PROFILING RENAL VARIATIONS AND PLASMA PROTEOME IN HUMAN LEPTOSPIROSIS

By

LOW CHENG YEE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

May 2024

FPSK (p) 2024 17

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PROFILING RENAL VARIATIONS AND PLASMA PROTEOME IN HUMAN LEPTOSPIROSIS

By

LOW CHENG YEE

May 2024

Chairman : Zamberi bin Sekawi, PhD
Faculty : Medicine and Health Sciences

Leptospirosis, a notifiable endemic disease in Malaysia, is associated with a higher mortality rate than another prevalent disease in the region, dengue fever. However, underreporting due to asymptomatic cases, misdiagnosis, and co-infections obscures the actual disease burden. Diverse clinical symptoms and limited diagnostic methods complicate leptospirosis diagnosis. The demand for accurate biomarker-based diagnostics is increasing. This study examined renal profile variations between leptospirosis and non-leptospirosis patients, revealing that leptospirosis patients exhibited hypocalcaemia and hypochloraemia. Additionally, the plasma proteome of leptospirosis patients with leptospiraemia and seroconversion was compared to dengue patients and healthy subjects using isobaric tags for relative and absolute quantitation (iTRAQ)-mass spectrometry (MS). The initial iTRAQ analysis identified a total of 450 proteins and then refined them to a list of 290 proteins after a series of exclusion criteria were applied. Among these 290 proteins, a subset was

differentially expressed in the leptospiraemia (85 proteins) and seroconversion (113 proteins) groups. The protein-protein interaction network identified three main protein clusters, which are associated with reverse cholesterol transport, platelet aggregation, cell-matrix adhesion, fibroblast migration, and acute-phase response. The plasma proteome of leptospirosis patients compared to the control groups identified 11 proteins that showed significant expression variations, which are apolipoprotein A-II (APOA2), C-reactive protein (CRP), fermitin family homolog 3 (FERMT3), leucine-rich alpha-2-glycoprotein 1 (LRG1), lipopolysaccharide-binding protein (LBP), myosin-9 (MYH9), platelet basic protein (PPBP), platelet factor 4 (PF4), profilin-1 (PFN1), serum amyloid A-1 protein (SAA1), and thrombospondin-1 (THBS1). Following verification in a verification cohort, a panel of eight plasma protein biomarkers has been identified for leptospirosis, which are CRP, LRG1, LBP, MYH9, PPBP, PF4, SAA1, and THBS1. These protein biomarkers align with the leptospirosis biological processes, enhancing their potential as leptospirosis diagnostic tools. In conclusion, combining renal profile differences and a panel of eight protein biomarkers offers a promising approach for leptospirosis diagnosis, addressing the limitations of the "one disease, one biomarker" concept.

Keywords: biomarker, leptospirosis, plasma proteome, renal profile

SDG: GOAL 3: Good Health and Well-Being

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMPROFILAN VARIASI RENAL DAN PROTEOM PLASMA DALAM LEPTOSPIROSIS MANUSIA

Oleh

LOW CHENG YEE

Mei 2024

Pengerusi : Zamberi bin Sekawi, PhD
Fakulti : Perubatan dan Sains Kesihatan

Leptospirosis diwartakan sebagai penyakit wajib notifikasi di Malaysia, dikaitkan dengan kadar kematian yang lebih tinggi berbanding penyakit lain yang lebih umum di rantau ini, iaitu demam denggi. Namun demikian, kekurangan notifikasi disebabkan oleh kes-kes tanpa gejala, kesilapan dalam diagnosis, dan jangkitan serentak mengaburkan beban penyakit sebenar. Gejala-gejala klinikal yang pelbagai dan kaedah diagnostik yang terhad mempersulitkan diagnosis leptospirosis. Permintaan atas ujian diagnostik berasaskan biopenanda yang tepat semakin meningkat. Kajian ini mengkaji variasi profil renal antara pesakit leptospirosis dan pesakit bukan leptospirosis, menunjukkan bahawa pesakit leptospirosis mengalami hipokalsemia dan hipokloremia. Di samping itu, proteom plasma pesakit leptospirosis dengan leptospiraemia dan serokonversi dibandingkan dengan pesakit demam denggi dan subjek sihat menggunakan tag isobarik untuk kuantiti relatif dan mutlak (iTRAQ)-spektrometri jisim (MS). Analisis iTRAQ mengenal pasti sejumlah 450

protein dan kemudiannya menyaringkannya kepada senarai 290 protein selepas satu siri kriteria pengecualian digunakan. Di antara 290 protein ini, terdapat subset menunjukkan perbezaan dalam ekspresi pada kumpulan leptospiraemia (85 protein) dan serokonversi (113 protein). Rangkaian interaksi protein-protein mengenal pasti tiga kelompok protein utama yang berkaitan dengan pengangkutan kolesterol terbalik, penggumpalan platelet, perlekatan sel-matriks, migrasi fibroblas, dan tindak balas fasa akut. Proteom plasma pesakit leptospirosis berbanding kumpulan kawalan mengenal pasti 11 protein yang menunjukkan variasi ekspresi yang signifikan, iaitu apolipoprotein A-II (APOA2), C-reactive protein (CRP), fermitin family homolog 3 (FERMT3), leucine-rich alpha-2-glycoprotein 1 (LRG1), lipopolysaccharide-binding protein (LBP), myosin-9 (MYH9), platelet basic protein (PPBP), platelet factor 4 (PF4), profilin-1 (PFN1), serum amyloid A-1 protein (SAA1), dan thrombospondin-1 (THBS1). Berikutan pengesahan dalam kohort pengesahan, satu panel biopenanda protein plasma terdiri daripada lapan biopenanda telah dikenal pasti untuk leptospirosis, iaitu CRP, LRG1, LBP, MYH9, PPBP, PF4, SAA1, dan THBS1. Biopenanda protein ini sejajar dengan proses-proses biologi leptospirosis, meningkatkan potensi mereka sebagai alat diagnosis leptospirosis. Kesimpulannya, gabungan perbezaan profil renal dan panel biopenanda yang terdiri daripada lapan biopenanda protein menawarkan pendekatan yang menjanjikan untuk diagnosis leptospirosis, menangani batasan konsep "satu penyakit, satu biomarker".

Keywords: biopenanda, leptospirosis, profil renal, proteom plasma

SDG: MATLAMAT 3: Kesihatan yang Baik dan Sejahtera

ACKNOWLEDGEMENTS

The completion of this study involved the help of many individuals. I could only have achieved the objectives with them.

I express my deepest gratitude to my supervisor, Prof. Dr. Zamberi Sekawi, who has mentored me tremendously. I thank him for his patience, guidance, and encouragement. “Leaders do not force people to follow; they invite them on a journey”, and this is the way he has been leading me throughout the journey. I thank him for lending me adequate freedom, trust, and flexibility.

I am indebted to my co-supervisors, who have generously shared their invaluable knowledge. Thanks to Assoc. Prof. Dr. Leslie Than Thian Lung for his insightful comments and ideas that significantly improved the study. Through his broad research networks, I had the opportunity to learn from various experts in the relevant fields who are genuinely professional and inspiring. Thanks to Assoc. Prof. Dr. Ling King Hwa, even while on sabbatical leave in the United States, for sparing valuable time to participate in my comprehensive exam via video call and to provide feedback on our research and manuscripts.

In particular, I convey my utmost indebtedness to Dr. Muhamad Yazli Yuhana for generously sharing the plasma samples collected by his team at Hospital Teluk Intan. I appreciate Dr. Lin Qingsong, Mr. Lim Teck Kwang, and Dr. Lee Yew Mun from the National University of Singapore for sharing their

knowledge and technical skills related to labelling mass spectrometry, which greatly enhanced the quality of this study.

I acknowledged the dedicated medical personnel at Hospital Serdang, Hospital Tengku Ampuan Rahimah, and Hospital Teluk Intan. Their invaluable assistance in collecting blood samples from the study subjects has significantly contributed to the advancement of this research.

My heartfelt thanks go to the study cohorts, whose participation was crucial in advancing this study. Their willingness to share detailed health information and provide valuable blood samples has not gone unnoticed. I am truly grateful, even though I felt guilty for inconveniencing them during their illness.

Furthermore, I recognise the unwavering efforts and contributions of all the researchers in the Malaysia Leptospirosis Research Network (MyLepto). Their collective efforts were essential in recruiting study subjects and collecting blood samples.

Last but not least, I owe a debt of gratitude to Assoc. Prof. Dr. Chee Hui Yee for steering me from a production laboratory to the field of research during a critical junction in my life. I could never thank my fellow labmates, parents, family members, and friends enough for their eternal support, love, and encouragement. Their infallible love and support have always been my strength.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Zamberi bin Sekawi, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Leslie Than Thian Lung, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Ling King Hwa, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

_____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 7 November 2024

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AKI	acute kidney injury
APOA2	apolipoprotein A-II
CRP	C-reactive protein
DDA	data-dependent acquisition
DEN-CTRL	dengue case enrolled in this study
DIA	data-independent acquisition
ELISA	enzyme-linked immunosorbent assay
FDR	false discovery rate
FERMT3	fermitin family homolog 3
FMHS	Faculty of Medicine and Health Sciences
GCP	Good Clinical Practices
GO	Gene Ontology
HLTY-CTRL	healthy subject enrolled in this study
HRP	horseradish peroxidase
HSDG	Hospital Serdang
HTAR	Hospital Tengku Ampuan Rahimah
HTI	Hospital Teluk Intan
HUPO-PPP	Human Proteome Organization Plasma Proteome Project
ICU	intensive care unit
iTRAQ	isobaric tags for relative and absolute quantification
LBP	lipopolysaccharide-binding protein
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry

LEPTO	leptospirosis case enrolled in this study
LEPTO-Ab	MAT-confirmed LEPTO
LEPTO-DNA	positive blood/urine culture or PCR-confirmed LEPTO
LPS	lipopolysaccharides
LRG1	leucine-rich alpha-2-glycoprotein 1
MAT	microscopic agglutination test
MMTS	methyl methanethiosulfonate
MREC	Medical Research Ethics Committee
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem MS
MYH9	myosin-9
m/z	mass-to-charge ratio
non-LEPTO	case other than LEPTO, DEN-CTRL, and HLTY-CTRL enrolled in this study
NS1	non-structural protein 1
OD	optical density
PCR	polymerase chain reaction
PFN1	profilin-1
PF4	platelet factor 4
PPBP	platelet basic protein
PPI	protein-protein interactions
r_s	Spearman correlation coefficient
SAA1	serum amyloid A-1 protein
SCX	strong cation exchange

SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SILAC	stable isotope labelling with amino acids in cell culture
SPSS	Statistical Package for Social Sciences
SRM	selected reaction monitoring
TCEP	tris(2-carboxyethyl)phosphine
THBS1	thrombospondin-1
TMT	tandem mass tag
UPM	Universiti Putra Malaysia
2-DE	two-dimensional gel electrophoresis

CHAPTER 1

INTRODUCTION

Leptospirosis, a zoonotic disease transmitted between animals and humans, is caused by *Leptospira* species. These bacteria are excreted in the urine of infected animals and subsequently contaminate soil or water for transmission. It is prevalent in tropical and subtropical regions and poses a significant global health threat. Leptospirosis is endemic in Malaysia, becoming a notifiable disease in 2010. According to the statistics from the Ministry of Health Malaysia (2023), its fatality rate has consistently exceeded that of dengue fever from 2011 to 2020.

Despite its endemic status, leptospirosis cases are likely underreported due to asymptomatic cases, misdiagnosis, and co-infections with other febrile illnesses. Leptospirosis can manifest as either asymptomatic or symptomatic, with symptoms often resembling those of other endemic febrile illnesses. The disease presents a wide range of clinical signs in humans, making it challenging to diagnose based solely on clinical presentation. Laboratory diagnosis of leptospirosis primarily relies on serological tests, which detect agglutinating antibodies. However, serological tests may not be sensitive during the early acute phase of the disease or delayed seroconversion. Direct pathogen or antigen detection is more accurate but requires stricter specimen collection timing.

Leptospires initially spread throughout various tissues in the body but are primarily cleared from most except the kidneys, where they persist due to immune privilege and potential biofilm formation. The kidneys can exhibit symptoms of leptospirosis such as acute kidney injury, proteinuria, and renal inflammation. This ongoing infection and inflammation are influenced by factors like the presence of anti-leptospiral antibodies and the ability of leptospires to form biofilms.

Biomarker studies in leptospirosis have become increasingly significant due to their potential to improve diagnostic decisions. Many of these biomarker investigations for leptospirosis focused on the perturbations in the host proteome profile proteome, particularly in blood fractions, making them valuable sources for proteomic biomarker investigations.

Mass spectrometry (MS) enables the comprehensive analysis of proteins in biological samples. It allows for identifying and quantifying proteins, facilitating the discovery of novel biomarkers for various diseases. It is widely used in studying disease mechanisms, diagnostic accuracy, and treatment monitoring. Moreover, it has a crucial role in biomarker validation owing to its high precision and sensitivity. MS enables simultaneous analysis of multiple samples when coupled with isotope labelling techniques. Labelling-MS facilitates the comparison of protein expression levels between different biological conditions, which increases throughput and reduces experimental variability.

We hypothesised that leptospirosis infection would induce perturbations in the levels of renal electrolytes, renal waste products, and blood proteins, resulting in identifiable changes in renal and plasma proteome profiles. These changes may serve as potential diagnostic biomarkers for human leptospirosis.

This study analysed clinical data from a retrospective cohort to identify differences in renal electrolyte and waste product levels between leptospirosis and non-leptospirosis patients. Additionally, plasma proteome profiles of leptospirosis patients, including those with leptospiraemia and those with seroconversion were examined using labelling-MS. These proteome profiles were compared to two control groups: dengue patients and healthy individuals matched by gender. The identified differentially expressed proteins were subsequently verified in an independent verification cohort. This study proposes combining renal profile variations and plasma protein biomarkers to improve the accuracy of leptospirosis diagnosis.

General Objective:

To identify renal profile variations and potential plasma protein biomarkers in leptospirosis patients.

Specific Objectives:

1. To compare renal electrolyte and waste product levels in leptospirosis and non-leptospirosis patients.

2. To determine plasma proteome biomarkers in leptospirosis patients with leptospiraemia and seroconversion compared to dengue and healthy subjects.

3. To verify the differentially expressed proteins in leptospirosis patients using an independent verification cohort.

CHAPTER 2

LITERATURE REVIEW

2.1 Leptospirosis

Leptospirosis is a zoonotic disease that infects both animals and humans. It exhibits a global presence, with a more pronounced prevalence in tropical and subtropical regions. The primary transmission mode for leptospirosis is exposure to water or soil tainted with the urine of diseased animals, particularly rodents like rats.

2.1.1 Aetiology

Leptospira species cause leptospirosis. *Leptospira* is characterised by two historically recognised species within the genus: *L. interrogans* encompassing pathogenic serovars and *L. biflexa* comprising saprophytic serovars (Adler, 2015). With over 250 distinct *Leptospira* serovars identified, the primary distinguishing factor among them lies in the heterogeneity of their carbohydrate component within the lipopolysaccharides (LPS). Serovars sharing common antigenic properties are grouped into serogroups (Levett, 2001). Serovar differentiation hinges on variations in the O-antigen polysaccharide of LPS determined by the microscopic agglutination test (MAT) (Bulach et al., 2000). MAT employs a panel of live leptospires representing diverse serogroups as antigens to detect agglutinating antibodies (Adler, 2015). However, it is important to note that the antigen-antibody reactions in the MAT assay do not reliably predict the specific causative serovars (Levett,

2003; Smythe et al., 2009). Direct isolation and molecular identification are the more appropriate methods for identifying the infecting serovars precisely. Alongside the serological classification scheme, *Leptospira* species are also classified based on genetic relatedness, initially by DNA-DNA hybridisation and latterly supported by 16s rRNA phylogenetic inference (Morey et al., 2006). Even though the genotypic classification system is taxonomically legitimate, the serological classification provides epidemiological data for clinicians and microbiologists. The coexistence of two classification systems for *Leptospira* is necessary for evolutionary and epidemiology purposes.

2.1.2 Clinical Symptoms

Human leptospirosis can result from direct or indirect exposure to the urine of infected animals. A wide range of clinical expressions has been observed in human leptospirosis cases (World Health Organization, 2003). Leptospiral infections can manifest as either asymptomatic or symptomatic illnesses, spanning from mild febrile conditions that resolve on their own to severe multi-organ dysfunction leading to fatal outcomes. The clinical presentation of leptospirosis is frequently non-specific, often resembling symptoms seen in other prevalent febrile illnesses like dengue, influenza, and malaria.

Generally, leptospirosis occurs as two distinguishable syndromes: anicteric and icteric syndrome (Levett, 2001; Izurieta et al., 2008). The clinical course of anicteric leptospirosis typically follows a biphasic pattern (Adler, 2015). The biphasic pattern involves two overlapping phases after an asymptomatic incubation period of 7-10 days, ranging from 2-20 days. First, the acute or

septicaemic phase which lasts for about one week is characterised by leptospiraemia (Adler, 2015), during which *Leptospira* can be detected in the blood (Agampodi et al., 2012) and cerebrospinal fluid (Waggoner et al., 2015). Non-specific symptoms during the septicaemic phase including fever, headache, myalgia, chills, abdominal pain, and conjunctival suffusion, are common (Levett, 2001; Fraga et al., 2015), and skin rash (Iragorri and Tullus, 2009) is infrequently reported. Muscle pains, especially in the calves or lower back and conjunctival suffusion are suggested as significant symptoms to recognise leptospirosis from other infectious diseases (Haake and Levett, 2015).

The immune phase then follows the septicaemic phase. In some cases, a one- to four-day defervescence period (Levett, 2001; Fraga et al., 2011) may present between the septicaemic and immune phases. The immune phase is best described by the rising of anti-*Leptospira* antibodies together with leptospiruria (Adler, 2015). Earlier symptoms including fever, may recur during the latter phase (Fraga et al., 2011), and aseptic meningitis may develop (Wang et al., 2016). Most patients recover spontaneously from mild leptospirosis without seeking medical treatment (Taylor et al., 2015) or after appropriate antibiotic treatment (Suputtamongkol et al., 2010).

However, about 10-15% of leptospirosis cases may develop as a fulminant, monophasic illness (Spichler et al., 2005; Maroun et al., 2011) that presents icteric syndrome (Levett, 2001; Izurieta et al., 2008), which is also known as

Weil's disease (Adler, 2015). The icteric syndrome is characterised by jaundice, kidney failure, and haemorrhage.

In leptospirosis, jaundice is not directly associated with hepatocellular necrosis, and liver functions may recover in weeks after the symptoms are resolved (Costa et al., 2015). Elevation of serum bilirubin, transaminase, and alkaline phosphatase levels is typical (Bharti et al., 2003). Acute kidney failure is frequently presented accompanied by oliguria or anuria in severe leptospirosis cases (Iragorri and Tullus, 2009; Pothuri et al., 2016). Pulmonary haemorrhage has been identified as a predominant cause of fatal leptospirosis (Yersin et al., 2000; Croda et al., 2010). Cardiac involvement in leptospirosis is presented by electrocardiographic abnormalities, myocarditis, and endocardial inflammation (Shah et al., 2010). It is believed that the cardiac manifestations in leptospirosis are usually masked by pulmonary haemorrhage (Yersin et al., 2000; Shah et al., 2010).

Table 1 summarises the typical clinical symptoms and observations of leptospirosis based on other clinical studies and case reports.

Table 1: Clinical symptoms associated with leptospirosis

Syndrome	Anicteric Syndrome		Icteric Syndrome / Weil's Disease	
Phase/ pattern	Biphasic		Monophasic, fulminant	
	Septicaemic phase	Immune phase		
Description	Leptospiraemia.	Rising of anti-*Leptospira* antibodies together with leptospiruria.	Severe form of leptospirosis may involve multi-organ dysfunction.	
Clinical symptoms/ observations	Fever Headache Myalgia Chills Abdominal pain Conjunctival suffusion Nausea Vomiting Cough Dyspnoea Thrombocytopenia	Lin et al. (2008) Turhan et al. (2006) Katz et al. (2001) Hochedez et al. (2013)	Jaundice/liver dysfunction Acute renal failure Oliguria or anuria Thrombocytopenia	Pothuri et al. (2016) Gancheva and Karcheva (2013) Bourquin et al. (2011) Ghasemian et al. (2016)
			Chest roentgenography abnormalities / multiple nodular densities pattern	Lin et al. (2008) Herrmann-Storck et al. (2010)
	Skin rash	Berman et al. (1973) Iragorri and Tullus (2009)	Severe rhabdomyolysis Pulmonary failure Thrombosis	Turhan et al. (2006)
	Aseptic meningitis	Wang et al. (2016) Romero et al. (2010) van Samkar et al. (2015)	Alveolar haemorrhage Acute Respiratory Distress Syndrome (ARDS) Clotting disorders Pancreatitis Gastrointestinal bleeding Intracranial bleeding	Najafi et al. (2015)
			Severe cardiogenic shock	Forbat et al. (2018)
			Altered Mental state	Ghasemian et al. (2016)

2.1.3 Renal Involvement

Leptospires colonise renal tubules in a variety of mammalian reservoirs. Research conducted by Athanazio et al. (2008) in an *in vitro* study involving rats demonstrated that pathogenic leptospires disseminated widely throughout the body during the early stages of infection. The leptospires were cleared from most tissues in a week, except the kidneys, where dense aggregations of leptospires persisted within the renal tubules. The kidneys were not specifically targeted due to tropism since the leptospires initially spread to various tissues and not exclusively to the kidneys (Athanazio et al., 2008). Monahan et al. (2008) proposed that the clearance was aided by the circulation of anti-leptospiral immunoglobulins, which became detectable in rats around seven days after infection. This finding supports the idea that the kidneys possess a degree of immune privilege, allowing for the persistent colonisation of leptospires (Monahan et al., 2008). It is hypothesised that the anti-leptospiral antibodies in the tubular and urinary bladder may not clear the bacteria due to the absence of complement (Adler and Faine, 2006) or due to the ability of leptospires to form biofilms (Ristow et al., 2008), which is immunologically favourable for *Leptospira* colonisation.

Renal involvement in leptospirosis can manifest in various ways, such as anuria, oliguria, microscopic haematuria, proteinuria, and acute kidney injury (AKI), requiring dialysis in severe cases (Ghasemian et al., 2016). AKI typically presents with a rapid increase in serum urea and creatinine levels accompanied by hypokalaemia (Katz et al., 2001; Ghasemian et al., 2016), leading to mild persistent renal dysfunction. Additionally, research has

demonstrated that leptospiral outer membrane proteins can trigger renal inflammation and interstitial nephritis (Yang et al., 2001). The combination of bacterial invasion, inflammation, hemodynamic alterations, and the pathogenicity of bacterial products collectively plays a role in developing leptospiral nephropathy (Visith and Kearkiat, 2005).

2.1.4 Laboratory Diagnosis

MAT is acknowledged as the benchmark for serological leptospirosis diagnosis (World Health Organization, 2003). MAT employs a collection of live leptospires that serve as antigens, representing diverse serogroups, for identifying agglutinating antibodies (Adler, 2015). However, it is known to be time-consuming and less sensitive when applied to early acute-phase samples due to delayed seroconversion (Rajapakse et al., 2015). MAT has limitations for diagnosing leptospirosis, particularly during the septicaemic phase or in regions with limited access to diagnostic facilities. Despite the availability of various rapid-format serological and enzyme-linked immunosorbent assays (ELISA) tests, challenges persist with delayed diagnosis caused by delayed seroconversion and difficulties in detecting antibody titres. Furthermore, concerns remain regarding the specificity and sensitivity of these commercial tests for diagnostic purposes (Effler et al., 2002).

