The participant can consult any emergency room/clinic/doctor (preferably NKTI or SLH) if any of the above occurs. Less serious adverse events can be consulted through out-patient consultation in clinics. A portion of the project fund is allocated to support affected participants in the event of hospitalization and/or required treatment resulting from study-related adverse events or complications.

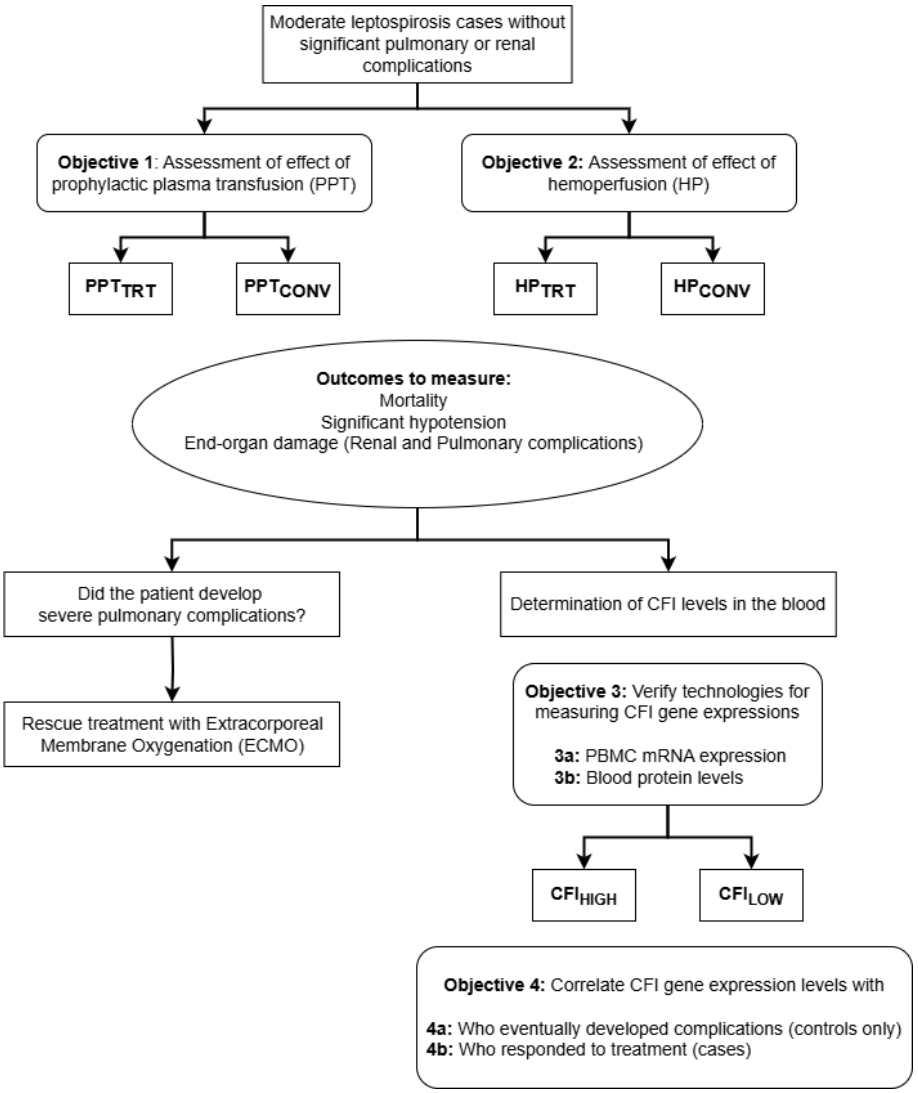
A participant will be withdrawn under the following conditions: (1) if s/he withdraws from the study for whatever reason, (2) if the samples are not feasible for testing, and (3) if the study is terminated prematurely. His/her participation in this study is voluntary, and s/he can withdraw anytime, for any reason. This will not affect his/her treatment provided his/her doctors. In case s/he withdraws permission, s/he will be withdrawn from the study, his/her record will be deleted from the database, and the sample collected from him/her will be properly disposed of.