Compared to indirect detection methods, direct pathogen or antigen detection may impose stricter demands on sampling timing and specimen type. Leptospiraemia may cease shortly after the symptoms appear (Adler, 2015). Direct visualisation of leptospires in blood or urine samples by darkfield

microscopy is unreliable given its low sensitivity and specificity (Vijayachari et al., 2001). While direct isolation of leptospires from the blood, urine, and cerebrospinal fluid may be a more precise confirmatory test, it is not a common practice in diagnosis since *Leptospira* are fastidious bacteria that take up to a few months to grow (Zuerner, 2006), often resulting a retrospective diagnosis. The effectiveness of molecular leptospiral DNA detection relies not solely on the bacterial load within clinical materials but also on the specificity and sensitivity of the employed probes or primer in the detection process.

Researchers have devised several scoring models to guide diagnosing suspected cases to address the absence of a readily available confirmatory diagnostic test for leptospirosis. Initially, Faine's criteria were formulated in three parts, incorporating clinical data, epidemiological factors, and bacteriological and laboratory findings that relied exclusively on MAT titres (Shivakumar and Shareek, 2004). A cumulative score of 26 or higher in either the clinical data part or the combined score of clinical data and epidemiological factors parts, or a total score of 25 or greater across all three parts, may suggest a presumptive diagnosis. Following this, these standards underwent modifications and expansions, resulting in modified Faine's criteria (Shivakumar and Shareek, 2004), which included additional serological test formats in Part C. Furthermore, to enhance their clinical utility, the modified Faine's criteria underwent further refinements and amendments, leading to the development of modified Faine's criteria with amendments (Shivakumar, 2013).

2.2 Leptospirosis in Malaysia

Leptospirosis is endemic in Malaysia. It has been a notifiable disease since December 2010. A probable or confirmed case must be reported to the Health District Office within seven days following the date of diagnosis (Ministry of Health Malaysia, 2011). Both the probable and confirmed cases are defined based on the immunological results.

Annual statistical reports related to endemic diseases in Malaysia are accessible online at the official portal of the Ministry of Health Malaysia (Ministry of Health Malaysia, 2023). The total cases and fatality rate of leptospirosis versus dengue from 2011 to 2020 are compared as shown in Figure 1.

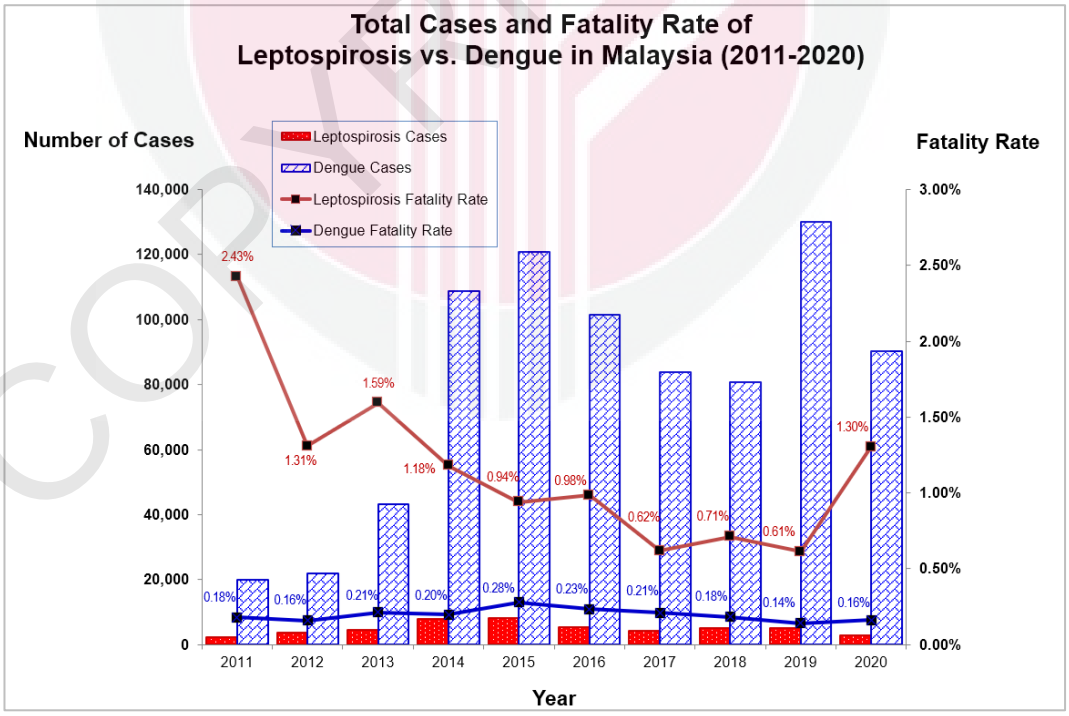


Figure 1: Total Cases and Fatality Rate for Leptospirosis and Dengue in Malaysia (2011-2020)
Data on the number of cases were retrieved from the Annual Reports of the Ministry of Health Malaysia, for the years 2011 to 2020.

Dengue consistently had a significantly higher number of reported cases compared to leptospirosis throughout the years. The gap in total cases between the two diseases remained substantial over the years. However, the fatality rate of leptospirosis was constantly higher than that of dengue. The fatality rate of leptospirosis in 2011 was relatively high at 2.43%, indicating a significant risk of death for those who contracted the disease.

Many countries experience underreporting leptospirosis cases (Victoriano et al., 2009; Rita et al., 2014; Fontes et al., 2015; Torgerson et al., 2015; Garba et al., 2017). Despite the degree of underreporting of human leptospirosis in Malaysia has not been determined, the cases are believed to be underreported due to several factors.

First, many leptospirosis cases go unnoticed or asymptomatic, and most patients recover spontaneously from mild leptospirosis without needing medical care. These cases are mostly discovered by chance during seroprevalence studies among high-risk cohorts (Ashford et al., 2000; Vijayachari et al., 2004; Cook et al., 2017). Asymptomatic leptospiruria in humans has also been reported when both pathogenic and intermediate pathogenic leptospires are found shed into the urine (Ganoza et al., 2010; Sivasankari et al., 2016). Hence, the cases of unnoticed and unrecorded leptospirosis are hardly to be estimated, especially in endemic regions and among high-risk individuals.

Furthermore, the primary contributor to underreporting is failing to diagnose or misdiagnose the disease. Leptospirosis often presents with symptoms resembling other febrile illnesses, particularly dengue fever. This similarity is compounded by the fact that both diseases share geographic distributions, including in Malaysia. Misdiagnosis of leptospirosis and missed diagnosis during co-infections with other febrile illnesses has been associated with dengue fever (Flannery et al., 2001; Bruce et al., 2005; Fontes et al., 2015), H1N1 influenza (Lo et al., 2011), hepatitis (Ismail et al., 2006), malaria (Yong and Koh, 2013), typhoid fever (Othman et al., 2007; Noor Rafizah et al., 2012), pulmonary-renal syndrome (Kishimoto et al., 2004), melioidosis (Saengjaruk et al., 2002), chikungunya (Nhan et al., 2016), rickettsia (Özdemir et al., 2008), and murine typhus (Gasem et al., 2009). Clinical manifestations are not sufficiently reliable to diagnose leptospirosis. Therefore, depending on specialised laboratory diagnostic tests to distinguish leptospirosis from other illnesses is imperative.

2.3 Biomarker Studies for Leptospirosis

Identifying biomarkers for leptospirosis that can potentially be used in making clinical decisions is necessary.

2.3.1 Selection of Specimens

In matching the study goals for most leptospirosis biomarker studies, urine, faeces, blood, and blood fractions (plasma, serum, blood cells, and buffy coat) were usually selected. Blood was typically the specimen of choice in biomarker

identification. Researchers have collected biopsy specimens from target organs such as the kidney (Małecki et al., 2019), liver (Wysocki et al., 2014), and skin (Doudier et al., 2010) for leptospirosis diagnosis and pathogenesis studies. However, these specimens have never been used in biomarker studies.

Various inflammatory mediators have been associated with disease severity in human leptospirosis. Most reported biomarkers were identified based on the host's immune response. Blood fractions, serum and/or plasma served as the data mines for fishing the disease biomarker. Serum and plasma contain blood proteins and proteins that are secreted from almost all tissues, making them an excellent source for proteomic biomarker studies.

Serum is commonly collected in clinical settings for leptospirosis diagnosis, as it is a readily accessible sample and favoured by researchers. However, in line with the Human Proteome Organization Plasma Proteome Project (HUPO-PPP) recommendations, several factors support the preference for plasma over serum in proteomic biomarker studies (Rai et al., 2005). Serum content can be influenced by uncontrollable variables such as temperature and clotting time, potentially altering its composition. Furthermore, there is a risk of proteins of interest binding to the clot and being removed during the separation process. Nevertheless, serum continues to be widely utilised in biomarker discovery efforts for leptospirosis.

2.3.2 Identified Biomarkers for Leptospirosis

Proteomic studies in patients with leptospirosis are vital during both the septicaemic and immune phases of the disease. Many of these biomarker investigations are centred on the proteome. Srivastava et al. (2012) conducted a study revealing differential protein expression in the serum proteome of leptospirosis-infected patients, identifying unique proteins like alpha-1-antitrypsin, vitronectin, ceruloplasmin, G-protein signalling regulator, and apolipoprotein A-IV. The study of Tan et al. (2017) found significant differences in the expression levels of haptoglobin, serum amyloid A, transthyretin, transferrin, and apolipoprotein A-I between mild and severe cases of leptospirosis. Conroy et al. (2014) proposed that interleukin-18 binding protein and soluble endoglin might serve as discriminatory markers between leptospirosis and dengue fever. Leptospirosis patients with acute kidney injury exhibited elevated levels of intercellular adhesion molecule-1 and syndecan-1, as observed by Libório et al. (2015). Additionally, high cytokine levels, including interleukin 6, interleukin 8, and interleukin 10, were associated with severe leptospirosis (Chirathaworn et al., 2016).

Table 2 summarises the specimens used and the potential biomarkers identified in leptospirosis biomarker studies.

Table 2: Specimens used and potential biomarkers identified in leptospirosis biomarker studies

Specimen	Leptospirosis Biomarker / Laboratory Characteristic	Reference
Serum	Down-regulated apolipoprotein A-I, transferrin & transthyretin in ML vs. SL. Up-regulated serum amyloid A & haptoglobin in ML vs. SL.	Ting et al. (2017)
	Differentially expressed inflammation-related biomarkers of leptospiral infection: α-1-antitrypsin, vitronectin, ceruloplasmin, G-protein signalling regulator & apolipoprotein A-IV in LP vs. malaria patients.	Srivastava et al. (2012)
	Serum protein carbonyl as an indicator of leptospirosis severity & as an oxidative stress biomarker in differentiating LP from DF.	Fernando et al. (2016)
	Soluble endoglin & interleukin-18 binding protein discriminate between LP and DF.	Conroy et al. (2014)
	Higher levels of intercellular adhesion molecule-1 & syndecan-1 in LP compared with exposed controls. Increased level of syndecan-1 & ICAM-1 in LP with AKI vs. LP without AKI.	Libório et al. (2015)
	Higher levels of interleukin-6, interleukin -8 & interleukin -10 in SL with organ involvement.	Chirathaworn et al. (2016)
	High levels of angiopoietin-2 & asymmetric and symmetric dimethylarginine in SL with AKI, sepsis and intensive care unit treatment.	Lukasz et al. (2014)
	Increased lactate, total bilirubin, lipase, & aspartate aminotransferase/alanine aminotransferase ratio; or a decreased cytokines interleukin-10/tumour necrosis factor-alpha ratio in SL.	Mikulski et al. (2015)
Plasma	Higher granzyme B, interferon-alpha inducible protein-10 & monokine induced by interferon-alpha in LP vs. HC.	De Fost et al. (2007)
	Increased soluble E-selectin (sCD62E) & soluble intercellular adhesion molecule 1 in LP vs. HC.	Raffray et al. (2017)
	Elevated plasma sST2 levels in SL. Soluble ST2 levels were associated with bleeding and mortality.	Wagenaar et al. (2009)
Whole blood, red blood cells, serum, erythrocytes	Higher reactive oxygen species production & lower antioxidant reduced glutathione levels in LP vs. HC. Oxidative stress occurs in SL and correlates with renal dysfunction & thrombocytopenia.	Araújo et al. (2014)
Plasma, urine	Neutrophil gelatinase associated lipocalin in plasma & urine as a marker for leptospirosis associated AKI.	Srisawat et al. (2015)
Urine	Elevated levels urinary defensin alpha 1 reflect kidney injury.	Chagan-Yasutan et al. (2016)

Abbreviations: ML = mild leptospirosis; **SL** = severe leptospirosis; **LP** = leptospirosis patient; **DF** = dengue fever; **AKI** = acute kidney injury; **HC** = healthy control

2.4 Human Plasma Proteome

Blood comprises plasma, platelets, red blood cells, and white blood cells circulating throughout the body. Plasma accounts for approximately 55% of the total blood volume and consists of water, electrolytes, enzymes, antibodies, hormones, coagulation factors, and a multitude of other proteins. The protein concentration of human plasma is about 60-80 mg/mL (Barrett et al., 2019).

Anderson and Anderson (2002) divided the plasma proteins, which span ten orders of magnitude, into three major classes: plasma proteins at the high abundance end, tissue leakage proteins in the middle, and cytokines at the low abundance end. Schiess et al. (2009) provided a comprehensive visual representation in Figure 2 to define these protein classes, coupled with data from the HUPO-PPP study (States et al., 2006) and cancer biomarkers that are currently in clinical use (Polanski and Anderson, 2007).

Albumin, the predominant transport protein in the blood, constitutes 55% of the total blood proteins with a concentration of approximately 45 mg/mL (Smith et al., 2013). About 38% of blood proteins are globulins, 7% are fibrinogen, and the remaining plasma proteins include regulatory elements like enzymes, proenzymes, hormones, and low-abundance cytokines such as interleukin-1 present in concentrations below 5 pg/mL (Smith et al., 2013).

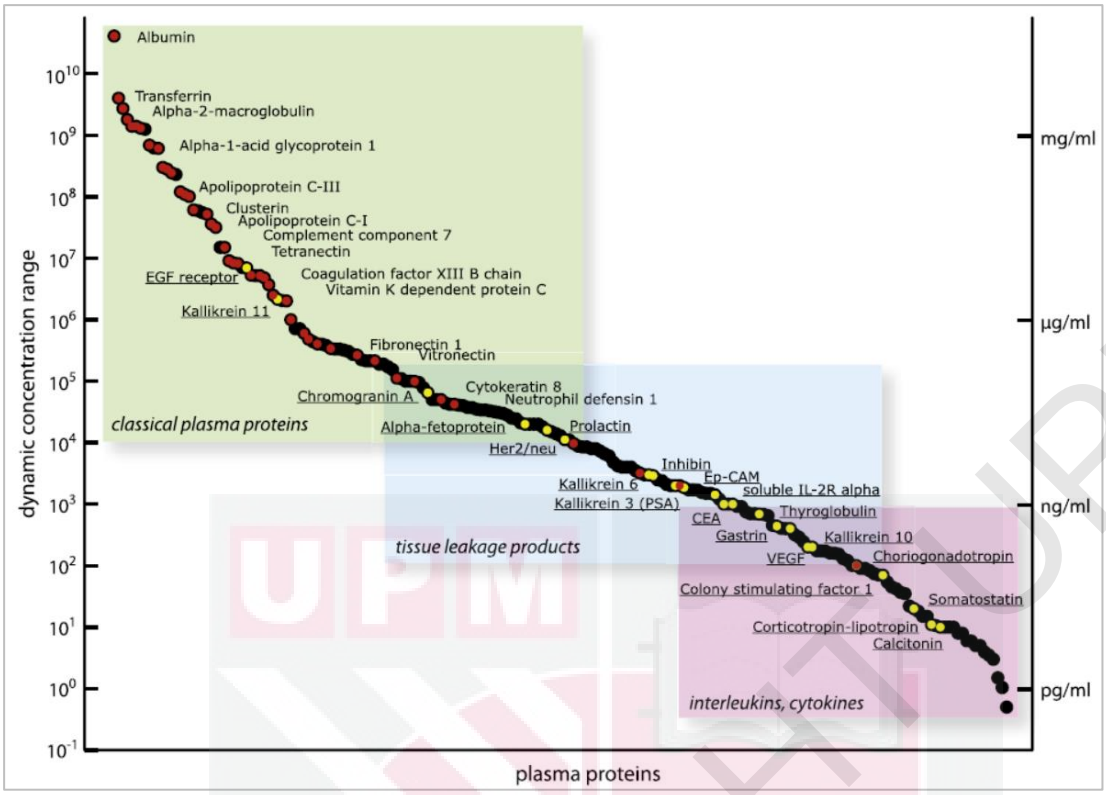


Figure 2: Spectrum of Plasma Protein Concentrations
Plasma proteins are classified into three primary categories: classical plasma proteins, tissue leakage products, and interleukins/cytokines (Anderson and Anderson, 2002). Within this depiction, red dots correspond to proteins identified through the HUPO-PPP (States et al., 2006), while yellow dots denote the cancer biomarkers in current clinical use. [Source: Schiess et al. (2009)]

A total of 784 proteins secreted to the blood have been annotated according to the 2022 Report on the Human Proteome from the HUPO-PPP (Omenn et al., 2023). A total of 438 out of the 784 blood proteins have reference plasma or serum concentrations available, as presented in Figure 3 (Uhlén et al., 2019).

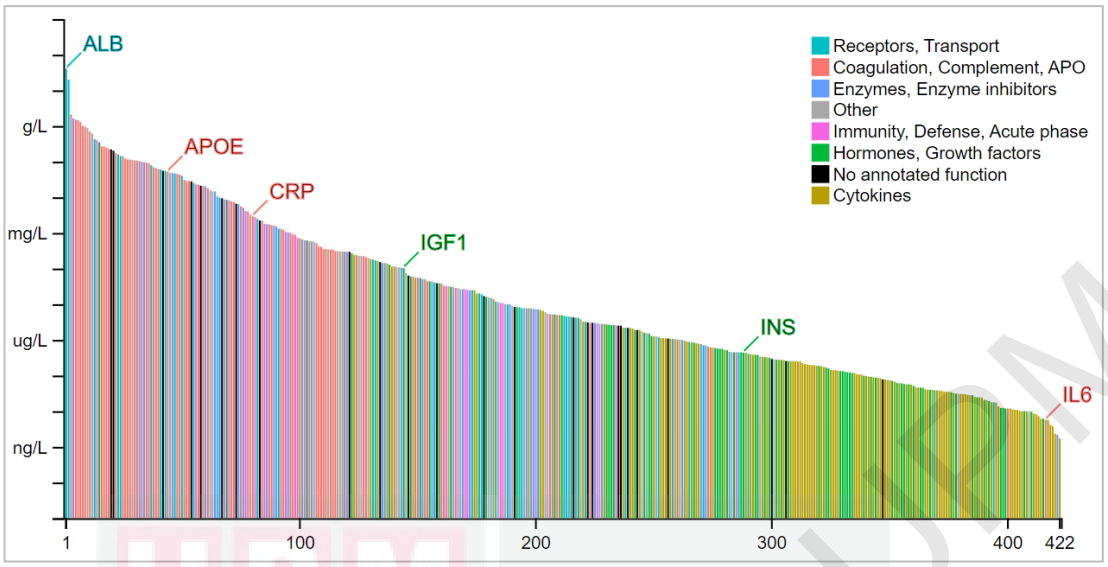


Figure 3: Concentrations of Proteins Secreted to Blood
Reference plasma concentrations are available for 438 blood proteins. These proteins are categorised by functional annotations, with coagulation and complement factors displaying high abundances, while cytokines, hormones, and growth factors tend to have lower concentrations.
[Source: Uhlén et al. (2019)]

Plasma proteome analyses are useful for discovering biomarkers and understanding disease mechanisms (Deutsch et al., 2021). The dynamic changes in the plasma proteome as diseases progress and in response to treatment enable the identification of potential biomarkers. These changes may reveal disease-specific pathways, cellular processes, and signalling networks contributing to disease development.

2.5 Phases of Biomarker Discovery Research

A biomarker discovery workflow may involve multiple stages. The process typically comprises three phases: discovery, verification, and validation (Rifai et al., 2006; Geyer et al., 2017). Figure 4 illustrates the number of samples and candidate biomarkers that may be involved in each phase.

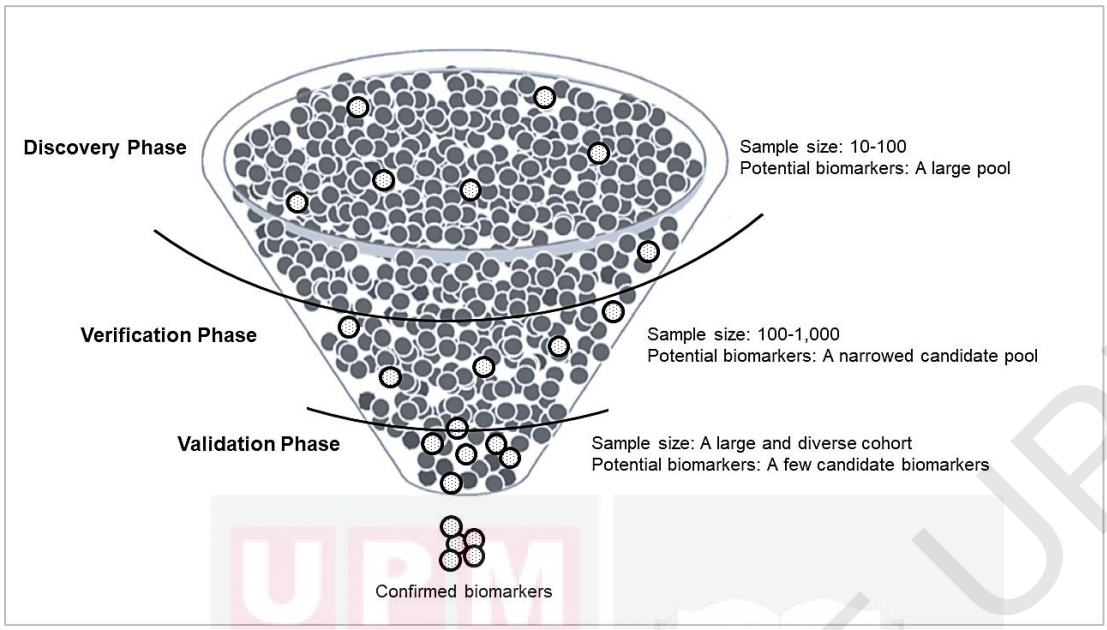


Figure 4: Three Phases in Biomarker Discovery Research
[Source: Adapted from Carey et al. (2010)]

2.5.1 Discovery Phase

The discovery phase aims to identify a pool of candidate biomarkers potentially related to a specific disease or condition. High-throughput technologies, including genomics, proteomics, metabolomics, or transcriptomics, are employed to analyse large sets of biological data (Rifai et al., 2006). These technologies generate comprehensive datasets containing information about genes, proteins, metabolites, or other biomolecules. This phase targets to produce a list of candidate biomarkers based on their statistical significance and biological relevance. The discovery phase may include false positives (Rifai et al., 2006).

2.5.2 Verification Phase

In the verification phase, the focus shifts from a broad pool of candidates to a more refined selection. The goal is to confirm the findings from the discovery phase and reduce the number of false positives.

Independent datasets or samples validate the candidate biomarkers identified in the discovery phase, which helps assess the reproducibility of the findings. Quantitative assays like ELISA, quantitative polymerase chain reaction (PCR), or MS are used to measure the levels of candidate biomarkers in biological samples (Geyer et al., 2017). It generates a narrowed candidate pool consistently showing associations with the disease or condition across multiple datasets or samples. By reducing false positives, the verification phase enhances the specificity of the potential biomarkers.

2.5.3 Validation Phase

The validation phase is the most rigorous and critical step in biomarker development. Its primary goal is to establish the clinical utility of the selected biomarkers for practical use in patient care and research. The performance of biomarkers is analysed in large and diverse clinical cohorts or patient populations (Geyer et al., 2017).