Considering the risk associated with leptospiral infection, while handling infected samples, precautions will be taken to protect the research staff and other people s/he will interact with. First, research personnel will wear recommended protective personnel equipment (PPEs) at all times in

compliance with institutional protocols. Staff who will be facing participants should have training on integrating with patients, donning and doffing procedures of PPEs, and sample handling and disposal. Budget will be allocated for testing for leptospirosis using latex agglutination tests. In case of hospitalization, a budget will be allocated for emergent care, in addition to the universal health care insurance by the government.

Dr. Nevado declares a potential conflict of interest as he has a patent for a CFI quantification kit for the prognostication of leptospirosis for its pulmonary complications. Otherwise, Dr. Danguilan, Dr. Chavez, Dr. Arakama, Dr. Solante, Dr. Sayo-Abungan, Dr. Verona, Dr. Trifalgar-Arches and Dr. Lee do not have any conflict of interest to declare. Drs. Chavez, Arakama, Solante, Sayo-Abungan, Verona, Trafalgar-Arches and Danguilan may serve as both investigators and the participants' attending physicians, but Dr. Nevado and Dr. Lee will not.

8. **Theoretical Framework:** *Description of the theory on why the research problem exists. It can also be a diagram; just put the image here*




9. **Expected Outputs (6Ps)**
    a. **Publication:** *published aspect of the research, or the whole of it, in a scientific journal or conference proceeding for peer review or in a popular form*
    This project will hopefully produce data on the clinical effectiveness of prophylactic plasma

transfusion, hemoperfusion, and ECMO for leptospirosis. In addition, it can provide evidence of the use of CFI as a prognostic biomarker and of the different preemptive medical procedures that might prevent the development of complications in leptospirosis. Insights gained from this data will be published in at least one (1) peer-reviewed indexed international journal.

b. **Patent:** *proprietary invention or scientific process for potential future*
The investigators are currently applying for a patent for CFI as a potential prognostic marker for pulmonary complications in leptospirosis and related technologies. For now, no additional patents will be sought.

c. **Product:** *invention with a potential for commercialization*
Quantitative diagnostic and prognostic kits in the form of optimized qPCR and possible ELISA kits for the early risk assessment of leptospirosis will hopefully be developed.

d. **People Service:** *people or groups of people who receive technical knowledge and training*
This project will provide opportunities for pulmonary fellows to be trained on pre-emptive medical procedures such as ECMO and HP. Medical technologists in hospitals could also benefit from learning qPCR and ELISA techniques that will be important in assessing CFI gene expression levels.

e. **Place and Partnership:** *linkage forged because of the study*
A multi-site approach will be implemented in this project, with NKTI and San Lazaro Hospital responsible for the clinical aspects of the project, and IHG-NIH leading the molecular tests and analysis. In addition, as part of the DeLEPTO program, these institutions will also be in close collaboration with labs from UP Diliman and UP Manila, which are responsible for the basic science and pre-clinical aspects of the program.

f. **Policy:** *science-based policy crafted and adopted by the government or academe as a result of the study*
The clinical insights to be gained from this study will aid in informing clinical guidelines for the improved management of leptospirosis patients.

10. **Potential Outcome:** *refers to the result that the proponent hopes to deliver three (3) years after the successful completion of the project*
The procedure for ELISA and qPCR were mentioned in the methods. The study will generate quantitative prognostic kits in the form of optimized qPCR and possibly ELISA kits for the early risk assessment of leptospirosis, especially those who could develop pulmonary complications; these will serve as prototypes of the kits. This may be included in hospital and clinic routines to provide aid in admission and preemptive treatment. In addition, the development of digital droplet PCR kits is the foremost objective of Project 4-LIGTAS, to be done in close coordination with Project 1.

    The investigators will be able to deliver the results as long as the expected number of patients are admitted as planned. The NKTI is a renal referral center for leptospirosis patients with kidney failure requiring dialysis. San Lazaro Hospital is a tertiary care center which receives leptospirosis patients of varying severity even during off-seasons. Thus, as referral centers in the middle of Metro Manila, they have the catchment areas for the specific disease that is the focus of the study.

11. **Social Impact:** *refers to the effect or influence of the project on the reinforcement of social ties and the building of local communities.*

Leptospirosis is a devastating disease affecting the financially disadvantaged population. The male breadwinner of families is usually affected, and their death causes families extreme dysfunctionality. Investing in innovative treatments that can lead to saving the lives of these patients is evidence that the government considers the preservation of their health important to the community.

12. **Economic Impact:** *refers to the effect or influence of the project on the commercialization of its products and services, improvement of the competitiveness of the private sector, and local, regional, and national economic development*
    Leptospirosis has a very high mortality rate. Therapies aimed at lowering the mortality from leptospirosis from innovative therapies ultimately lead to the preservation of the workforce and a positive impact on the economy.

    Meanwhile, the kits may be produced by biotechnology companies that specialize in medical testing. With the prevalence of reported leptospirosis of about 5,000 a year and nearly three times that number of suspected cases, the potential coverage could be estimated at 20,000 patients who are admitted to large hospitals capable of HP or ECMO.

13. **Target Beneficiaries:** *Refers to groups/persons who will be positively affected by the conduct of the project.*
    a) The Filipino patients who can directly benefit from early prognostication and the companion early treatment;
    b) The clinicians, who can benefit from potential products and can help decide on the proper treatment of their patients;
    c) The health institutions, particularly the hospitals, in their wise allocation of resources and workforce to treat higher-risk individuals;
    d) The basic science researchers in both private and public sectors, as they further their technologies to develop better testing as well as create preemptive innovations, not only for leptospirosis but for other similar conditions as well (such as dengue and sepsis).
    e) The biological/medical industry, as any product related to this study, could be exploited economically and socially for the benefit of the global audience.

14. **Sustainability Plan:** *refers to the continuity of the project or how it shall be operated amidst financial, social, and environmental risks.*
    The main challenge is the inherent seasonal occurrence of leptospirosis. By involving NKTI and SLH, which typically have more than 20 patients with leptospirosis even in off-season, the project has a good possibility of completion.

    Another challenge is the logistics of the hospital. Seemingly, this can be overcome by the backup facilities in NKTI and SLH, such as the qPCR machines and freezers that had been operational before due to the COVID-19 pandemic. These, as well as the newly established research center, may serve as counterpart facilities during the implementation.

15. **GAD Score:** *refers to the result of accomplishing GAD checklists for project monitoring and evaluation/project management and implementation) to highlight the contribution of the project in the achievement of the objectives of Republic Act 7192, "Women in Development and Nation Building Act," interpreted as gender-responsive gender-sensitive, has promising GAD concepts, or GAD is invisible.*

    **GAD score = 15.49**

16. **Limitations of the Project:** *refers to restrictions or constraints in the conduct of the project.*
Since protocol development was jointly prepared by consultants of the NKTI and the other members of the DeLEPTO team, there are no major constraints foreseen. The objective of the study will be clearly explained to possible participants.

17. **Risk:** *refers to an uncertain event or condition that its occurrence has a negative effect on the project.*
Suppose patients who are affected by floods are given timely prophylaxis. In that case, this will result in a decreased number of patients who will develop complications from leptospirosis. This is considered an ideal outcome for patients. However, this will also result in a decrease in patient enrollment, and in turn will lead to a longer recruitment period for the study.

18. **Assumption:** *refers to an event or circumstance that its occurrence will lead to the success of the project*
The rainy season every year starts in June, and if there has been no substantial improvement in the drainage system of Metro Manila's streets, then floods are to be expected. If those who are exposed to floods are not provided with timely prophylaxis then they are at high risk for developing leptospirosis. Patients exhibiting symptoms of leptospirosis are brought to SLH, while patients who develop renal failure from leptospirosis are usually brought to the NKTI for dialysis. These patients are the participants in the present study.