The assessment includes evaluating the biomarkers' capacity to predict clinical outcomes, diagnose diseases, inform treatment choices, or monitor disease progression to ensure that the biomarkers meet regulatory standards for

accuracy, reliability, and safety if intended for clinical use (Rifai et al., 2006). Biomarkers that demonstrate consistent and clinically meaningful associations with the disease or condition are validated. Validated biomarkers may be translated into clinical practice for diagnosis, prognosis, or treatment decisions.

In summary, the three phases of biomarker discovery (discovery, verification, and validation) represent a sequential process from identifying potential candidates to establishing their clinical utility.

2.6 Mass Spectrometry-based Proteomics

Mass spectrometry-based proteomics is a powerful analytical technique used to study the proteome, which refers to the complete set of proteins expressed by an organism, cell, tissue, or biological sample (Paulo et al., 2012).

In many proteomics workflows, peptides are separated using liquid chromatography (LC) before entering the mass spectrometer. LC separates peptides based on chemical properties, typically by gradient elution from a chromatography column. Once separated, peptides enter the mass spectrometer, which is ionised and sorted based on their mass-to-charge ratio (m/z) (Drabik and Silberring, 2016).

In data-dependent acquisition (DDA), the mass spectrometer selects the most intense precursor ions (peptides) for fragmentation (tandem MS or MS/MS) (Zhang et al., 2013). Fragmentation spectra are produced and used for peptide identification. On the other hand, data-independent acquisition (DIA) isolates

and fragments all precursor ions within a predefined m/z range (Zhang et al., 2013). DIA data can be used for both peptide identification and quantification.

Identified MS/MS spectra are matched to known peptide sequences in protein databases using bioinformatics tools. These tools assign peptide sequences to experimental spectra and calculate statistical scores to assess the confidence of the identifications. After peptide identification, software tools aggregate peptide-level information to infer protein identifications. Quantitative data is also integrated to determine relative protein abundances.

2.6.1 Untargeted MS-based Proteomics

Untargeted proteomics aims to comprehensively identify and quantify as many proteins as possible in a biological sample without specific prior knowledge of the proteins of interest (Liu et al., 2013). It may use a shotgun proteomics approach by digesting proteins in the sample into peptides, separating them, and subjecting them to mass spectrometry analysis (Parker and Borchers, 2014). The generated mass spectra are subsequently utilised to identify and quantify peptides and infer the presence of their parent proteins. DDA may also identify peptides but may not cover all proteins in the sample (Parker and Borchers, 2014). Untargeted proteomics is used to discover novel proteins, study protein expression changes in response to various conditions (e.g., disease, treatment), and identify potential biomarkers.

2.6.2 Targeted MS-based Proteomics

Targeted proteomics focuses on precisely measuring and quantifying specific predefined proteins or peptides of interest, often with known sequences (Paulo et al., 2012). Specific peptides associated with the target proteins are selected for analysis in targeted proteomics. A predefined set of transitions (precursor-to-fragment ion pairs) is monitored in the mass spectrometer, allowing for highly sensitive and quantitative measurement of those peptides (Paulo et al., 2012). Selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) are the techniques that are commonly used in targeted proteomics (Zhang et al., 2014).

Targeted proteomics is primarily used for accurate protein quantification, making it suitable for verifying candidate biomarkers or tracking changes in specific protein levels with high precision (Parker and Borchers, 2014). This approach is commonly used in clinical assays aiming to measure specific proteins accurately and reproducibly for disease diagnosis or therapeutic drug monitoring. Targeted proteomics offers high sensitivity and specificity for the selected proteins or peptides, making it suitable for precise quantification. It provides excellent reproducibility, allowing for reliable comparison of protein levels across different samples and experiments.

2.7 Protein Quantification by LC-MS

Label-free and labelling MS are quantitative proteomics approaches to measure protein abundance in biological samples.

2.7.1 Label-free Quantification

Label-free MS relies on directly measuring peptides or proteins without incorporating chemical or isotopic labels (Bubis et al., 2017). Protein quantification is based on the intensity of peptide ion signals in the mass spectrometer. Relative protein abundance is determined by comparing the ion intensities of the same peptide across different samples.

Sample preparation in label-free MS typically involves protein extraction, digestion into peptides, and LC separation of peptides (Bubis et al., 2017). Peptides are analysed in the mass spectrometer, and their ion intensities are recorded in data-dependent or data-independent acquisition modes (Zhang et al., 2014).

Label-free MS does not require the complex and costly process of labelling samples with isotopes or chemical tags. It is suitable for various applications, including biomarker discovery, protein expression profiling, and comparative proteomics. It may have limitations in quantifying proteins that span a wide range of abundance levels in a single experiment.

2.7.2 Isotope-labelling Quantification

Labelling MS involves the introduction of isotopic or chemical labels into proteins or peptides to distinguish them in the mass spectrometer. Quantification is based on comparing isotopic or labelled peptides in the mass spectra (Drabik and Silberring, 2016).

Sample preparation for labelling MS includes the labelling step, which can involve chemical labelling, such as isobaric tags for relative and absolute quantification (iTRAQ) or tandem mass tag (TMT), or metabolic labelling, such as stable isotope labelling with amino acids in cell culture (SILAC) (Drabik and Silberring, 2016). Labelled samples are mixed, and subsequent steps include protein digestion, LC separation, and MS analysis.

Labelling methods offer high quantitative precision and accuracy because labelled peptides can be easily distinguished in the mass spectra (Feist and Hummon, 2015). It is suitable for large-scale quantitative proteomics studies with high-throughput capabilities. Some labelling methods allow for the simultaneous quantification of multiple samples, enhancing experimental efficiency. However, labelling procedures can be more complex, time-consuming, and expensive than label-free methods (Feist and Hummon, 2015).

In summary, label-free MS is a more straightforward and cost-effective approach for quantifying protein abundance, making it suitable for various applications. Labelling MS methods offer high precision and multiplexing capabilities, making them ideal for large-scale quantitative studies but may require more extensive sample preparation. The choice between these two approaches depends on the research objectives, resources, and the desired level of quantitative accuracy.

CHAPTER 3

MATERIALS AND METHODS

Figure 5 presents the research workflow, illustrating the sequential phases that guided the study from inception to completion.

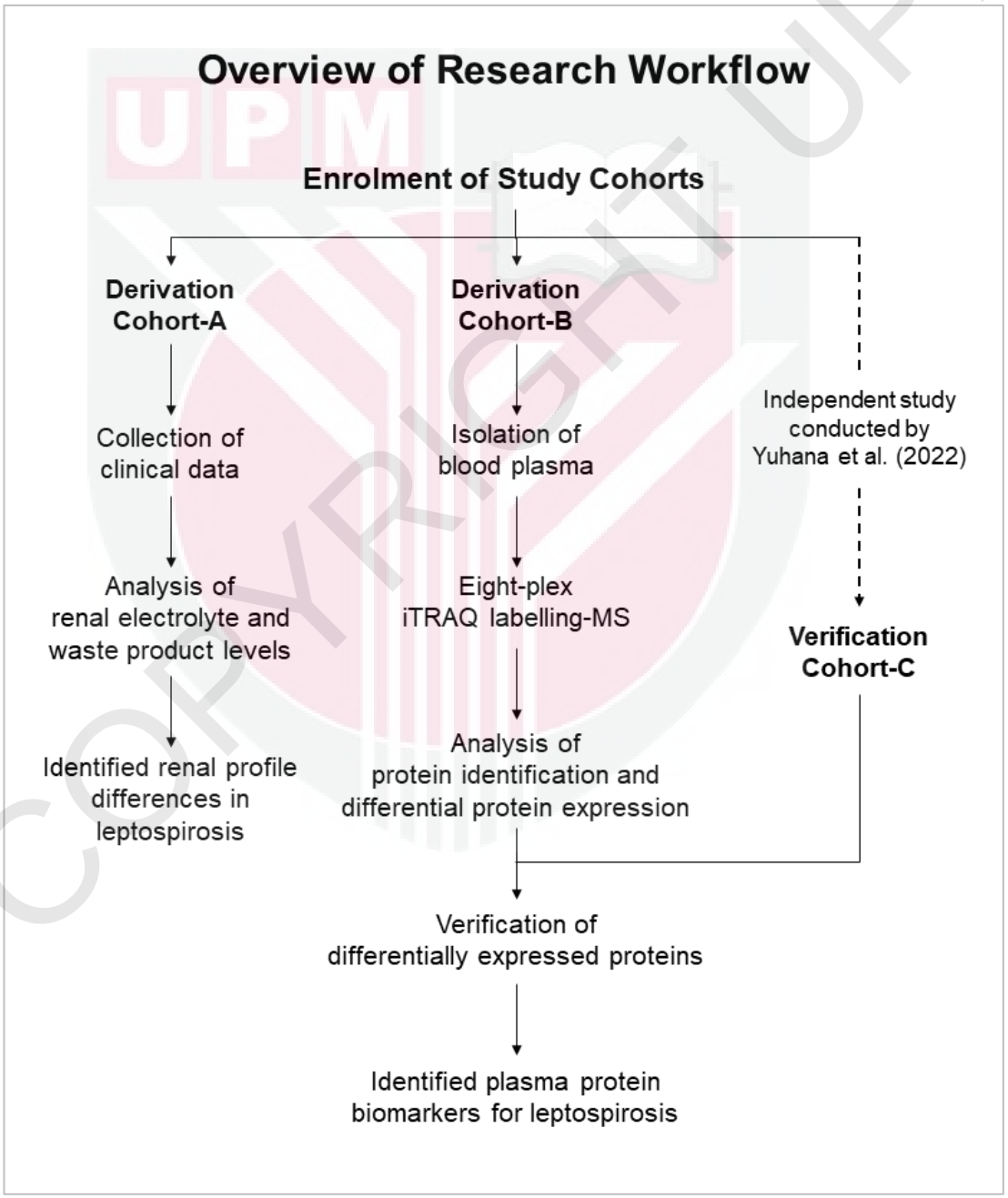


Figure 5: Overview of Research Workflow

3.1 Research Ethics

This research adhered to strict ethical guidelines. The study protocol received approval from both the Institutional Review Board of the Faculty of Medicine and Health Sciences at Universiti Putra Malaysia and the Medical Research Ethics Committee (MREC) under the Ministry of Health Malaysia (NMRR-15-2148-27536) (Appendix A and B). All participated subjects provided written informed consent before data and sample collection. The ethics clearance NMRR-15-756-25320 included provisions for informed consent regarding the secondary use of samples collected in an independent study by Yuhana et al. (2022). The author acquired certification in Good Clinical Practices (GCP) and implemented GCP principles in alignment with ethical and scientific standards.

3.2 Enrolment of Study Cohorts

This host-derived biomarker discovery study encompassed three distinct cohorts. Cohort-A and Cohort-B were designated derivation cohorts, while Cohort-C served as the verification cohort. Participants within the paediatric age category (below 18 years old) and those with a prior history of autoimmune diseases or any known comorbidities were excluded from the study.

3.2.1 Derivation Cohorts

In Cohort-A, cases were retrospectively identified by including individuals with clinical suspicion of leptospirosis who were admitted to Hospital Serdang, Selangor (HSDG) between 2011 and 2017.

On the other hand, Cohort-B was assembled prospectively and comprised individuals with clinical suspicion of leptospirosis and dengue. They were admitted to HSDG from June 2016 to October 2017 and Hospital Tengku Ampuan Rahimah, Selangor (HTAR) from April to July 2017. Healthy volunteers were recruited as the control group from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (FMHS, UPM).

3.2.2 Verification Cohort

Verification Cohort-C was recruited during an independent study conducted by Yuhana et al. (2022) from January to December 2016. The study participants were selected by enrolling hospitalised febrile patients at Hospital Teluk Intan, Perak (HTI), who had a tympanic temperature exceeding 38 °C for less than 14 days.

3.2.3 Sample Size

For the renal profile analysis, the minimal sample size (n) was determined using the one-proportion formula, as recommended by Cross and Daniel (2018). The prevalence (P) of leptospirosis was estimated at 0.084 based on previous research conducted in Malaysia (Rafizah et al., 2013). The Z-statistic

was set at 1.96 to correspond with a 95% confidence level, and a precision level (d) of 0.05 was applied, following the guidelines of Naing et al. (2006). Considering a 90% eligibility rate for the study population, the calculation indicated that a minimum of 133 subjects was necessary. Details of the calculations are shown as follows.

$$Sample\ size\ (n) = \frac{Z^2 \cdot P \cdot (1-P)}{d^2}$$

$$= \frac{(1.96)^2 \times 0.084 \times (1-0.084)}{(0.05)^2}$$

$$= 118.2$$

Rounding up the minimum required sample size before adjustment = 119.

$$Adjusting\ for\ 90\%\ eligibility: \frac{119}{0.9} = 132.2$$

Rounding up the minimum required sample size after adjustment = 133.

For plasma proteome profiling, which utilised an iTRAQ MS-based biomarker discovery workflow, a sample size of six individuals per group was deemed sufficient to achieve adequate statistical power (Zhou et al., 2012). This sample size estimation was supported by both an a priori power analysis conducted prior to the experiment and a post hoc power analysis validated after the experiment, ensuring robustness in the identification of potential biomarkers.

3.3 Collection of Clinical Data

A structured proforma was employed to gather clinical data from all study cohorts. Clinical presentation information was recorded using a binary scale (Yes or No), whereas laboratory findings were documented as continuous variables in their original numerical format.

3.4 Isolation of Blood Plasma

A 4 mL blood sample was aseptically drawn from individuals in Cohort-B by trained medical personnel using a 21-gauge butterfly needle. The collected blood was dispensed into a vacutainer tube containing 7.2 mg of EDTA and gently inverted upside-down ten times. Subsequently, the blood samples were kept upright and placed on ice until centrifuged at 1,300 $\times$ *g* for 15 minutes at 4 °C. Any haemolysed blood samples or visually lipemic plasma were excluded from further analysis. Aliquots of the plasma were then preserved at -80 °C until they were utilised in subsequent experiments. The entire process, from blood collection to plasma isolation, was completed within an hour, with consistent collection, processing, and storage conditions maintained for all samples to minimise pre-analytical variations.

3.5 Diagnostic Tests and Case Definitions

Enrolled subjects were categorised based on the results of diagnostic tests, as summarised in Table 3. Leptospirosis tests were conducted at the Microbiology Laboratory of FMHS, UPM. A confirmed leptospirosis case was defined as having a single serum titre of 1:400 or at least a four-fold rise in titre

of paired sera in the MAT and/or a positive result in quantitative PCR, and/or a positive blood or urine culture, following the criteria outlined by the World Health Organization (2003). All confirmed leptospirosis cases in this study are referred to as LEPTO patients. LEPTO patients with a MAT-confirmed diagnosis (with seroconversion) are further denoted as LEPTO-Ab. In contrast, those with a diagnosis confirmed through positive blood/urine culture or PCR (with leptospiraemia) are called LEPTO-DNA.

Table 3: Case definitions of study subjects based on diagnostic test results

Case Definition		*Leptospira*		Dengue Test	Aerobic & anaerobic blood culture
		Culture and/or DNA detection	MAT		
LEPTO	LEPTO-Ab	Negative	Positive	Negative	Negative
	LEPTO-DNA	Positive	Negative	Negative	Negative
Non-LEPTO		Negative	Negative	Negative	Positive / Negative
DEN-CTRL		Negative	Negative	Positive	Negative
HLTY-CTRL		Not Available (N/A) Volunteers were free from any diseases for two months and had no known medical illnesses at the time of blood collection.			

Dengue tests were conducted at the Microbiology Laboratory of HSDG. Dengue cases were confirmed by the positive detection of non-structural protein 1 (NS1) antigen and/or IgM. Enrolled dengue cases are designated as DEN-CTRL.

Healthy subjects, hereafter referred to as HLTY-CTRL, were volunteers free from any diseases for two months and had no known medical illnesses at the time of blood collection.

Subjects other than those falling into the LEPTO, DEN-CTRL, and HLTY-CTRL categories are classified as non-LEPTO.

3.6 Analysis of Renal Electrolyte and Waste Product Levels

Data on renal electrolytes, including sodium, potassium, calcium, chloride, and waste products, including creatinine and urea, were collected. The data was analysed using IBM Statistical Package for Social Sciences (SPSS) version 22. The laboratory findings were categorised as usual, low, or high based on the hospital's predefined cut-off values. A comparative analysis was performed between LEPTO cases (n=30) and non-LEPTO cases (n=107). A bivariate analysis employing the Chi-square test was conducted on all variables to determine differences between the two groups (Agresti, 2013). Variables that demonstrated significance in the bivariate analysis and those with a *p*-value <0.25 were included in a multivariate logistic regression using the Backward-LR method. The inclusion of variables with a *p*-value <0.25 ensured potentially significant predictors were considered and not prematurely excluded (Bursac et al., 2008). The best model was selected to identify predictors of leptospirosis infection.

3.7 Eight-plex iTRAQ Labelling-MS

The iTRAQ experiment was designed to compare plasma proteome profiles among four biological conditions (as explained in Section 3.5), including the LEPTO-Ab, LEPTO-DNA, DEN-CTRL, and HLTY-CTRL groups.

3.7.1 Case Selection

Patients in the LEPTO-Ab, LEPTO-DNA, DEN-CTRL, and HLTY-CTRL groups were selected to ensure gender matching. A total of five LEPTO-Ab patients (age range: 18-37; mean: 25.4; SD: 7.2), eight LEPTO-DNA patients (age range: 19-40; mean: 26.5; SD: 7.6), five DEN-CTRL individuals (age range: 22-37; mean: 29.0; SD: 5.9), and seven HLTY-CTRL individuals (age range: 21-35; mean: 29.3; SD: 5.4), were selected.

3.7.2 Pooling of Plasma Samples

The chosen subjects within each category (LEPTO-Ab, LEPTO-DNA, DEN-CTRL, and HLTY-CTRL) were randomly divided into two separate biological replicate groups. Subsequently, an equal volume of plasma samples from individuals within the same group was combined to create a sample pool, which would then be labelled with distinct iTRAQ reagents. As a result of this experimental setup, each category consisted of 2 sample pools, thereby serving as the technical replicates (Figure 6).

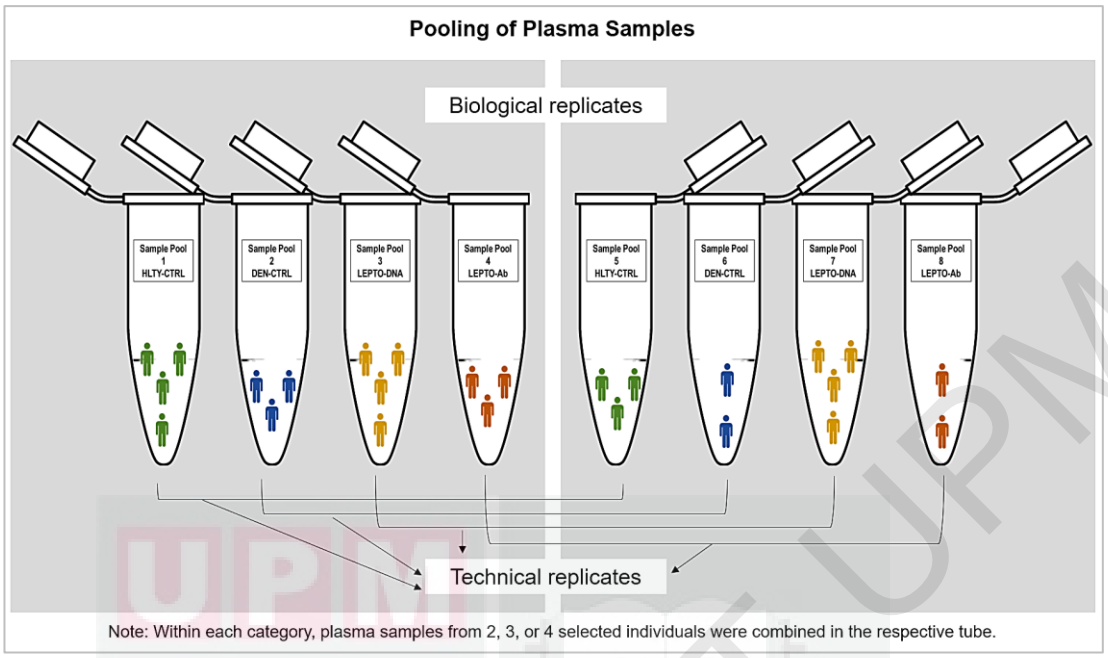


Figure 6: Experiment Design for Pooling Plasma Samples

3.7.3 Protein Quantification

Protein quantification was performed to assess the concentration of proteins in the sample pools. The protein concentration within these pools was determined using *RC DC* Protein kit from Bio-Rad, USA (Bio-Rad Laboratories, n.d.). This process involved the preparation of protein standards and sample pools, each executed in two technical replicates. Initially, the protein standards and sample pools underwent successive rounds of addition and aspiration of *RC* Reagent I and II. Subsequently, a mixture of alkaline copper tartrate (*DC* Reagent A) and surfactant (*DC* Reagent S) solutions was added. After introducing a dilute Folin reagent (*DC* Reagent B) and an incubation period at room temperature lasting 15 minutes, the absorbance was measured at 750 nm. The readings obtained from the technical replicates were averaged, and a standard curve was constructed based on the absorbance

values of the protein standards. Consequently, the protein concentration of each sample pool was estimated by referring to this standard curve.

3.7.4 Protein Gel Electrophoresis

Each sample pool containing approximately 20-40 μg of proteins was first prepared in Laemmli sample buffer with β-mercaptoethanol and then boiled at 95 °C for 5 minutes. These protein samples were subsequently separated on 4-12% Mini-PROTEAN® TGX™ Precast Protein Gel SDS-PAGE gels parallel with the Precision Plus Protein All Blue Standards from Bio-Rad, USA, using in 1× TG-SDS buffer (Bio-Rad Laboratories, 2011). Following electrophoresis, the gels were subjected to an overnight wash and staining procedure using GelCode® Blue Stain Reagent (Thermo Fisher Scientific, 2011). Then, the gels underwent repeated destaining in deionised water to remove excess stain, and the results were visualised and documented.

3.7.5 Protein Digestion

For protein digestion, sample pools containing 50-70 μg of proteins in 500 mM TEAB at pH 8.5 were initially denatured using 0.1% (w/v) sodium dodecyl sulfate (SDS) and reduced with 5 mM tris(2-carboxyethyl)phosphine (TCEP) through incubation at 60 °C for an hour (Lee et al., 2018). Subsequently, the samples were alkylated with 10 mM methyl methanethiosulfonate (MMTS) at room temperature for 30 minutes. The protein digestion was carried out using trypsin at a ratio of 1:40 (trypsin:protein) and incubated at 37 °C for 16 hours (Lee et al., 2018). The extent of digestion was assessed by comparing 2 μL of

protein-trypsin mixtures before and after the 16-hour incubation using SDS-PAGE (following the protocol described in Section 3.7.4. The protein digests were maintained at -80 °C until complete trypsin digestion was confirmed.

3.7.6 iTRAQ Labelling

Eight sample pools were prepared, with two sample pools representing each of the four subject categories. To label these sample pools, iTRAQ Reagents-8plex (AB SCIEX, USA) were employed, as depicted in Figure 7.