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## 20. Line-Item Budget

(Find attached LIB form)

## 21. List of Personnel

| Position/ Designation | Salary Grade | Number of Positions | Duration | Percent Time Devoted to Project | Responsibilities |
|---|---|---|---|---|---|
| Science Research Specialist II | 16 | 5 | 36 months | 100% | 1. Provide hands-on assistance on the experiments;<br>2. Report progress of the study to the project leader;<br>3. Present the results of the study in meetings; and<br>4. Regularly attend meetings for feedback relevant to the experiments |
| Project Development Officer III | 18 | 1 | 36 months | 100% | 1. Establish/organize the schedules of meetings and representation relevant to the projects<br>2. Prepare the administrative needs necessary for continuing operation of the projects<br>3. Coordinate the smooth flow of acquisition and procurement of supplies and equipment of the project<br>4. Prepare and archive documentary requirement for administrative purposes, such as financial reports, progress reports, annual reports, attendance sheets, and worksheets<br>5. Liaise with sponsors and |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | various stakeholders and collaborators<br>6. Record and document relevant communications<br>7. Assist in other administrative concerns as deemed necessary |
| Project Leader | Hono | 1 | 36 months | 15% | 1. Directly provide supervision to the research staff;<br>2. Submit progress and financial reports to research sponsors regularly;<br>3. Coordinate activities of various collaborators/centers involved in the study;<br>4. Keeps records and backup files for the study;<br>5. Ensure that guidelines are followed in their respective projects, including the consent forms;<br>6. Organize meetings and presentations pertinent to the study;<br>7. Ensure proper training of research staff;<br>8. Approve final write-ups prior to publication.<br>9. Analyze and interpret results;<br>10. Actively participate in the publication and protection of data/intellectual property. |
| Project Staff Level III | Hono | 4 | 36 months | 15% | 1. Assists the research staff;<br>2. Assist in sample collection;<br>3. Keeps records and backup files for the study;<br>4. Ensure that guidelines are followed in their respective projects, including the consent forms;<br>5. Organize meetings and presentations pertinent to the study;<br>6. Approve final write-ups prior to publication. |
| S&T Consultant | Hono | 2 | 36 | 15% | Refer patients for consideration for recruitment |

**22. List of Official Project Team Members**

| Title | First Name | Middle Name | Last Name | Sex | Expertise | Institutional Affiliation | Role |
|---|---|---|---|---|---|---|---|
| Dr. | Romina | A. | Danguilan | F | Nephrology | NKTI | Project Leader |
| Dr. | Joselito | R. | Chavez | M | Pulmonology | NKTI | Project Staff |
| Dr. | Jose | B. | Nevado | M | Internal Medicine/ Molecular Biology and Biotechnology | IHG-NIH | Project Staff |
| Dr. | Mel-hatra | I. | Arakama | F | Nephrology | NKTI | Project Staff |
| Dr. | Rontgene | M. | Solante | M | Infectious Diseases | SLH | Project Staff |
| Dr. | Ana Ria | | Sayo-Abungan | F | Infectious Diseases | SLH | Project Staff |
| Dr. | Jeffrey | A. | Verona | M | Infectious Diseases | SLH | Project Staff |
| Dr. | Jaime | D. | Trifalgar-Arches | F | Infectious Diseases | SLH | Project Staff |
| Dr. | Nathaniel | | Lee | M | Infectious Diseases | SLH | Project Staff |

**23. List of Equipment for Project**

| Name of Equipment | Existing Equipment in the Agency | To be purchased | Justification for Purchase |
|---|---|---|---|
| Qualitative PCR machine | 0 | 1 | For reverse transcription and melting curve analysis |
| Ultra Low Freezer | None for the project | 1 | For storage of patient samples |
| ELISA Reader | 1 (in the cooperating agency, MRDU lab of IHG NIH UPM) | 0 | Needed to perform and detect signals from ELISA kits |
| Nanodrop Spectrophotometer | 0 | 1 | For RNA quantification and OD 260/280 determination |