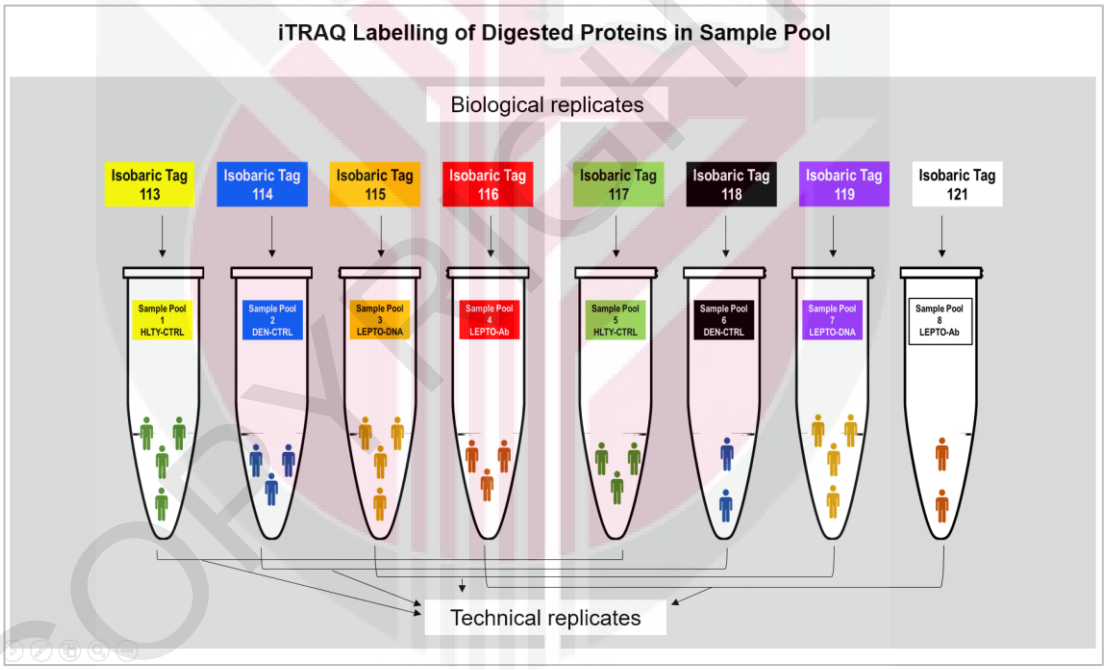


Figure 7: Experiment Design for iTRAQ Labelling

The protein digests underwent labelling using 8-plex iTRAQ Reagents following the manufacturer's instructions (AB SCIEX, 2010). The vials containing iTRAQ Reagents were allowed to reach room temperature, and then 50 μL of isopropanol was added to each vial. Simultaneously, the protein digests were freeze-dried to remove moisture and reconstituted with 30 μL of

500 mM TEAB buffer. Each freshly prepared iTRAQ Reagent was added to its corresponding tube of protein digests, as designated in Figure 6, and the samples were thoroughly mixed. The pH of each sample was tested with pH paper and adjusted to fall within the pH range of 7.5 to 8.5, if needed, using up to 5 μL of 500 mM TEAB buffer. The samples were then left to incubate at room temperature for 2 hours. Finally, all eight tubes containing iTRAQ-labelled protein digests were combined into a new tube and mixed thoroughly to ensure homogeneity.

3.7.7 Strong Cation Exchange Chromatography Purification

The iTRAQ-labelled sample pools underwent purification using the Cation-Exchange Cartridge System (AB SCIEX, USA) following the manufacturer's instructions (AB SCIEX, 2017). The sample mixture was diluted with Cation-Exchange Buffer-Load to reach a final volume of 5 mL, and the pH was adjusted to a range of 2.8-3.3 by adding 1 N phosphoric acid. The strong cation exchange (SCX) cartridge was conditioned by injecting 1 mL of Cation-Exchange Buffer-Clean, followed by 2 mL of Cation-Exchange Buffer-Load. The diluted sample mixture was slowly introduced (~1 drop/second) onto the cartridge. Subsequently, the cartridge was washed by injecting 1 mL of Cation-Exchange Buffer-Load. Peptides were then eluted by gradually injecting (~1 drop/second) 500 μL of Cation-Exchange Buffer-Elute. The eluted peptides were collected as a single fraction into a fresh tube and freeze-dried to remove any remaining solvent.

3.7.8 Desalting Procedure

The SCX chromatography eluent was desalted using C18 Pipette Tips-columns with a 100 μL bed size (Thermo Scientific, USA) (Lee et al., 2018). Three mobile phase solvents were prepared: Solvent A (2% acetonitrile [ACN] and 0.05% formic acid [FA] in deionised water), Solvent B (0.05% FA in ACN), and Solvent C (0.05% FA and 80% ACN in deionised water). C18 Pipette Tips-column was activated with 200 μL of Solvent B and equilibrated with 200 μL of Solvent A. The dried SCX eluent, reconstituted in 100 μL of Solvent A, was bound to the tip-column by aspirating and dispensing repeatedly. The tip-column was then washed with 200 μL of Solvent A, and the peptides were eluted using 100 μL of Solvent C (Lee et al., 2018).

3.7.9 Two-dimensional LC-tandem MS

The two-dimensional LC-tandem MS (2D LC-MS/MS) analysis was performed at the Protein and Proteomics Centre at the National University of Singapore (PPC, NUS). In summary, the desalted eluent was lyophilised to dryness and then reconstituted in 20 μL of LC-MS grade water. The sample was fractionated first using the Agilent 1290 Infinity LC system (Agilent Technologies, USA), followed by the cHiPLC-nanoflex system (Eksigent, USA). Twenty fractions obtained from the 2D LC separation were subsequently analysed using the TripleTOF 5600 system (AB SCIEX, USA).

3.8 Analysis of Protein Identification

The MS/MS spectra of peptides were extracted from the raw files and subjected to a database search using ProteinPilot™ software version 5.0 (AB SCIEX, USA). The database employed was a composite of protein sequences from UniProtKB for *Homo sapiens*, *Leptospira* and Dengue. The analysis parameters used with the Paragon Algorithm were configured (Table 4).

Table 4. Parameters employed in Paragon Algorithm analysis

Search Settings	Parameter Used
Sample Type	iTRAQ 8plex (Peptide-labelled)
Cysteine Alkylation	MMTS
Digestion	Trypsin
Instrument	TripleTOF 5600
Special Factors	None
Species	None
ID Focus	Biological modifications
Search Effort	Thorough
FDR Analysis	Yes
Bias Correction	No
Background Correction	No

Following the initial database search, the Pro Group Algorithm conducts a statistical analysis of the identified peptides to cluster them into proteins, taking into account shared peptide sequences. A stringent threshold of unused ProtScore >1.3 (95% confidence level) and the requirement of at least two or more unique peptides was applied to compile the definitive list of identified proteins.

3.9 Analysis of Differential Protein Expression

The list of identified proteins from Section 3.8 underwent additional analysis following the criteria outlined by Gan et al. (2007). Proteins without iTRAQ quantitation, those with fewer than two matched peptides, immunoglobulins, keratins, and those exceeding the 1% false discovery rate (FDR) threshold were excluded from further assessment. Proteins with iTRAQ ratios greater than 1.2 compared to the control groups were categorised as up-regulated, while those with iTRAQ ratios less than 0.8 were classified as down-regulated (Pierce et al., 2008). Proteins with *p*-values greater than 0.05 were excluded from the list of differentially expressed proteins.

3.10 Gene Ontology Annotation

The functional annotation of differentially expressed proteins in the Leptospiraemia Group (LEPTO-DNA compared to HLTY-CTRL and DEN-CTRL) and Seroconversion Group (LEPTO-Ab compared to HLTY-CTRL and DEN-CTRL) was performed. The protein identifiers were submitted to the UniProt online platform (https://www.uniprot.org) for mapping to UniProtKB entries. The Gene Ontology (GO) annotations for each protein were extracted from the UniProt database. These annotations included the GO terms associated with each protein, including molecular functions, biological processes, and cellular components.

3.11 Analysis of Protein-protein Interactions

Protein-protein interaction (PPI) network analysis was performed using the STRING v.12.0 web resource (https://string-db.org/). The list of proteins with significant differential expression in the Leptospiraemia and Seroconversion Groups was submitted to the STRING platform. An interaction score threshold of 0.7 was applied for network analysis. Subsequently, network clustering was conducted with Cytoscape v.3.10.1 to reveal functional groups of closely interacting proteins.

3.12 Validation of iTRAQ Results

Sandwich ELISAs were conducted to validate the iTRAQ results for the overlapping proteins across Leptospiraemia and Seroconversion Groups. Details of the primary antibodies, secondary antibodies, and recombinant proteins used as the protein standard for each protein can be found in Appendix C.

A standard ELISA protocol (Bio-Techne, 2018) was followed, which involved coating, blocking, and detection stages, with three thorough washes after each step. Initially, a 96-well microplate was coated with the Capture Antibody and left for overnight incubation at room temperature. Then, the plate was blocked using Reagent Diluent (R&D Systems, USA) and incubated for an hour at room temperature. Serial dilutions of protein standards and diluted plasma from each sample pool were added per well in duplicate reactions and incubated for two hours at room temperature. The horseradish peroxidase (HRP)-

conjugated detection antibody was added for a one-hour incubation at room temperature. Then, the working dilution of Streptavidin-HRP was applied for detection and incubated for 20 minutes in the dark. The substrate solution was added and incubated in darkness for 20 minutes at room temperature. Finally, 2 N sulfuric acid was added to stop the reaction. The optical density (OD) of each well was measured at 450 nm with a wavelength correction at 540 nm using a Multiskan GO UV/VIS spectrophotometer (Thermo Scientific, USA).

The average of duplicate readings for each standard and sample was calculated, and the average zero standard OD was subtracted from each value. A standard curve was created employing a four-parameter logistic (4-PL) curve fit by using SkanIt® Software version 4.0. The concentration obtained from the standard curve was then multiplied by the dilution factor. The concentration of the target protein in each sample pool was calculated, and the protein fold changes between the subject groups were compared to the iTRAQ data obtained in Section 3.9. The Spearman correlation coefficient was used to evaluate the correlation between ELISA and iTRAQ data.

3.13 Verification of Differentially Expressed Proteins

The identified overlapping differential proteins across Leptospiraemia and Seroconversion Groups were verified using ELISA in an independent study cohort. The ELISAs were conducted following the procedures detailed in Section 3.12. For this verification stage, plasma samples from the HLTY-CTRL group, which were not included in the iTRAQ experiment (as explained in Section 3.7), served as the HLTY-CTRL reference. In line with the study's primary objective of distinguishing between LEPTO and non-LEPTO cases,

the leptospiraemia (LEPTO-DNA) and seroconverted (LEPTO-Ab) samples from Cohort-C were collectively categorised as LEPTO. The Kruskal-Wallis test was employed to assess the distributions of ELISA data within the three distinct groups. Subsequently, post hoc comparisons were conducted using the Mann-Whitney U test for LEPTO vs. HLTY-CTRL and LEPTO vs. DEN-CTRL comparisons. Statistically significant differences between groups were determined based on a *p*-value below 0.05.

CHAPTER 4

RESULTS

4.1 Diagnostic Results of Study Cohorts

Table 5 provides a summary of the diagnostic findings for the study cohorts that were enrolled.

Table 5: Summary of diagnostic results of study cohorts

Cohort	Derivation				Verification
	Cohort-A	Cohort-B			Cohort-C
Enrolment	Retrospective	Prospective			Independent Study
	HSDG	HSDG	HTAR	FMHS, UPM	HTI
Total of Subjects	**137**	**58**	**16**	**21**	**233**
LEPTO-Ab	11	5	3	N/A	44
LEPTO-DNA	17	17	1	N/A	
LEPTO-Ab + LEPTO-DNA	2	1	0	N/A	
DEN-CTRL	N/A	13	0	N/A	45
HLTY-CTRL	N/A	N/A	N/A	21	N/A
Non-LEPTO	107	22	12	N/A	N/A

Note:
HSDG = Hospital Serdang, Selangor
HTAR = Hospital Tengku Ampuan Rahimah, Selangor
FMHS, UPM = Faculty of Medicine and Health Sciences, Universiti Putra Malaysia
HTI = Hospital Teluk Intan, Perak
LEPTO-Ab = Patient whose diagnosis confirmed by MAT
LEPTO-DNA = Patient whose diagnosis confirmed by blood/urine culture / qPCR
LEPTO-Ab + LEPTO-DNA = Patient whose diagnosis confirmed by MAT and by blood/urine culture / PCR
DEN-CTRL = Dengue patient
HLTY-CTRL = Healthy subject
Non-LEPTO = Cases other than LEPTO, DEN-CTRL, and HLTY-CTRL
N/A = Not Available

Cohort-A comprised a total of 137 suspected leptospirosis cases, with 72% being males. Their ages ranged from 18 to 86 years, with a mean age of 35.5 (14.8). The majority were of Malay ethnicity (68%), followed by Indian (13%), Chinese (7%), and others (12%). About 22% of them were diagnosed with confirmed leptospirosis. Among the 30 cases of confirmed leptospirosis, 37% (n=11) tested positive via the MAT, 53% (n=16) tested positive by PCR, 3% (n=1) was positive in blood cultures, and 7% (n=2) was positive in both MAT and cultures.

Over the 19 months of subject enrolment for Cohort-B, 74 suspected leptospirosis patients were enrolled from HSDG and HTAR. Among them, 27 subjects (36%) were diagnosed with leptospirosis (LEPTO), and 13 subjects (18%) served as dengue controls (DEN-CTRL). None of the LEPTO patients tested positive for dengue, and all DEN-CTRL subjects tested negative for leptospirosis. The remaining 34 subjects (46%) were non-LEPTO patients, presenting a broad spectrum of clinical manifestations, from mild to severe, requiring intensive care unit (ICU) treatment. However, all enrolled subjects eventually recovered and were discharged from the hospital.

Diagnosis of leptospirosis in 26 subjects was confirmed by a single type of test. Among them, 8 LEPTO subjects (31%) tested serologically positive using MAT (LEPTO Ab). On the other hand, 18 LEPTO subjects (69%) were seronegative but tested positive in blood culture, urine culture, or by PCR (LEPTO DNA). Only one subject tested positive in both the MAT and urine culture, likely

enrolled during the convalescent stage when leptospiruria and seroconversion coincided.

Cohort-C was enrolled in an independent study conducted by Yuhana et al. (2022). Out of the 233 subjects, 44 (18%) were diagnosed with leptospirosis and 45 (19%) were diagnosed with dengue.

4.2 Renal Profile Variations

Table 6 illustrates the variations in renal profiles between LEPTO patients (n=30) and non-LEPTO (n=107). It provides a comparison of renal electrolyte and waste product levels in the two groups, along with the frequency of each level and the corresponding p-values.

Table 6: Renal profile variations between LEPTO and non-LEPTO

Parameter	Non-LEPTO (n=107)		LEPTO (n=30)		*p*-value
	Frequency	(%)	Frequency	(%)	
Calcium <2.10 mmol/L	**43**	**(62.3)**	**20**	**(90.9)**	**0.014***
Calcium >2.55 mmol/L	1	(1.4)	0	(0.0)	1.000
Urea <2.5 mmol/L	8	(7.5)	2	(6.7)	0.767
Urea >9.2 mmol/L	**17**	**(15.9)**	**12**	**(40.0)**	**0.004***
Sodium <136 mmol/L	**66**	**(61.7)**	**26**	**(86.7)**	**0.010***
Potassium <3.5 mmol/L	23	(21.5)	8	(26.7)	0.640
Potassium >5.1 mmol/L	5	(4.7)	0	(0.0)	0.364
Chloride <98 mmol/L	**26**	**(24.3)**	**16**	**(53.3)**	**0.004***
Chloride >107 mmol/L	6	(5.6)	1	(3.3)	0.972
Creatinine <53 μmol/L	1	(0.9)	0	(0.0)	1.000
Creatinine >115 μmol/L	38	(35.8)	15	(50.0)	0.172

Note: *Statistically significant at $p<0.05$

LEPTO patients exhibited a higher prevalence of hypocalcaemia (calcium <2.10 mmol/L) and hyponatremia (sodium <136 mmol/L) compared to non-LEPTO patients, both with statistically significant *p*-values (*p*= 0.014 and *p*= 0.010, respectively). Additionally, hyperkalaemia (potassium >5.1 mmol/L) was observed in none of the LEPTO cases, contrasting with 4.7% of non-LEPTO cases. Hypochloraemia (chloride <98 mmol/L) was more common among LEPTO patients (53.3% vs 24.3%) with a significant *p*-value of 0.004. A higher percentage of LEPTO patients had elevated urea levels (40% vs. 15.9%, *p*= 0.004). The creatinine levels did not significantly differ, but a higher percentage of LEPTO patients had elevated creatinine levels above 115 µmol/L (50% vs. 35.8%, *p*= 0.172).

Significant differences were observed between the two groups in terms of low calcium levels (<2.10 mmol/L), high urea levels (>9.2 mmol/L), low sodium levels (<136 mmol/L), and low chloride levels (<98 mmol/L). The multivariate logistic regression model fits the sample well, as evidenced by a Hosmer-Lemeshow significance value of 0.979. The Nagelkerke R square was 0.426. Hypocalcaemia (<2.10 mmol/L) and hypochloraemia (<98 mmol/L) were identified as predictors for presumptive leptospirosis cases (Table 7).

Table 7: Renal profile variations as predictors for presumptive leptospirosis

Factors	B	SE	Wald	*df*	*p*-value	Adjusted OR	95% CI
Calcium							
Normal	-	-	-	-	-	1.00	-
Low (<2.10 mmol/L)	2.04	1.00	4.17	1	0.041	7.72	1.09-54.98
Chloride							
Normal	-	-	-	-	-	1.00	-
Low (<98 mmol/L)	1.70	0.77	4.82	1	0.028	5.45	1.20-24.79

Note:
B = beta coefficient
SE = standard error
df = degrees of freedom
OR = odd ratio
CI = confidence interval

4.3 Protein Qualification and Quantification

Figures 8-11 visually represent plasma protein profiles from the chosen study participants using SDS-PAGE before pooling, validating the absence of contamination or degradation.

Subsequently, an equal volume of plasma samples from each subject was combined into a single pool. After pooling, the protein concentration of the combined samples was measured and recorded. The sample pools underwent SDS-PAGE to assess their quality (Figure 12). The complete trypsin digestion was confirmed by comparing 2 μL of protein-trypsin mixtures before and after the 16-hour incubation using SDS-PAGE (Figure 13).

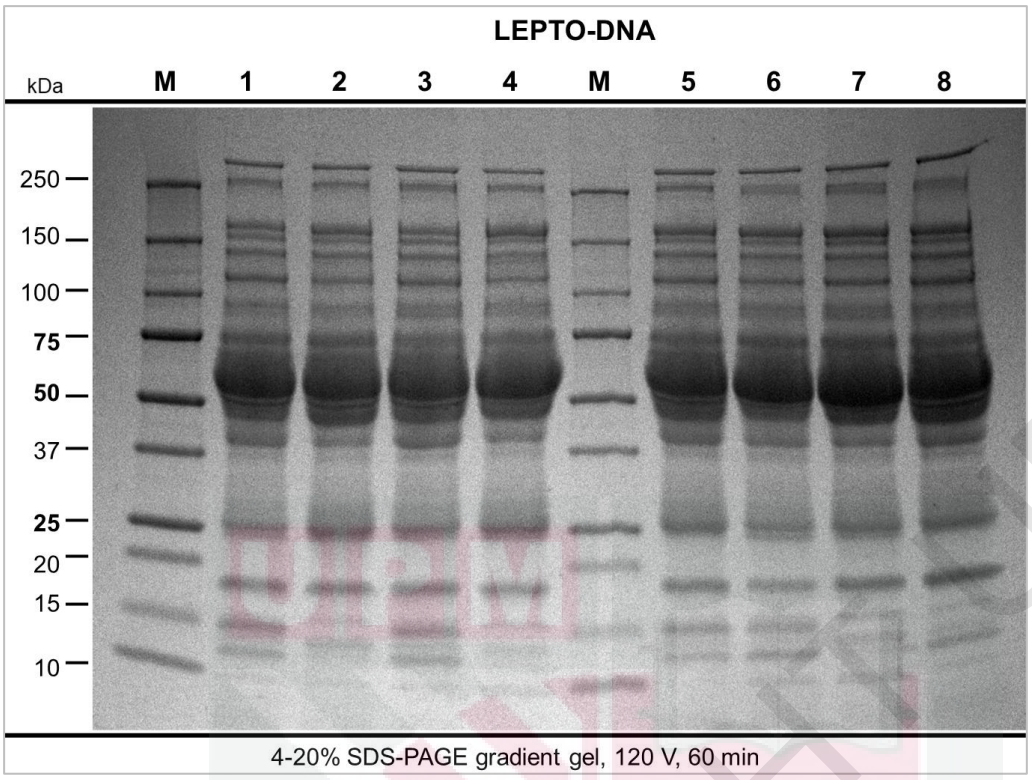


Figure 8: SDS-PAGE Profiling of Plasma Proteins in LEPTO-DNA Individuals

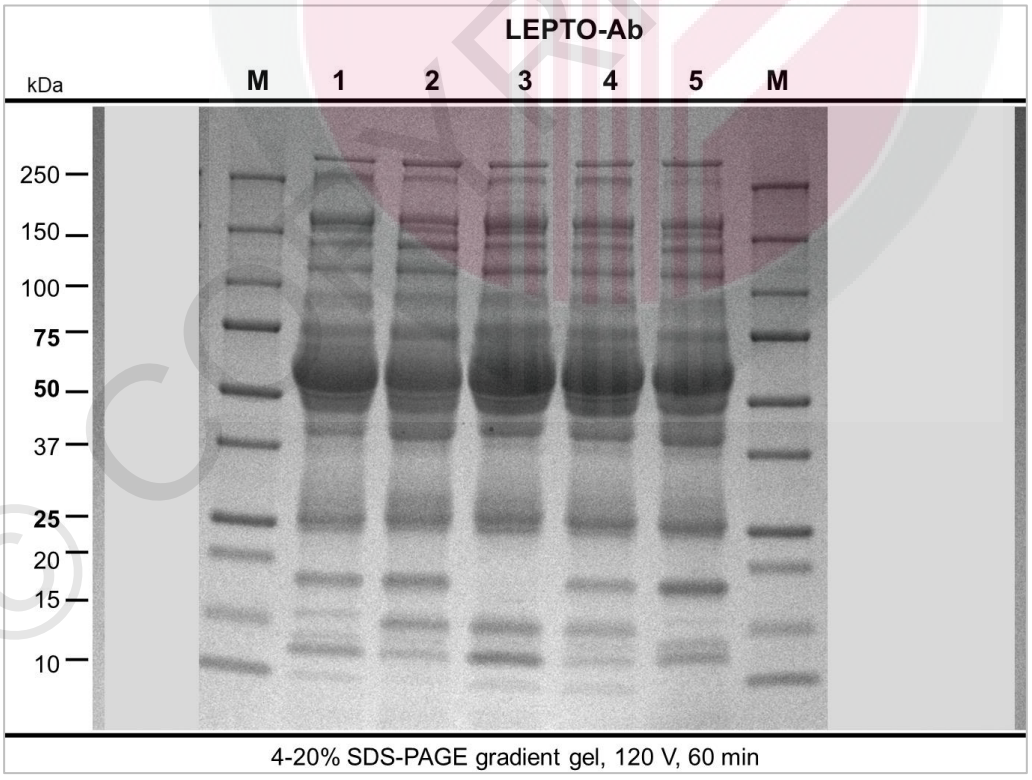


Figure 9: SDS-PAGE Profiling of Plasma Proteins in LEPTO-Ab Individuals

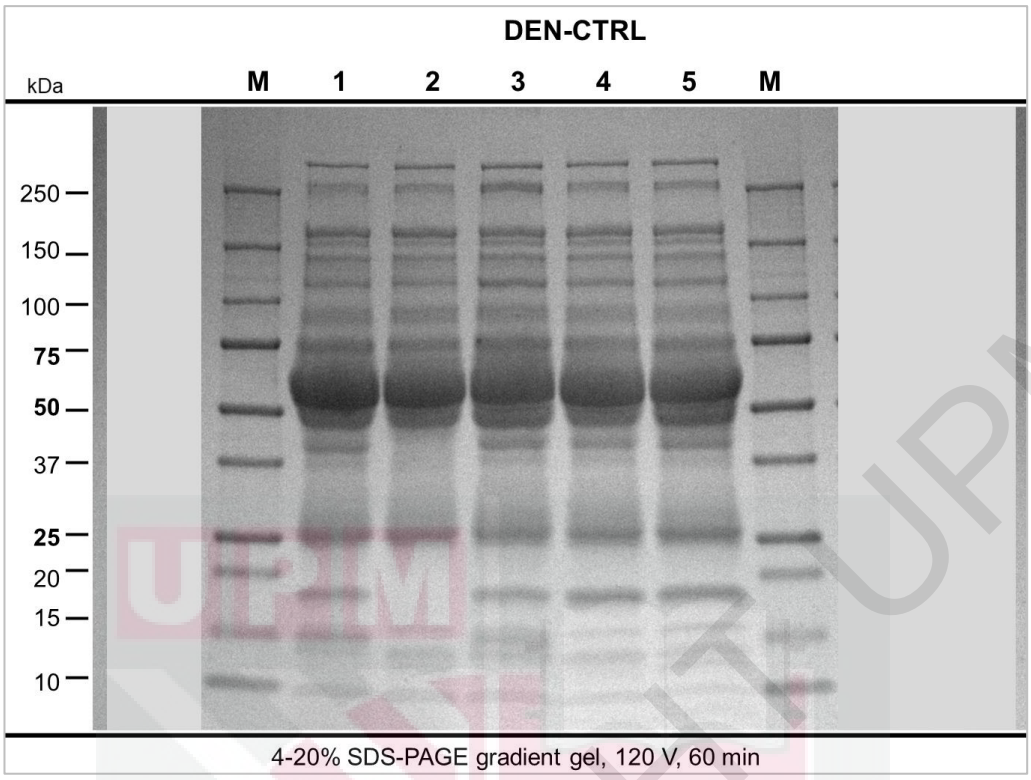


Figure 10: SDS-PAGE Profiling of Plasma Proteins in DEN-CTRL Individuals

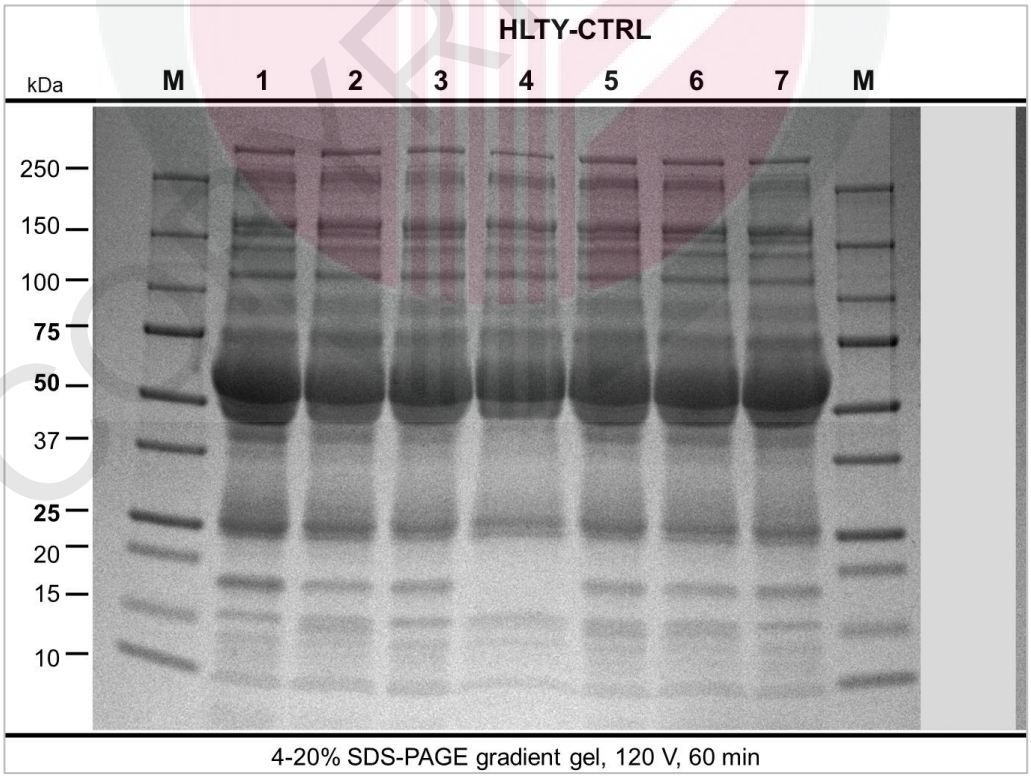
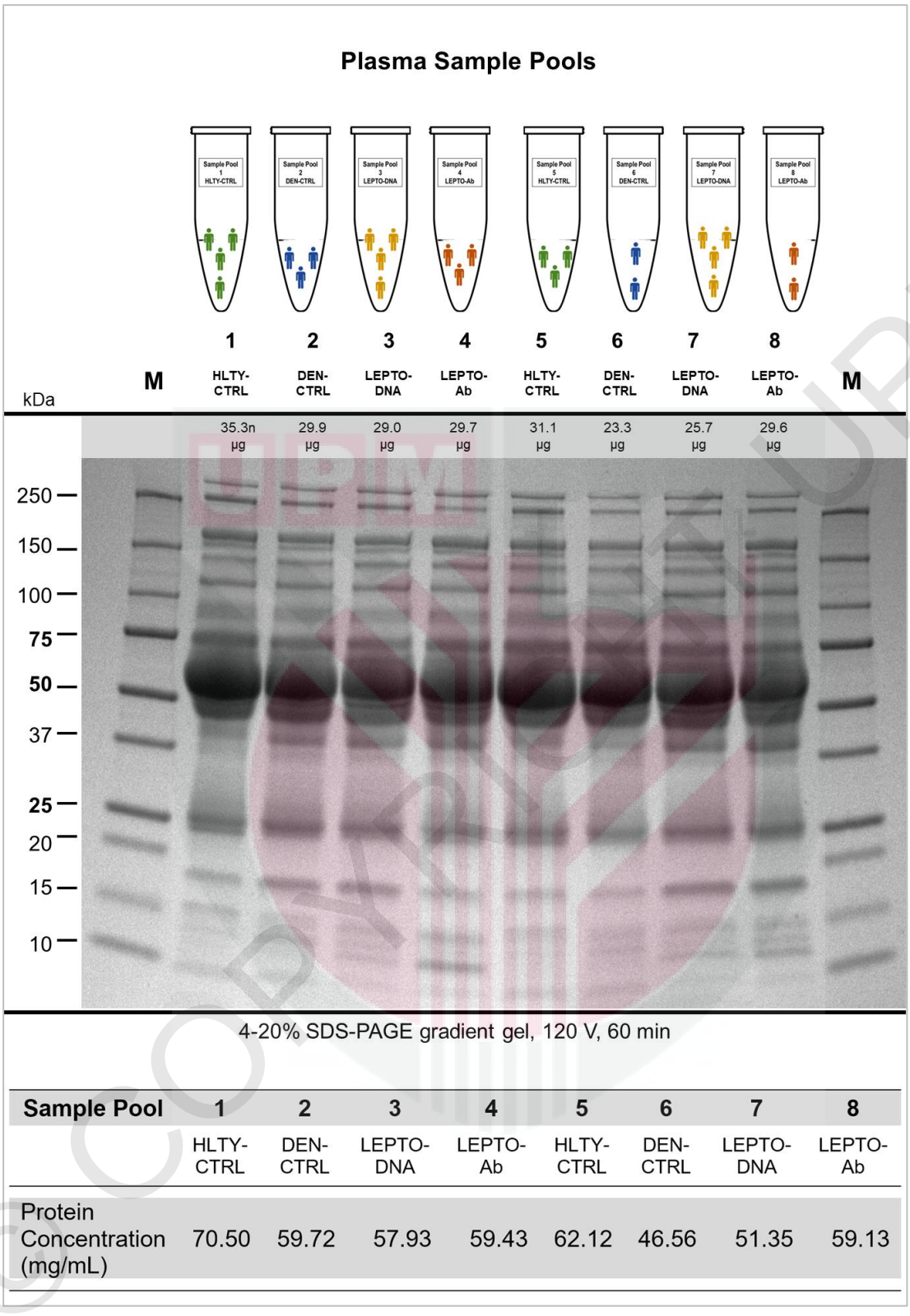


Figure 11: SDS-PAGE Profiling of Plasma Proteins in HLTY-CTRL Individuals



Sample Pool	1	2	3	4	5	6	7	8
	HLTY-CTRL	DEN-CTRL	LEPTO-DNA	LEPTO-Ab	HLTY-CTRL	DEN-CTRL	LEPTO-DNA	LEPTO-Ab
Protein Concentration (mg/mL)	70.50	59.72	57.93	59.43	62.12	46.56	51.35	59.13

Figure 12: SDS-PAGE Profiling of Plasma Proteins and Protein Concentrations in Sample Pools

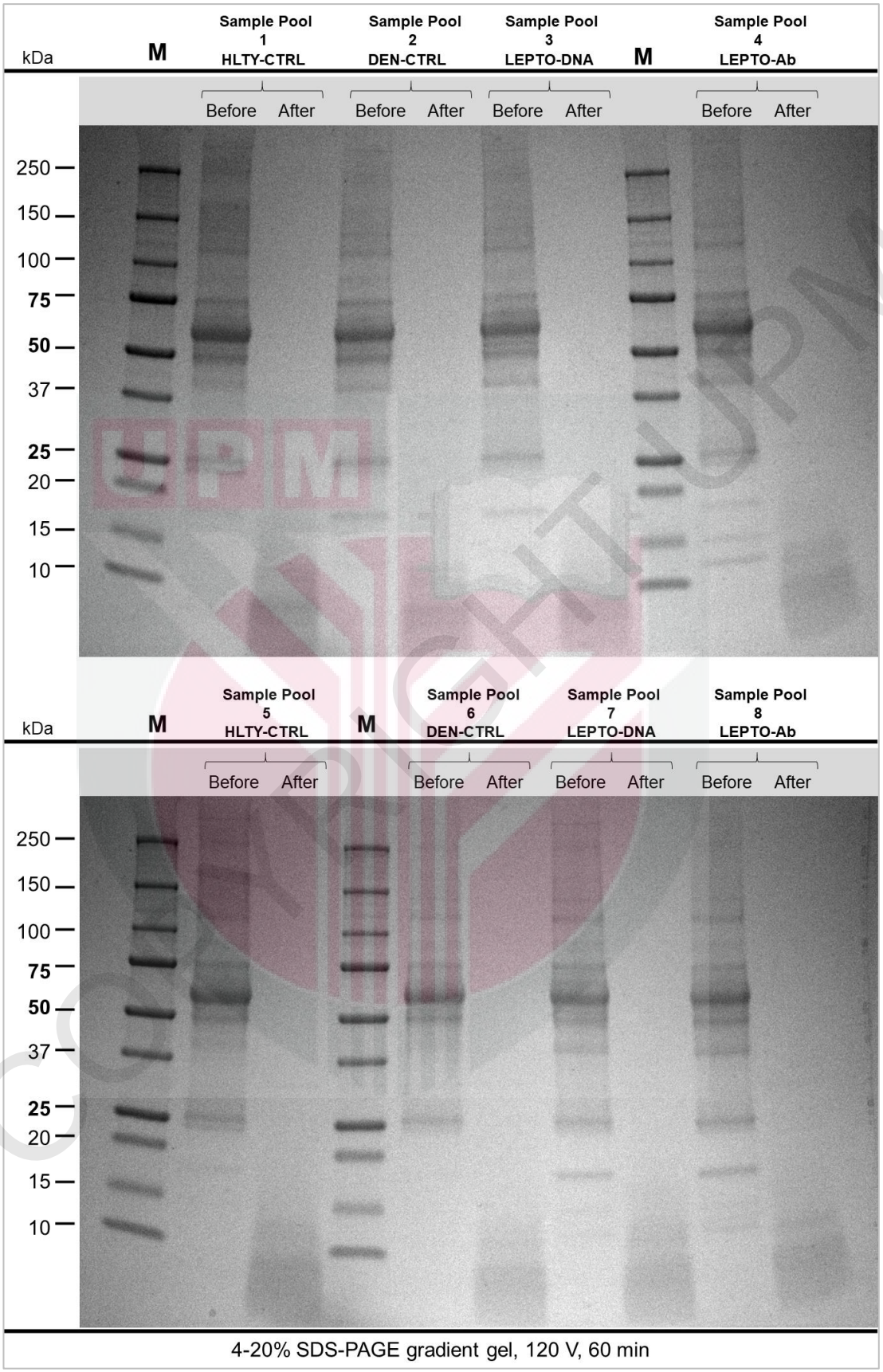


Figure 13: SDS-PAGE Analysis of Protein-Trypsin Mixtures Before and After 16-Hour Incubation for Complete Digestion Confirmation

4.4 Differential Plasma Proteome Profiles

By utilising a stringent criterion with an unused ProtScore >1.3 (at a 95% confidence level), the ProteinPilot™ software version 5.0 detected 450 proteins. Following this, a series of exclusions were applied, removing proteins without iTRAQ quantitation, those with only one matched peptide, immunoglobulins, keratins, and proteins that exceeded the 1% FDR threshold, resulting in a refined list of 290 proteins. The number of proteins that were down-regulated (iTRAQ ratios <0.8) and up-regulated (iTRAQ ratios >1.2) in each disease group compared to the control group, with a significance level of $p<0.05$, is detailed in Table 8.

Table 8: Total number of differentially expressed proteins

Subject Group	Total of Protein		Total
Disease versus (vs.) Control	Down-Regulated	Up-Regulated	
LEPTO-DNA vs. HLTY-CTRL	50	22	72
LEPTO-DNA vs. DEN-CTRL	10	48	58
LEPTO-Ab vs. HLTY-CTRL	73	22	95
LEPTO-Ab vs. DEN-CTRL	19	26	45

4.5 Functional Classification for Differentially Expressed Proteins

As depicted in the Venn diagrams (Figure 14), the Leptospiraemia and Seroconversion Groups exhibited 85 and 113 differentially expressed proteins, respectively.

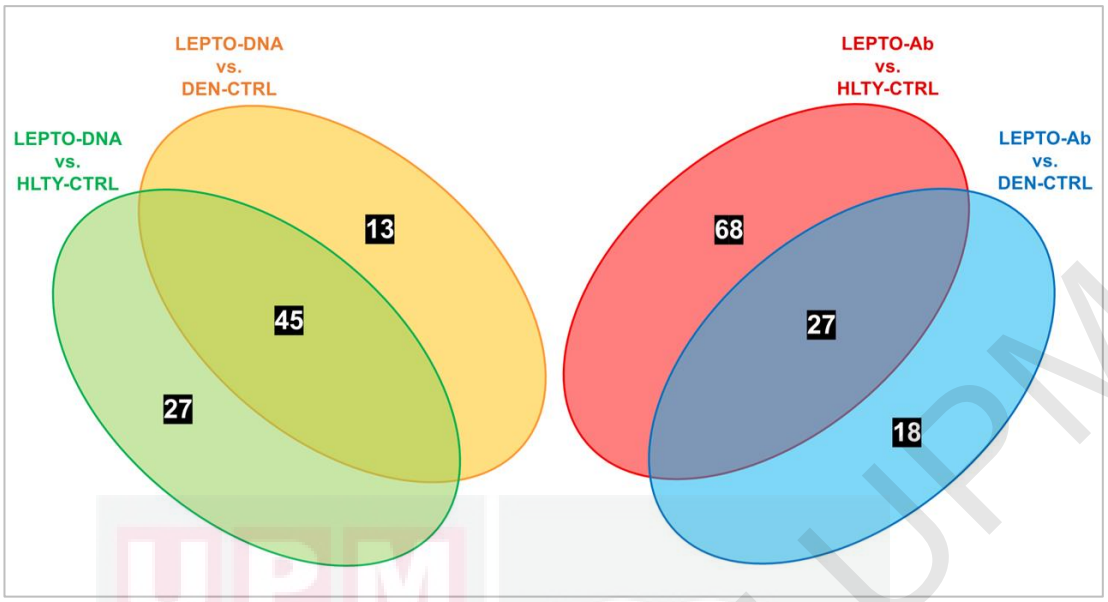


Figure 14: Total Differentially Expressed Proteins in Leptospiraemia and Seroconversion Groups

The Gene Ontology (GO) annotation data for Leptospiraemia (Figure 15) and Seroconversion (Figure 16) Groups are presented using clustered bar charts for three aspects: Molecular Function, Biological Process, and Cellular Component. The Seroconversion Group exhibits more proteins participating in most categories within these three aspects.

The comparison of Molecular Function categories between the Leptospiraemia and Seroconversion Groups reveals both commonalities and differences. Both groups exhibit common categories associated with binding, catalytic activity, molecular function regulator activity, structural molecule activity, antioxidant activity, cytoskeletal motor activity, and ATP-dependent activity. Notably, molecular transducer activity and transporter activity categories are unique to the Seroconversion Group, with two proteins each.

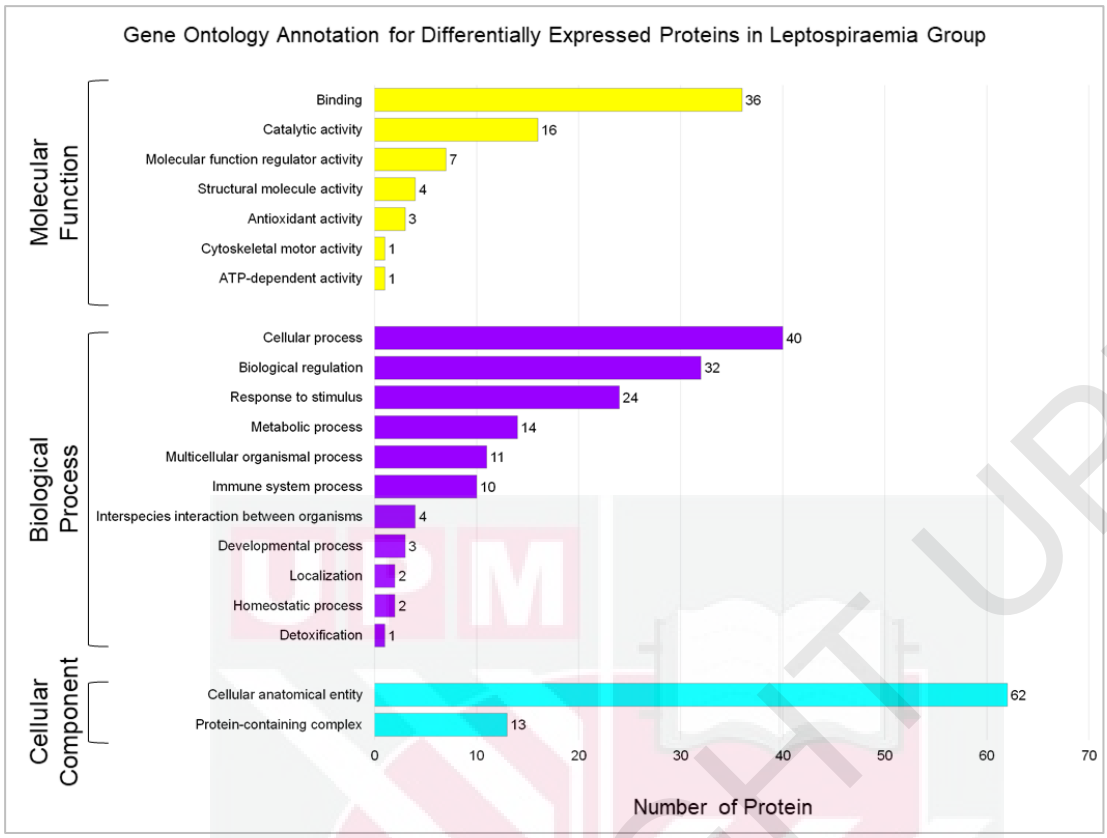


Figure 15: Gene Ontology Annotation for Differentially Expressed Proteins in Leptospiraemia Group

Fundamental Biological Process categories are shared between both Leptospiraemia and Seroconversion Groups. The cellular process is a common category with the highest number of proteins in both groups, featuring 40 proteins in Leptospiraemia and 47 in Seroconversion. A higher number of proteins is found in the Seroconversion group for many Biological Process categories, including biological regulation, response to stimulus, metabolic process, multicellular organismal process, developmental process, and localisation.

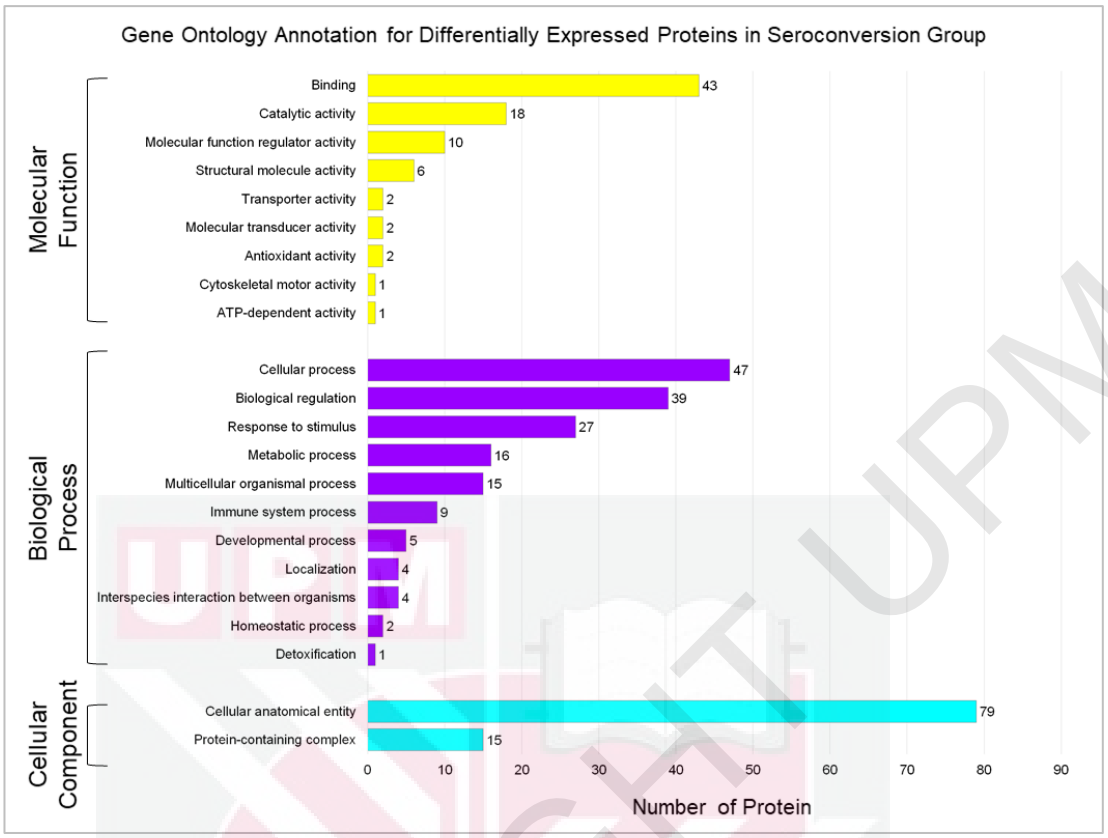


Figure 16: Gene Ontology Annotation for Differentially Expressed Proteins in Seroconversion Group

The comparison of Cellular Component categories in the Leptospiraemia and Seroconversion Groups reveals that the basic cellular structures and protein complexes remain consistent throughout the disease progression. However, there is an increase in the number of proteins associated with the categories of cellular anatomical entity and protein-containing complex in the Seroconversion Group. The total proteins related to cellular anatomical entity account for 73% (62 out of 85) in the Leptospiraemia Group and 70% (79 out of 113) in the Seroconversion Group.

4.6 Protein-protein Interaction Network

Protein-protein interactions (PPI) are visualised using the full STRING network in the evidence view, connecting proteins based on various interaction sources, including co-expression, co-occurrence, databases, experiments, gene fusion, neighbourhood, and text-mining. Out of the 128 differentially expressed proteins identified through iTRAQ in the Leptospiraemia and Seroconversion Groups, 127 proteins were integrated into the network, establishing 489 connections among them (Figure 17). The nodes within the network, which represent proteins uniquely differentially expressed in either the Leptospiraemia (highlighted as yellow nodes) or the Seroconversion Group (highlighted as red nodes), are depicted in Figure 18.

In the PPI network, several clusters of nodes are identified. Each cluster represents a group of proteins that interact more closely with each other than those outside the cluster. Cluster 1 consists of 15 nodes connected by 93 edges. The analysis of GO terms within Cluster 1 reveals that 99.03% of the functional annotations are related to reverse cholesterol transport, and 0.97% are associated with acute-phase response (Figure 19). Cluster 2 contains 16 nodes and 40 edges (Figure 20). Functional annotation analysis of Cluster 2 reveals that several functional annotations are prominent, with the majority (55.26%) associated with platelet aggregation. Additionally, 31.58% of the functional annotations are linked to cell-matrix adhesion, and 10.53% relate to fibroblast migration. There is also a minor association with an acute-phase response (2.63%).

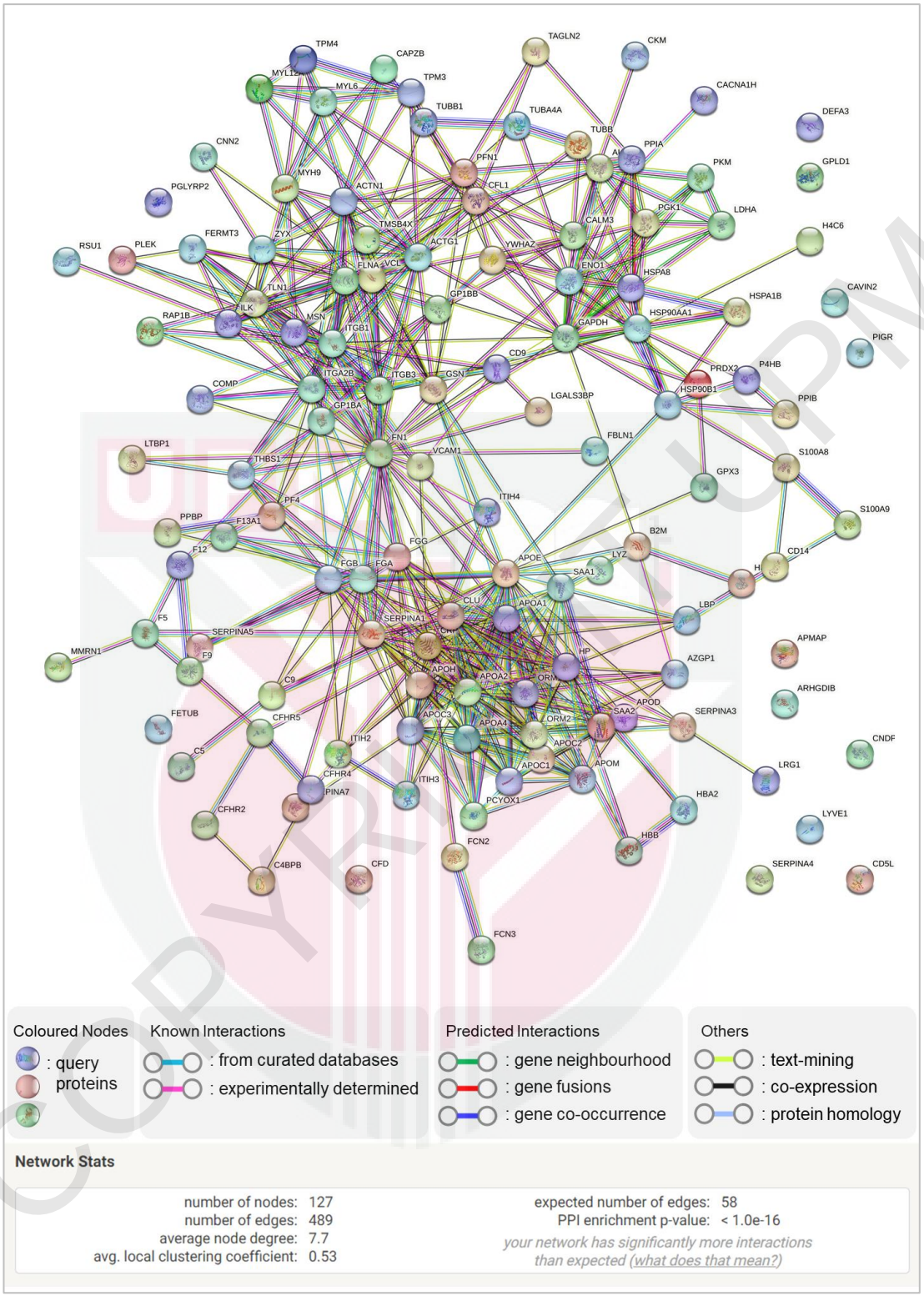


Figure 17: Protein-protein Interaction Network of Differentially Expressed Proteins in Leptospiraemia and Seroconversion Groups

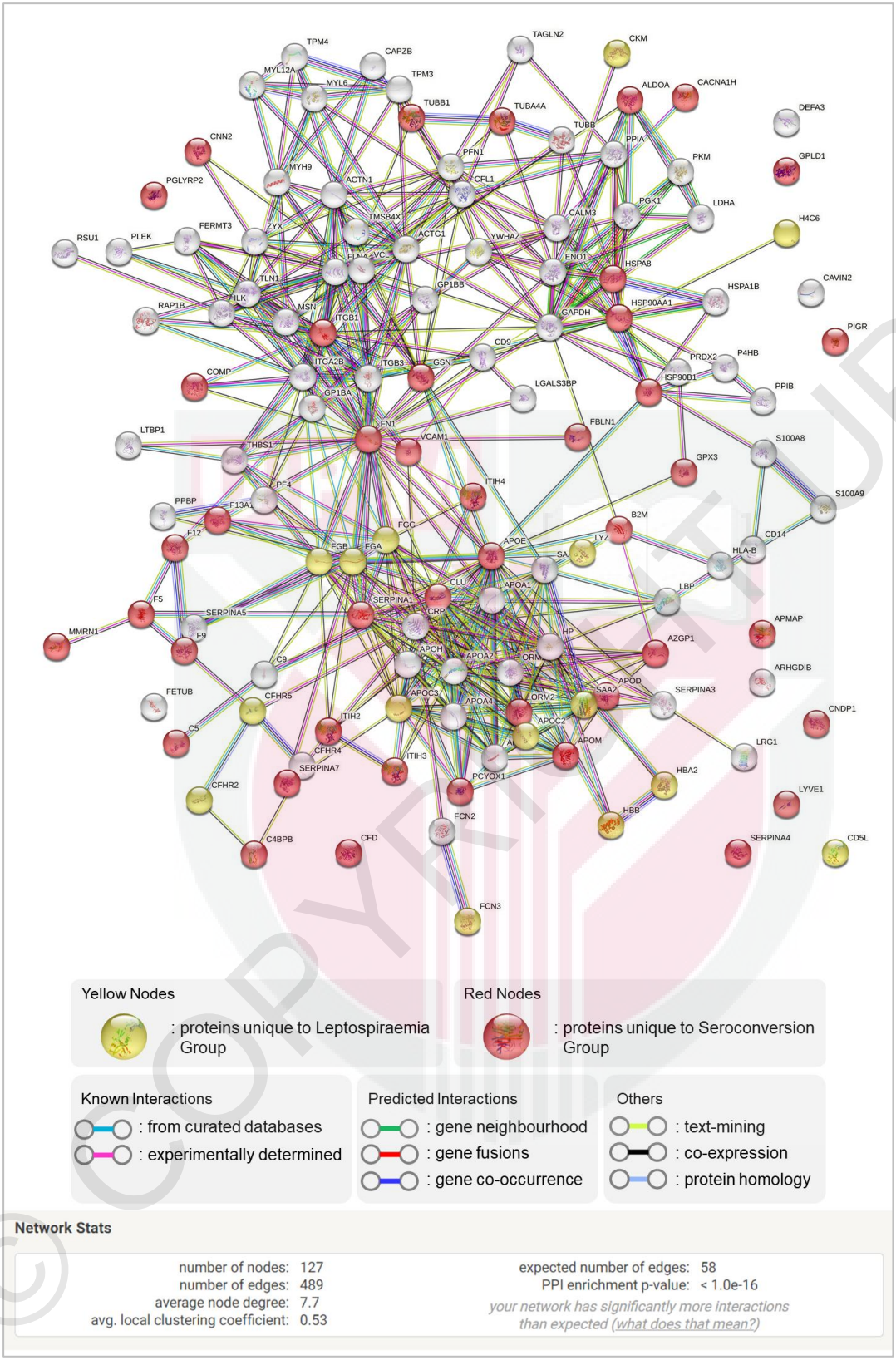


Figure 18: Exclusive Differentially Expressed Proteins in Leptospiraemia and Seroconversion Groups

[The protein abbreviation list for uniquely expressed proteins in Leptospiraemia (yellow nodes) or the Seroconversion (red nodes) Groups is in Appendix E and F.]

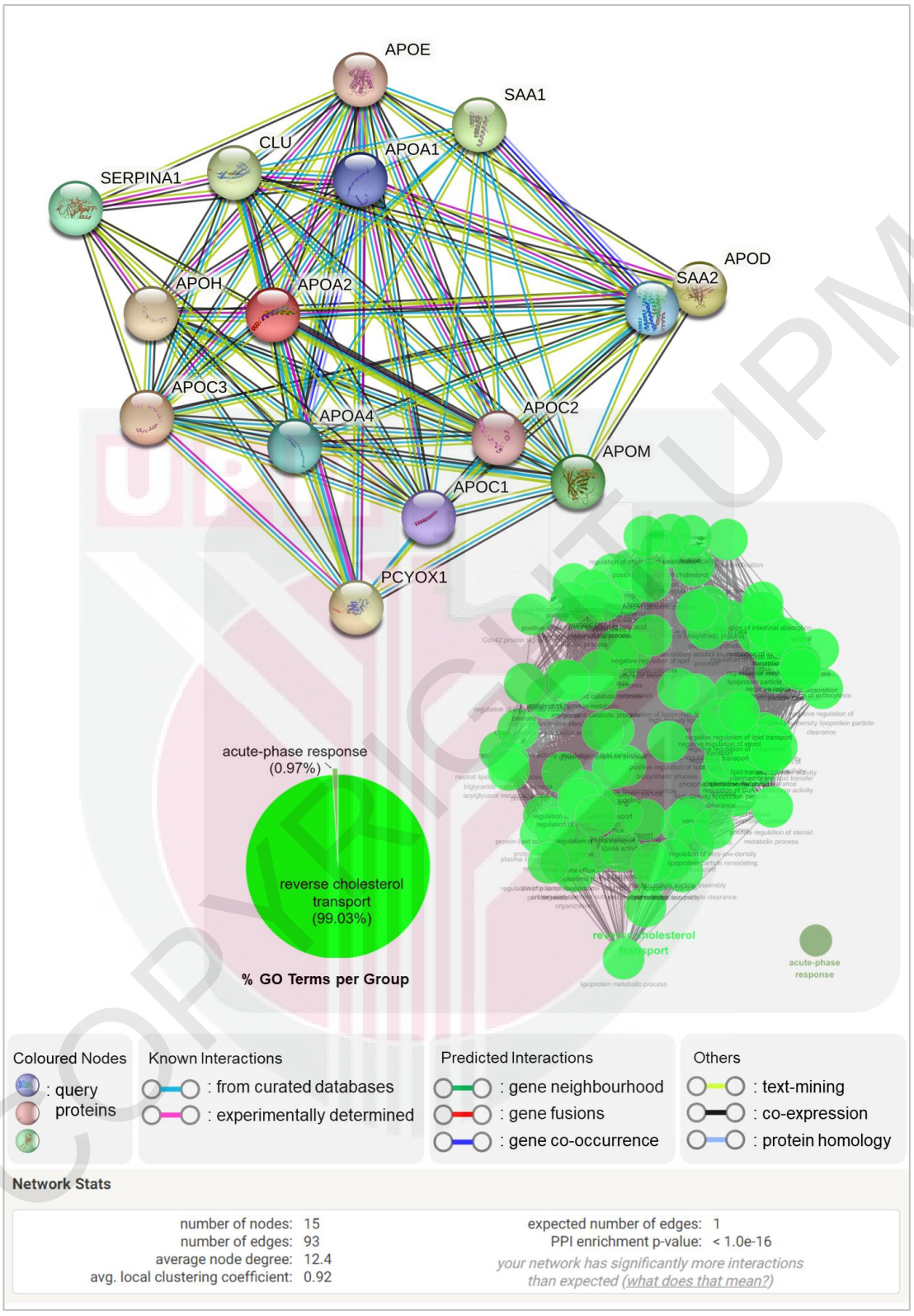


Figure 19: Cluster 1 of Protein-protein Interaction Network and Functional Annotations Summary
(The protein abbreviation list for Cluster 1 is in Appendix G.)

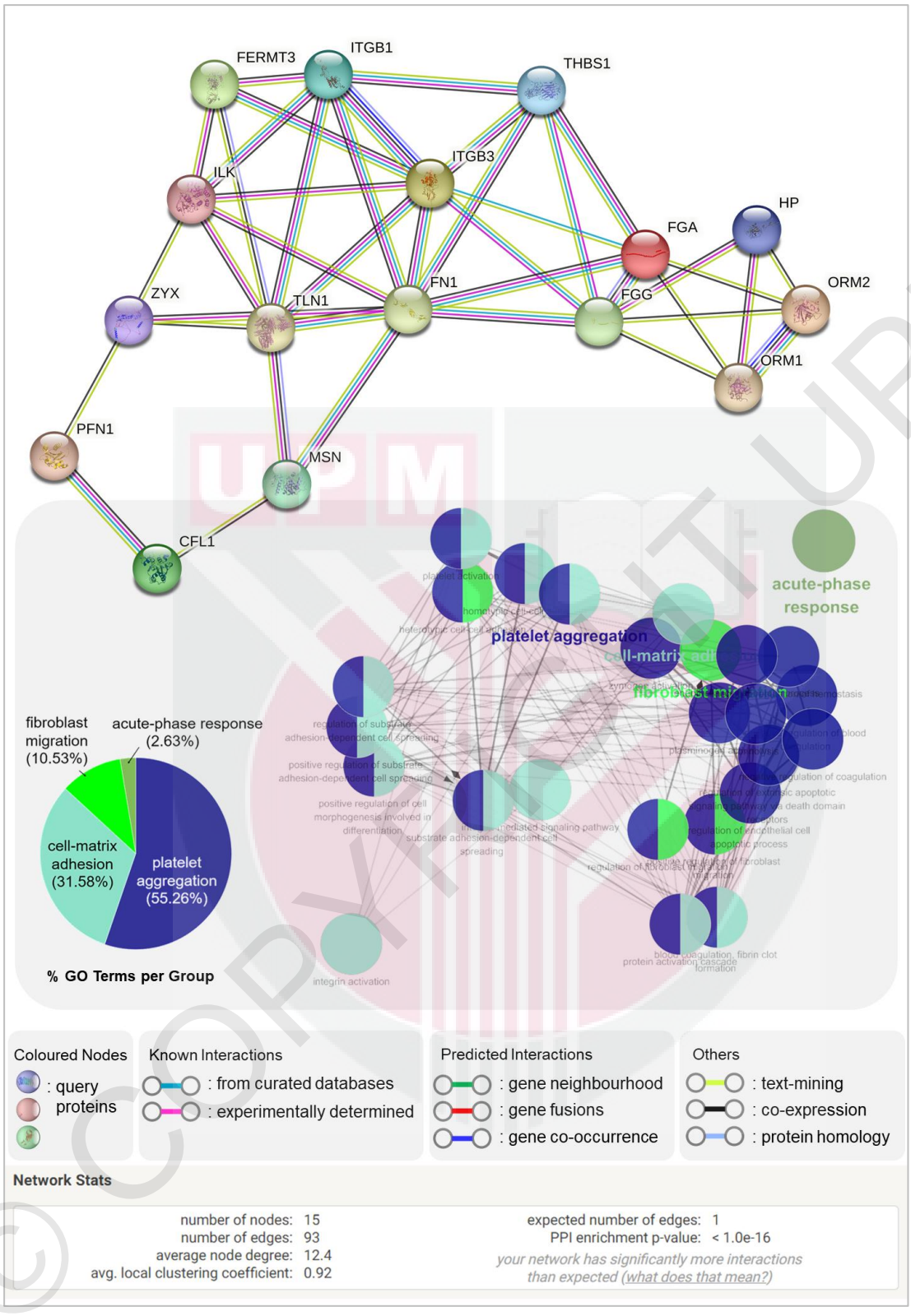


Figure 20: Cluster 2 of Protein-protein Interaction Network and Functional Annotations Summary
(The protein abbreviation list for Cluster 2 is in Appendix H.)

Cluster 3, a relatively small but interconnected group of proteins comprises 10 nodes and 21 edges. The functional annotations analysis of Cluster 3 shows that all (100%) are related to platelet aggregation (Figure 21).

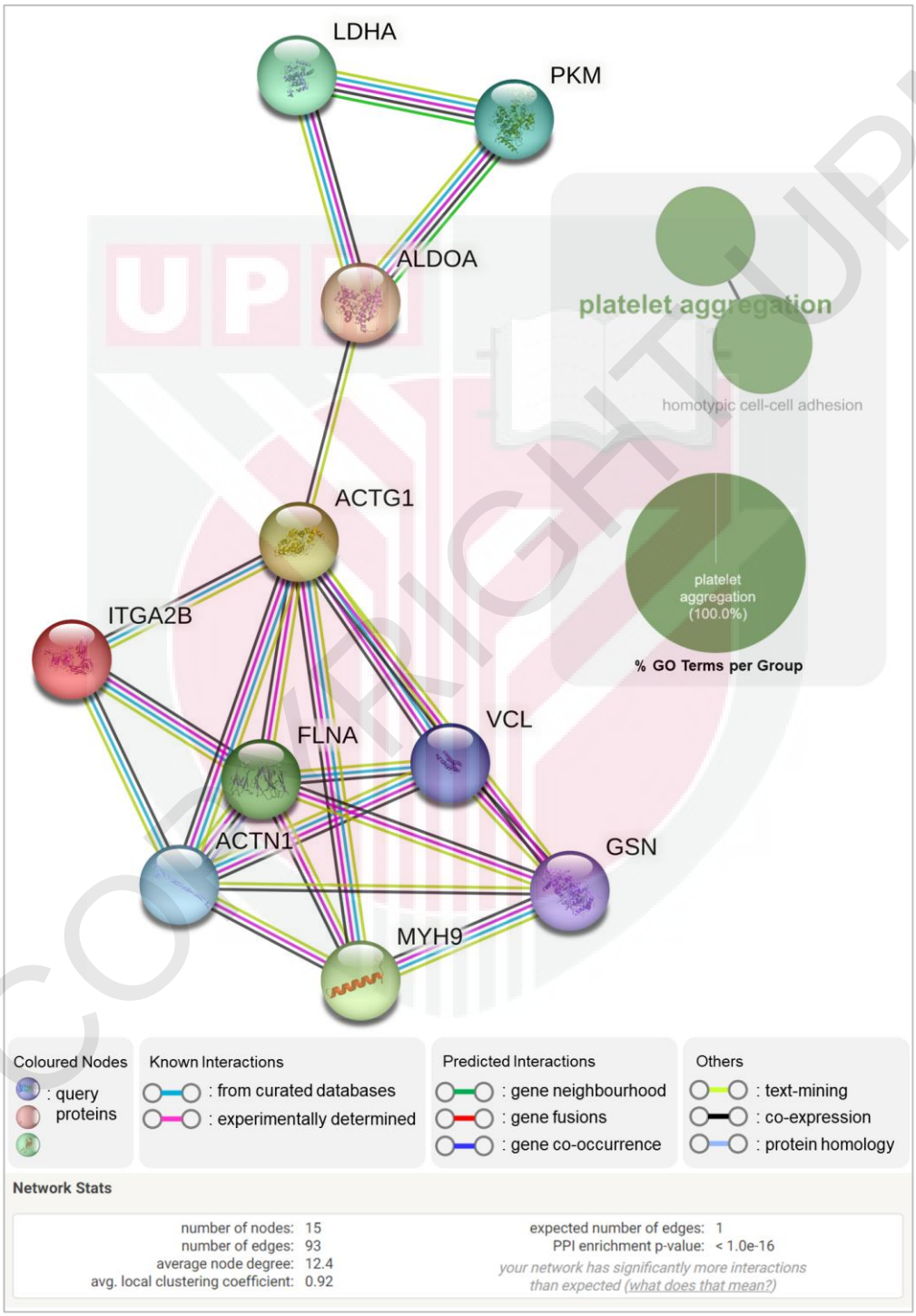


Figure 21: Cluster 3 of Protein-protein Interaction Network and Functional Annotations Summary
(The protein abbreviation list for Cluster 3 is in Appendix H.)

4.7 Validation of iTRAQ Data

A total of 11 proteins exhibited significant expression differences in LEPTO cases compared to HTLY-CTRL and DEN-CTRL. Their identification is visualised in Figure 22, showing an overlap in their presence. They include apolipoprotein A-II (APOA2), C-reactive protein (CRP), fermitin family homolog 3 (FERMT3), leucine-rich alpha-2-glycoprotein 1 (LRG1), lipopolysaccharide-binding protein (LBP), myosin-9 (MYH9), platelet basic protein (PPBP), platelet factor 4 (PF4), profilin-1 (PFN1), serum amyloid A-1 protein (SAA1), and thrombospondin-1 (THBS1). Notably, CRP, LBP, MYH9, and THBS1 exhibited differential expression between LEPTO-DNA and LEPTO-Ab cases. A summary of these findings is presented in Table 9.

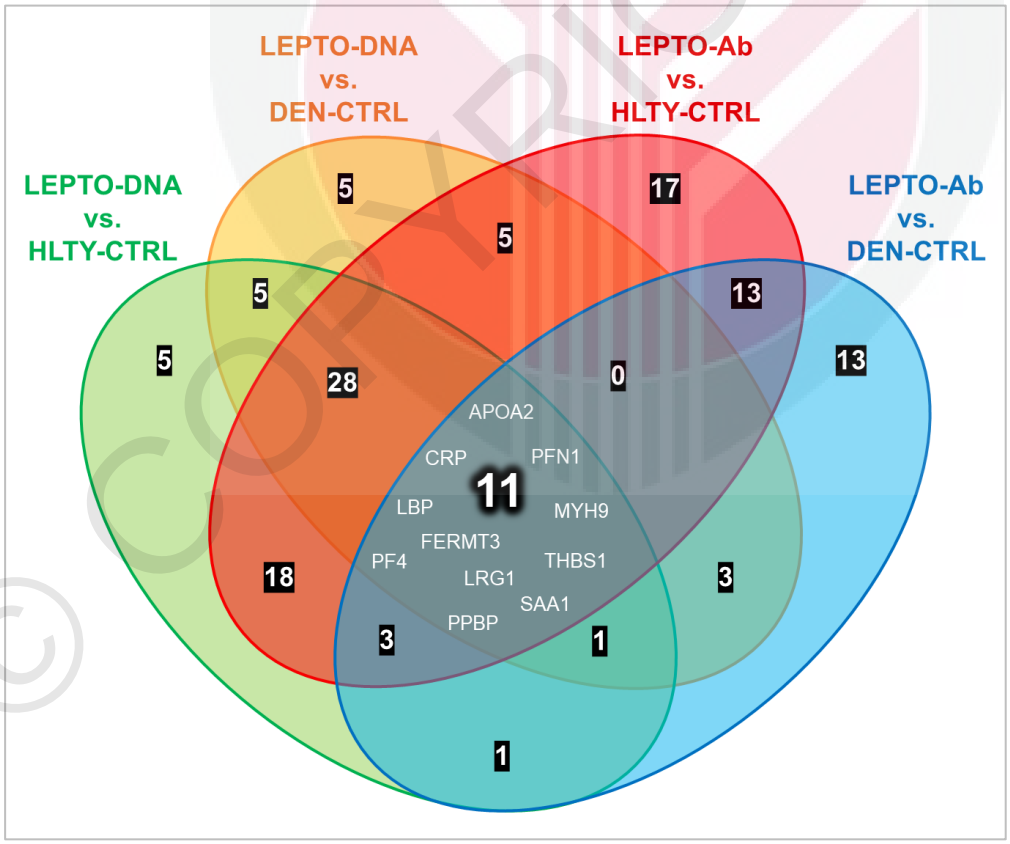


Figure 22: Overlapping Differentially Expressed Proteins in Leptospiraemia and Seroconversion Groups

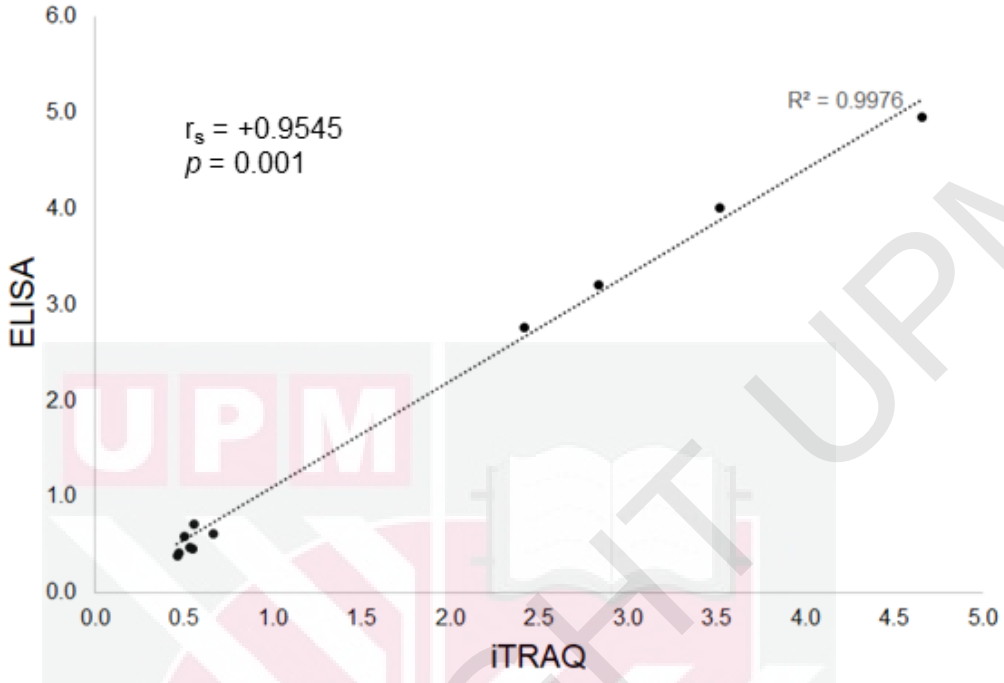
Table 9: Differentially expressed proteins in LEPTO groups compared to HTLY-CTRL and DEN-CTRL

UniProtKB	Protein Name	Peptides (95%)	LEPTO-DNA vs. HLTY-CTRL		LEPTO-DNA vs. DEN-CTRL		LEPTO-Ab vs. HLTY-CTRL		LEPTO-Ab vs. DEN-CTRL		LEPTO-DNA vs. LEPTO-Ab	
			iTRAQ Ratio	*p*-Value	iTRAQ Ratio	*p*-Value	iTRAQ Ratio	*p*-Value	iTRAQ Ratio	*p*-Value	iTRAQ Ratio	*p*-Value
P02652	Apolipoprotein A-II	9	0.4969	0.03862	0.5422	0.03725	0.5940	0.04226	0.6452	0.03497	N/A	N/A
P02741	*C-reactive protein	12	**4.6496**	**0.00046**	**4.3649**	**0.00211**	**8.2865**	**0.00037**	**7.7904**	**0.00119**	0.5454	0.02605
Q86UX7	Fermitin family homolog 3	5	0.5420	0.01252	**1.9335**	**0.00753**	0.4657	0.00059	**1.5896**	**0.00048**	N/A	N/A
P02750	Leucine-rich alpha-2-glycoprotein 1	14	**2.4084**	**0.00774**	**1.5342**	**0.01678**	**3.1812**	**0.00318**	**2.0392**	**0.00304**	N/A	N/A
P18428	*Lipopolysaccharide-binding protein	11	**2.8268**	**0.00047**	**1.2157**	**0.01519**	**4.3832**	**0.00122**	**1.8999**	**0.01116**	0.6398	0.03392
P35579	*Myosin-9	50	0.5297	0.00565	**1.8343**	**0.00316**	0.3545	0.00048	**1.2028**	**0.02103**	**1.5287**	**0.01044**
P02775	Platelet basic protein	8	0.4603	0.00780	**2.0321**	**0.00583**	0.3447	0.00163	**1.5548**	**0.00868**	N/A	N/A
P02776	Platelet factor 4	5	0.4626	0.00362	**2.9621**	**0.00061**	0.2957	0.00445	**1.8970**	**0.02105**	N/A	N/A
P07737	Profilin-1	6	0.5506	0.00431	**1.5633**	**0.00878**	0.5067	0.00039	**1.4469**	**0.00258**	N/A	N/A
P0DJI8	Serum amyloid A-1 protein	53	**3.5115**	**0.00590**	**2.5205**	**0.03243**	**7.2393**	**0.01243**	**5.4754**	**0.02176**	N/A	N/A
P07996	*Thrombospondin-1	12	0.6609	0.00783	**1.7034**	**0.00159**	0.4892	0.00251	**1.2505**	**0.03162**	**1.3883**	**0.01670**

Note:
*Differentially expressed proteins in LEPTO-DNA compared to LEPTO-Ab.
Proteins are **up-regulated (iTRAQ ratios >1.2)** or down-regulated (iTRAQ ratios <0.8).
N/A: Not Available

The iTRAQ results for these 11 proteins were validated using ELISAs. The correlation between iTRAQ and ELISA data was visualised through scatterplots (Figure 23-26). The Spearman correlation coefficient (r_s) was used to assess the strength and significance of the relationship between the two datasets. The r_s will consistently fall between 1.0 representing a perfect positive correlation, and -1.0 indicating a perfect negative correlation. A r_s of 0 signifies no association between the datasets. The interpretation of r_s strength is as follows: r_s >0.9 indicates a very strong correlation, r_s between 0.70 and 0.89 indicates a strong correlation, r_s between 0.40 and 0.69 indicates a moderate correlation, r_s between 0.20 and 0.39 indicates a weak correlation, and r_s <0.19 indicates a very weak correlation (Fowler et al., 2013).

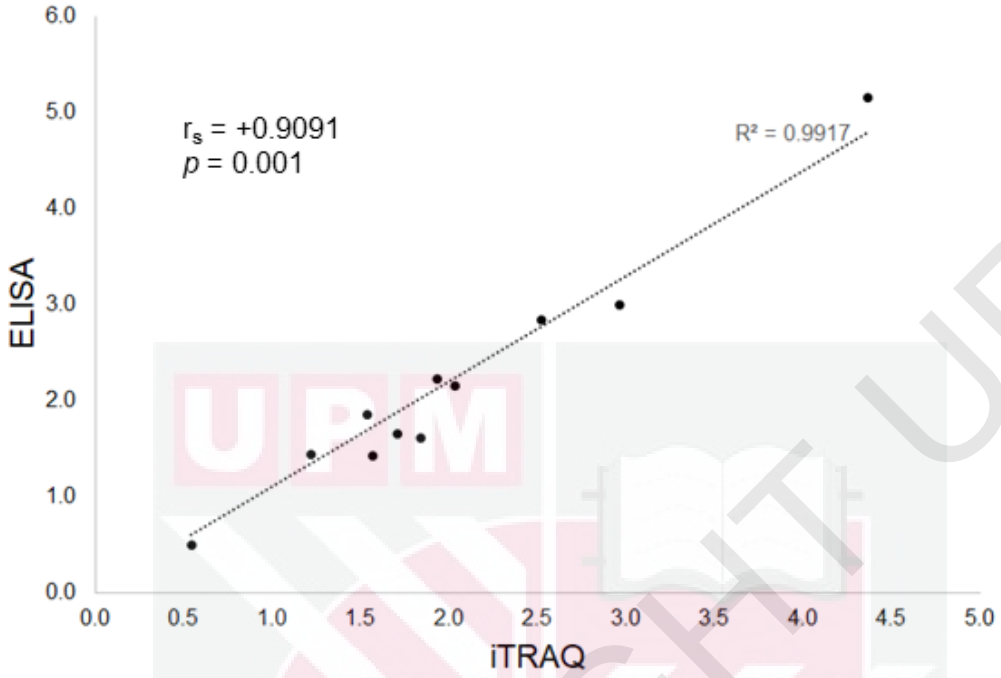
The r_s was calculated for each of the four groups representing LEPTO compared to HLTY-CTRL or DEN-CTRL, where each group contained data for 11 proteins. The strong positive correlations observed in all groups, with r_s values ranging from +0.9091 to +0.9818, suggest a strong positive association between the protein fold changes measured by iTRAQ and ELISA. These high r_s values imply the consistency between the two methods in quantifying protein fold changes.



UniProtKB	Protein Name	LEPTO-DNA vs. HLTY-CTRL Protein Fold Change	
		iTRAQ	ELISA
P02652	Apolipoprotein A-II	- 0.4969	- 0.6019
P02741	C-reactive protein	**+ 4.6496**	**+ 4.9611**
Q86UX7	Fermitin family homolog 3	- 0.5420	- 0.4630
P02750	Leucine-rich alpha-2-glycoprotein 1	**+ 2.4084**	**+ 2.7705**
P18428	Lipopolysaccharide-binding protein	**+ 2.8268**	**+ 3.2101**
P35579	Myosin-9	- 0.5297	- 0.4827
P02775	Platelet basic protein	- 0.4603	- 0.3958
P02776	Platelet factor 4	- 0.4626	- 0.4244
P07737	Profilin-1	- 0.5506	- 0.7185
P0DJI8	Serum amyloid A-1 protein	**+ 3.5115**	**+ 4.0200**
P07996	Thrombospondin-1	- 0.6609	0.6238

Note:
"+" indicates an increase in the direction of protein fold change.
"-" indicates a decrease in the direction of protein fold change.

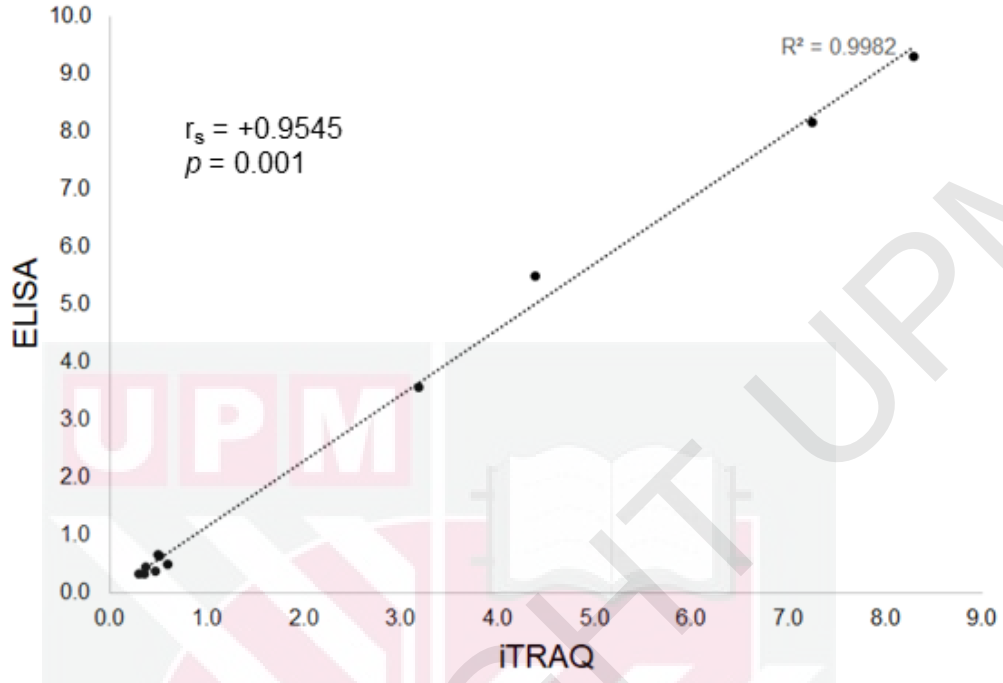
Figure 23: Scatterplot Comparing Protein Fold Changes Measured by iTRAQ and ELISA in LEPTO-DNA vs. HLTY-CTRL



UniProtKB	Protein Name	LEPTO-DNA vs. DEN-CTRL Protein Fold Change	
		iTRAQ	ELISA
P02652	Apolipoprotein A-II	- 0.5422	- 0.4976
P02741	C-reactive protein	**+ 4.3649**	**+ 5.1704**
Q86UX7	Fermitin family homolog 3	**+ 1.9335**	**+ 2.2351**
P02750	Leucine-rich alpha-2-glycoprotein 1	**+ 1.5342**	**+ 1.8640**
P18428	Lipopolysaccharide-binding protein	**+ 1.2157**	**+ 1.4484**
P35579	Myosin-9	**+ 1.8343**	**+ 1.6210**
P02775	Platelet basic protein	**+ 2.0321**	**+ 2.1649**
P02776	Platelet factor 4	**+ 2.9621**	**+ 3.0100**
P07737	Profilin-1	**+ 1.5633**	**+ 1.4357**
P0DJI8	Serum amyloid A-1 protein	**+ 2.5205**	**+ 2.8493**
P07996	Thrombospondin-1	**+ 1.7034**	**+ 1.6670**

Note:
"+" indicates an increase in the direction of protein fold change.
"-" indicates a decrease in the direction of protein fold change.

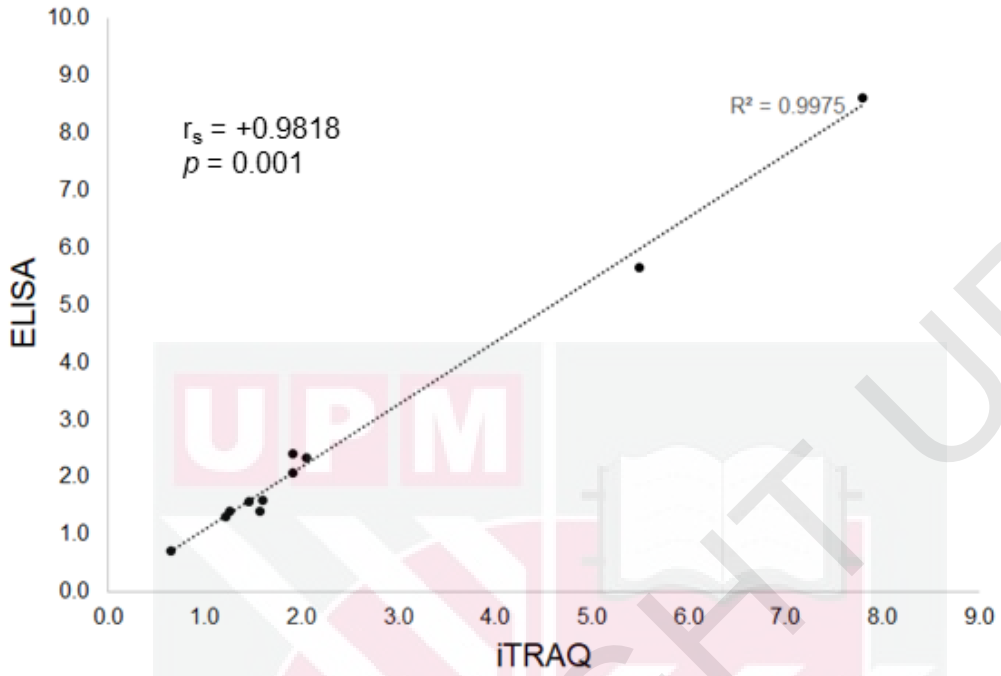
Figure 24: Scatterplot Comparing Protein Fold Changes Measured by iTRAQ and ELISA in LEPTO-DNA vs. DEN-CTRL



UniProtKB	Protein Name	LEPTO-Ab vs. HLTY-CTRL Protein Fold Change	
		iTRAQ	ELISA
P02652	Apolipoprotein A-II	- 0.5940	- 0.5041
P02741	C-reactive protein	**+ 8.2865**	**+ 9.3308**
Q86UX7	Fermitin family homolog 3	- 0.4657	- 0.3903
P02750	Leucine-rich alpha-2-glycoprotein 1	**+ 3.1812**	**+ 3.5679**
P18428	Lipopolysaccharide-binding protein	**+ 4.3832**	**+ 5.5162**
P35579	Myosin-9	- 0.3545	- 0.4460
P02775	Platelet basic protein	- 0.3447	- 0.3482
P02776	Platelet factor 4	- 0.2957	- 0.3312
P07737	Profilin-1	- 0.5067	- 0.6518
P0DJI8	Serum amyloid A-1 protein	**+ 7.2393**	**+ 8.1861**
P07996	Thrombospondin-1	- 0.4892	- 0.6694

Note:
"+" indicates an increase in the direction of protein fold change.
"-" indicates a decrease in the direction of protein fold change.

Figure 25: Scatterplot Comparing Protein Fold Changes Measured by iTRAQ and ELISA in LEPTO-Ab vs. HLTY-CTRL



UniProtKB	Protein Name	LEPTO-Ab vs. DEN-CTRL Protein Fold Change	
		iTRAQ	ELISA
P02652	Apolipoprotein A-II	- 0.6452	- 0.7258
P02741	C-reactive protein	**+ 7.7904**	**+ 8.6226**
Q86UX7	Fermitin family homolog 3	**+ 1.5896**	**+ 1.6140**
P02750	Leucine-rich alpha-2-glycoprotein 1	**+ 2.0392**	**+ 2.3442**
P18428	Lipopolysaccharide-binding protein	**+ 1.8999**	**+ 2.4215**
P35579	Myosin-9	**+ 1.2028**	**+ 1.3350**
P02775	Platelet basic protein	**+ 1.5548**	**+ 1.4175**
P02776	Platelet factor 4	**+ 1.8970**	**+ 2.0823**
P07737	Profilin-1	**+ 1.4469**	**+ 1.5930**
P0DJI8	Serum amyloid A-1 protein	**+ 5.4754**	**+ 5.6717**
P07996	Thrombospondin-1	**+ 1.2505**	**+ 1.4126**

Note:
"+" indicates an increase in the direction of protein fold change.
"-" indicates a decrease in the direction of protein fold change.

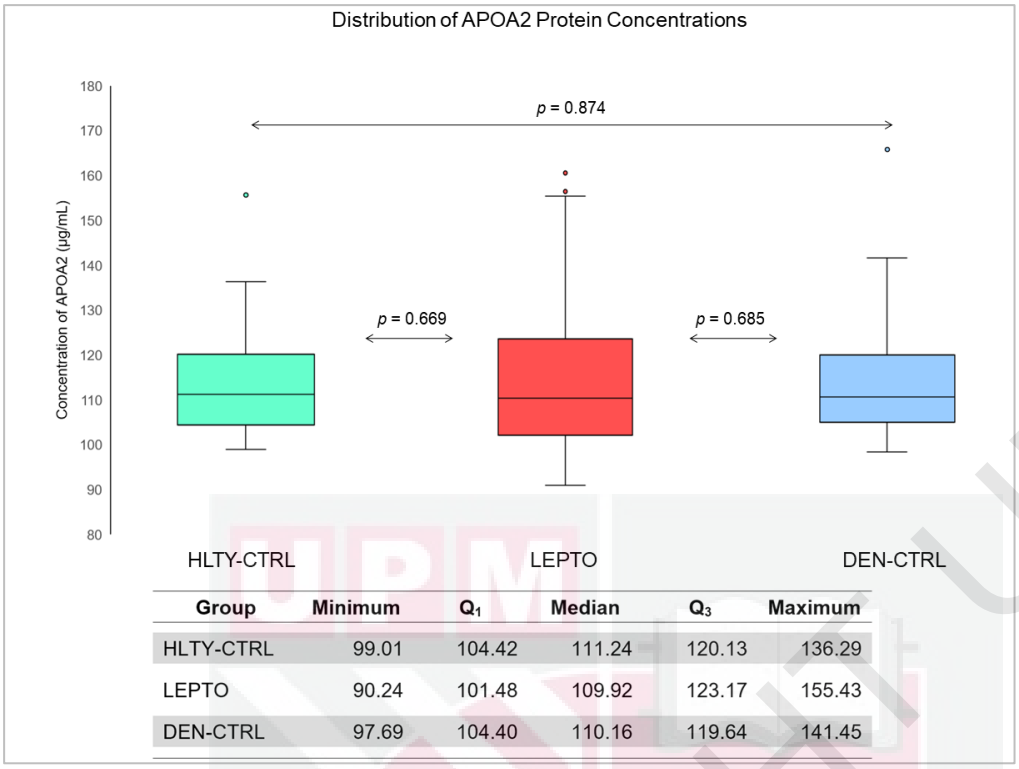
Figure 26: Scatterplot Comparing Protein Fold Changes Measured by iTRAQ and ELISA in LEPTO-Ab vs. DEN-CTRL

4.8 Verification of Differentially Expressed Proteins

The identified differentially expressed proteins were verified through ELISA within an independent study cohort, consisting of 44 individuals with LEPTO, 45 from the DEN-CTRL group, and 14 subjects classified as HLTY-CTRL.

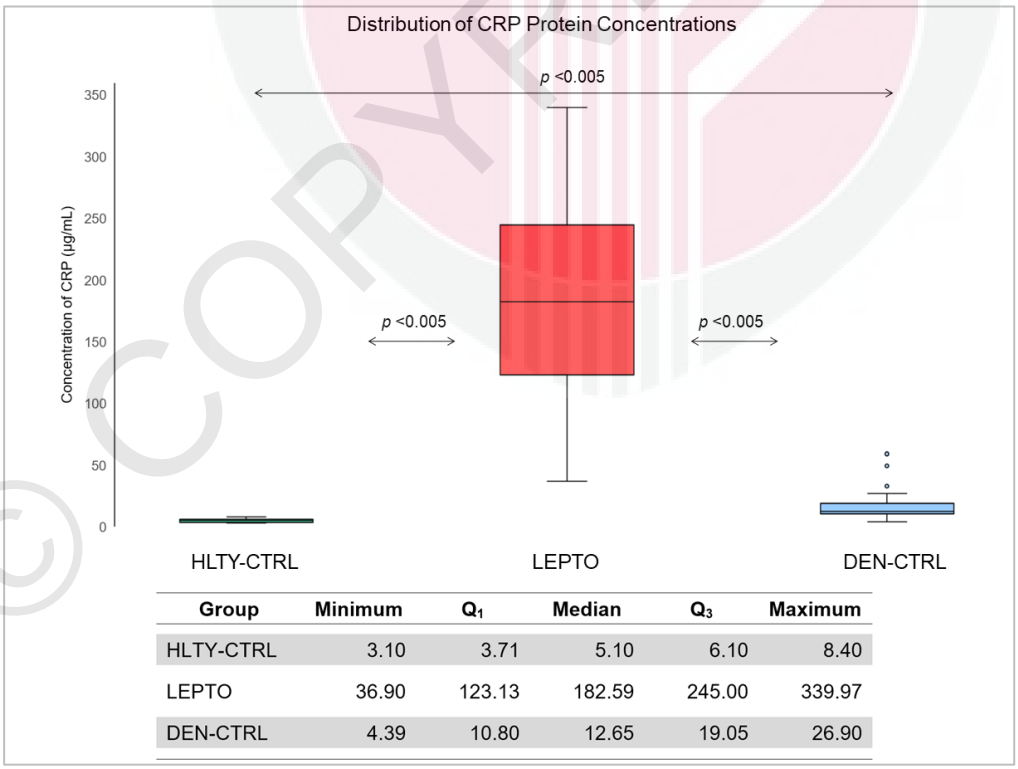
The directions of protein fold change for 8 out of the initial 11 proteins were consistent with those of the derivative cohort. Differential expression was conclusively confirmed using the Kruskal-Wallis and the Mann-Whitney U tests. Specifically, CRP, LRG1, LBP, MYH9, PPBP, PF4, SAA1, and THBS1 were established as proteins showcasing significant alterations in their expression levels among the study groups. In contrast, APOA2, FERMT3, and PFN1 were not associated with significant differential expression.

Referring to Figure 27-37, a series of box-and-whisker plots were generated for each protein to visually represent the ELISA data distribution among these groups. These plots provide an insightful visual overview of the protein concentration distributions. The protein concentrations estimated using ELISA for protein in this verification cohort can be referred to in Appendix D.



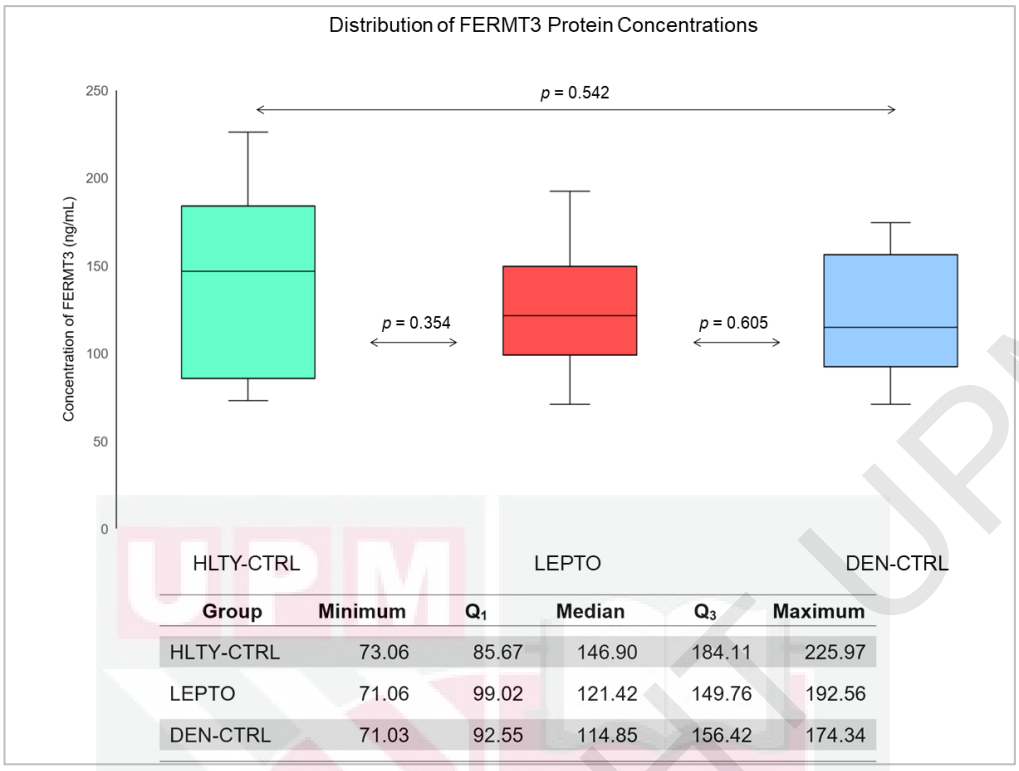
Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	99.01	104.42	111.24	120.13	136.29
LEPTO	90.24	101.48	109.92	123.17	155.43
DEN-CTRL	97.69	104.40	110.16	119.64	141.45

Figure 27: Distribution of APOA2 Protein Concentrations across Study Groups in Verification Cohort



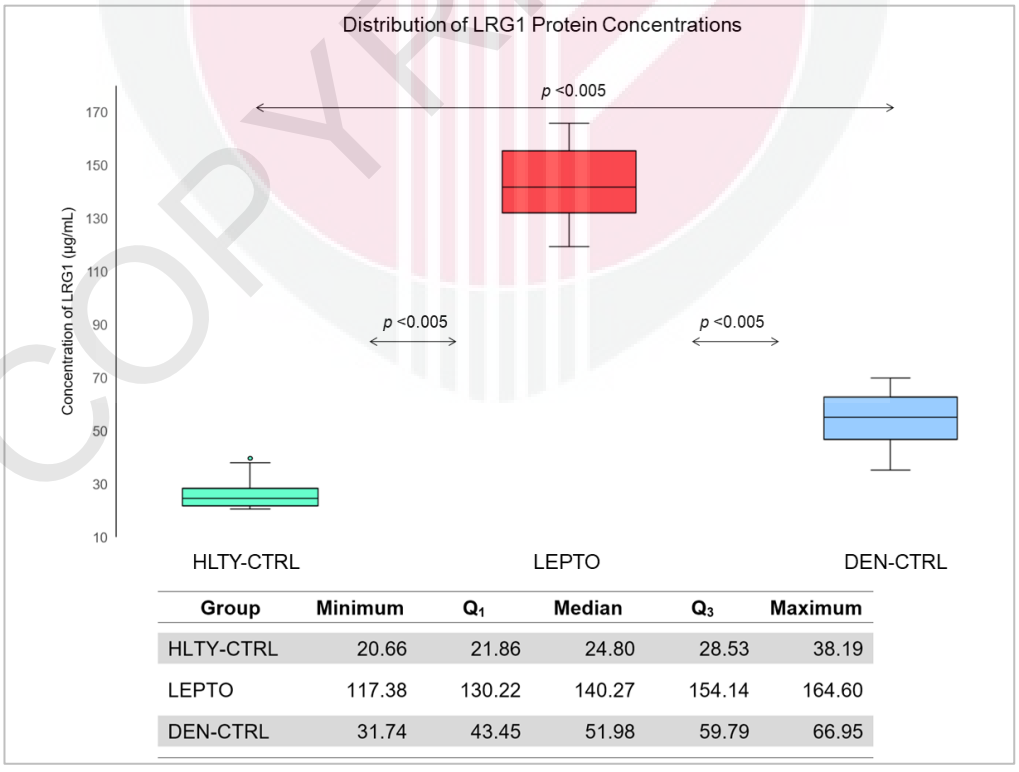
Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	3.10	3.71	5.10	6.10	8.40
LEPTO	36.90	123.13	182.59	245.00	339.97
DEN-CTRL	4.39	10.80	12.65	19.05	26.90

Figure 28: Distribution of CRP Protein Concentrations across Study Groups in Verification Cohort



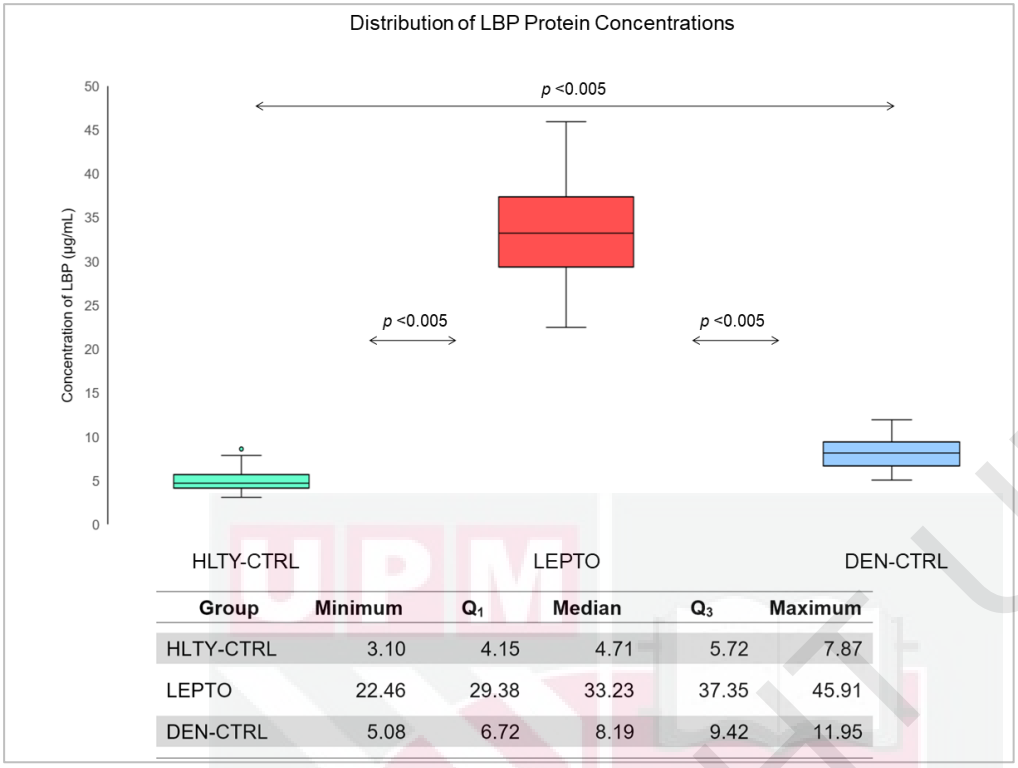
Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	73.06	85.67	146.90	184.11	225.97
LEPTO	71.06	99.02	121.42	149.76	192.56
DEN-CTRL	71.03	92.55	114.85	156.42	174.34

Figure 29: Distribution of FERMT3 Protein Concentrations across Study Groups in Verification Cohort



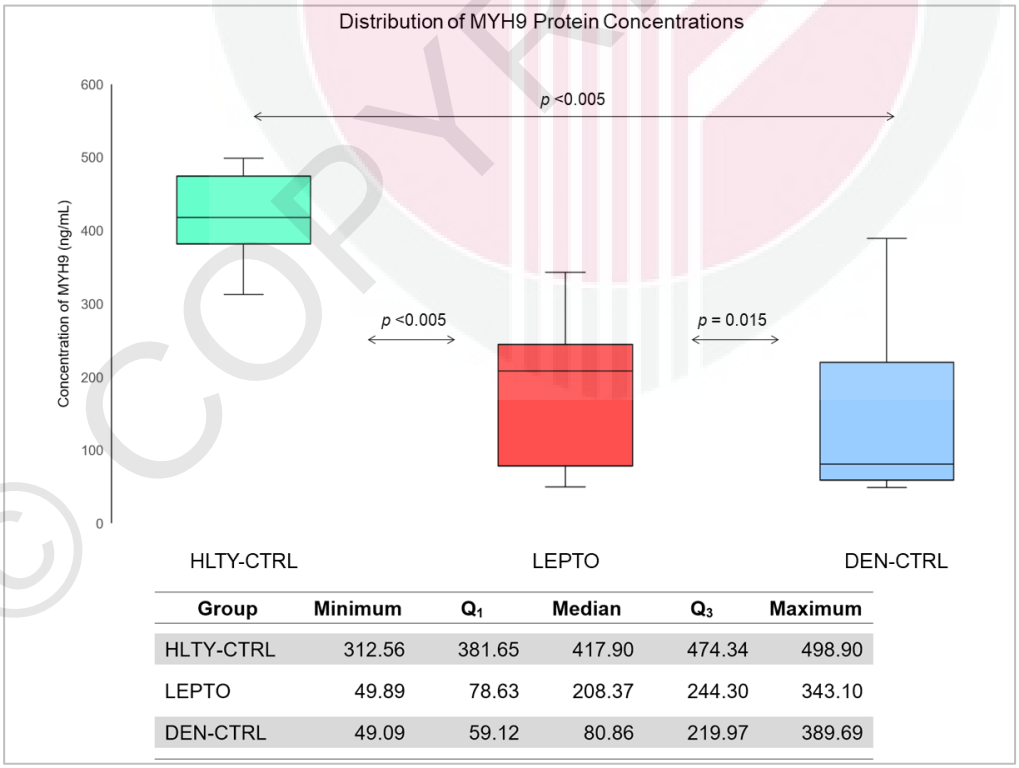
Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	20.66	21.86	24.80	28.53	38.19
LEPTO	117.38	130.22	140.27	154.14	164.60
DEN-CTRL	31.74	43.45	51.98	59.79	66.95

Figure 30: Distribution of LRG1 Protein Concentrations across Study Groups in Verification Cohort



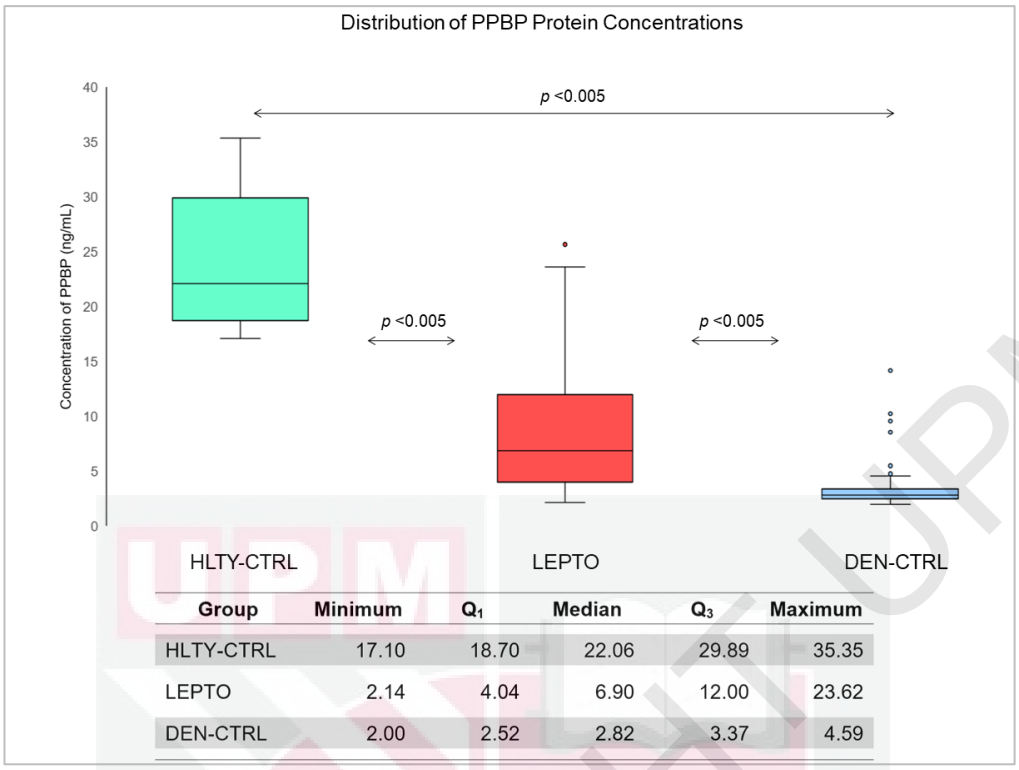
Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	3.10	4.15	4.71	5.72	7.87
LEPTO	22.46	29.38	33.23	37.35	45.91
DEN-CTRL	5.08	6.72	8.19	9.42	11.95

Figure 31: Distribution of LBP Protein Concentrations across Study Groups in Verification Cohort



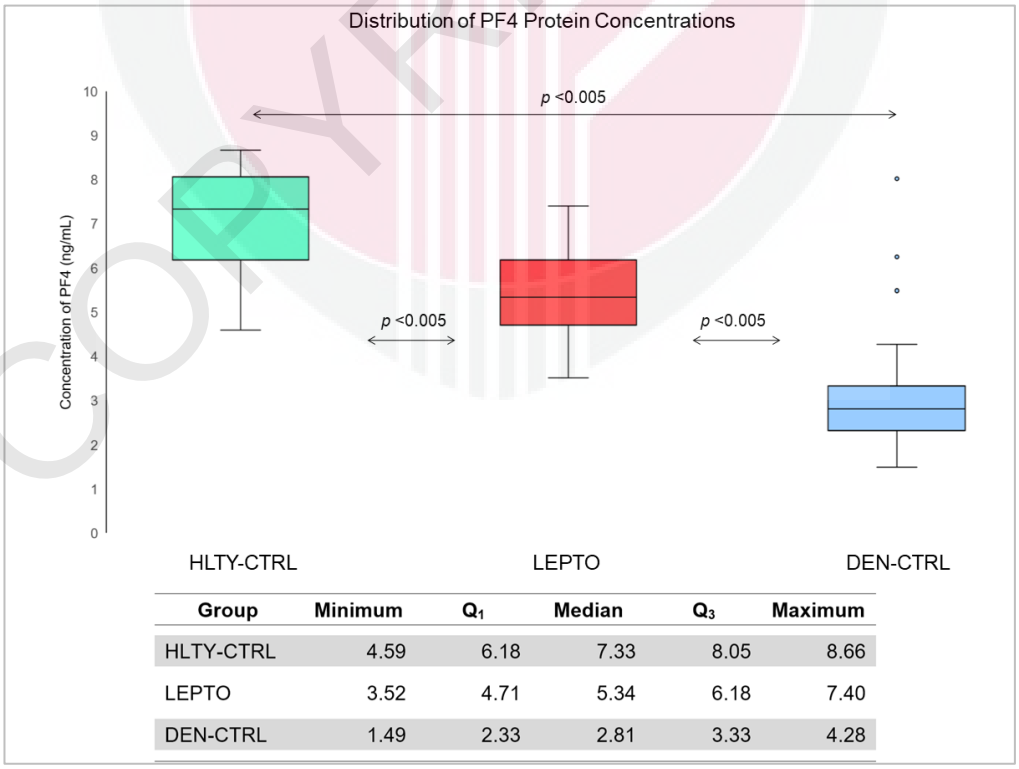
Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	312.56	381.65	417.90	474.34	498.90
LEPTO	49.89	78.63	208.37	244.30	343.10
DEN-CTRL	49.09	59.12	80.86	219.97	389.69

Figure 32: Distribution of MYH9 Protein Concentrations across Study Groups in Verification Cohort



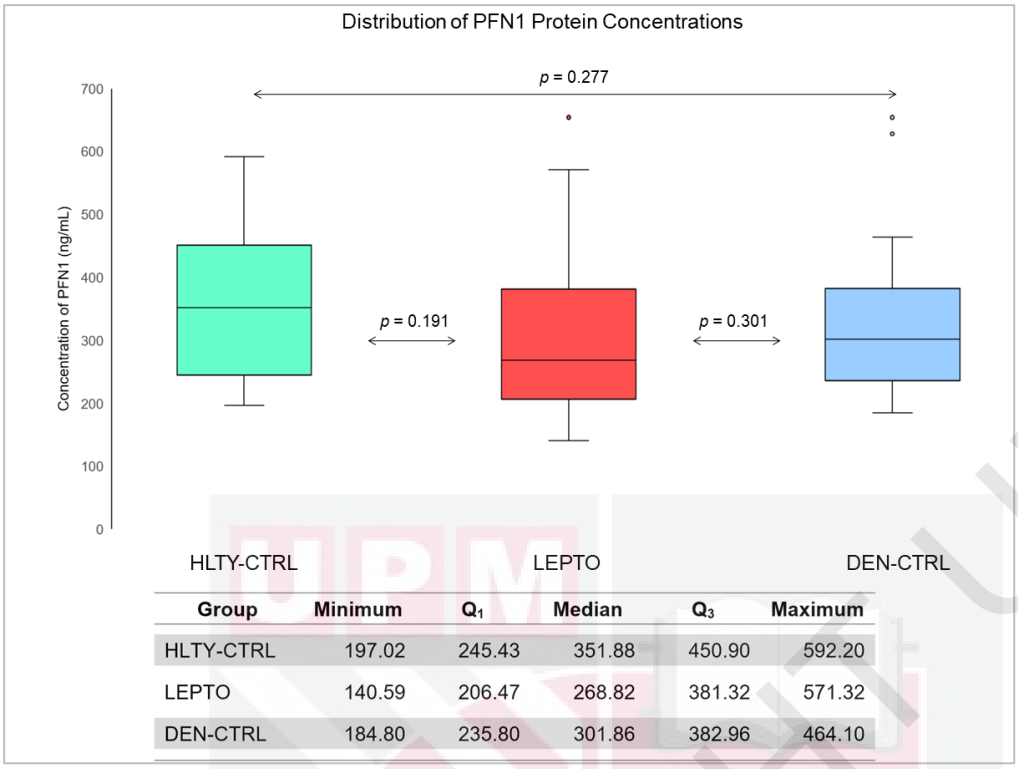
Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	17.10	18.70	22.06	29.89	35.35
LEPTO	2.14	4.04	6.90	12.00	23.62
DEN-CTRL	2.00	2.52	2.82	3.37	4.59

Figure 33: Distribution of PPBP Protein Concentrations across Study Groups in Verification Cohort



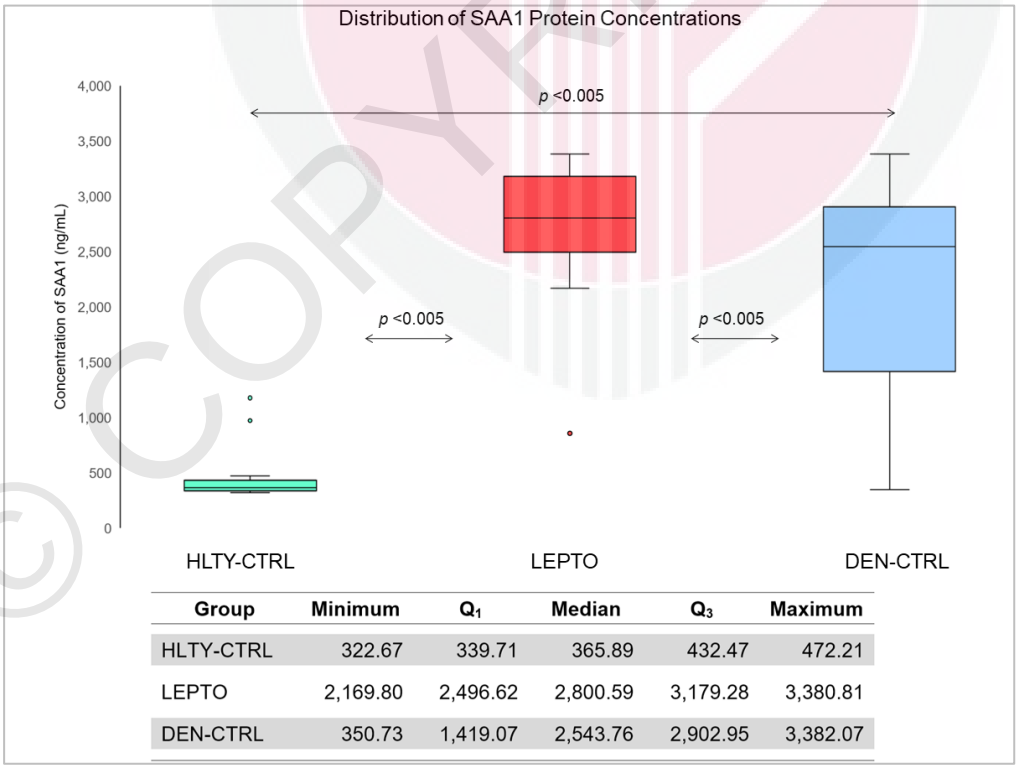
Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	4.59	6.18	7.33	8.05	8.66
LEPTO	3.52	4.71	5.34	6.18	7.40
DEN-CTRL	1.49	2.33	2.81	3.33	4.28

Figure 34: Distribution of PF4 Protein Concentrations across Study Groups in Verification Cohort



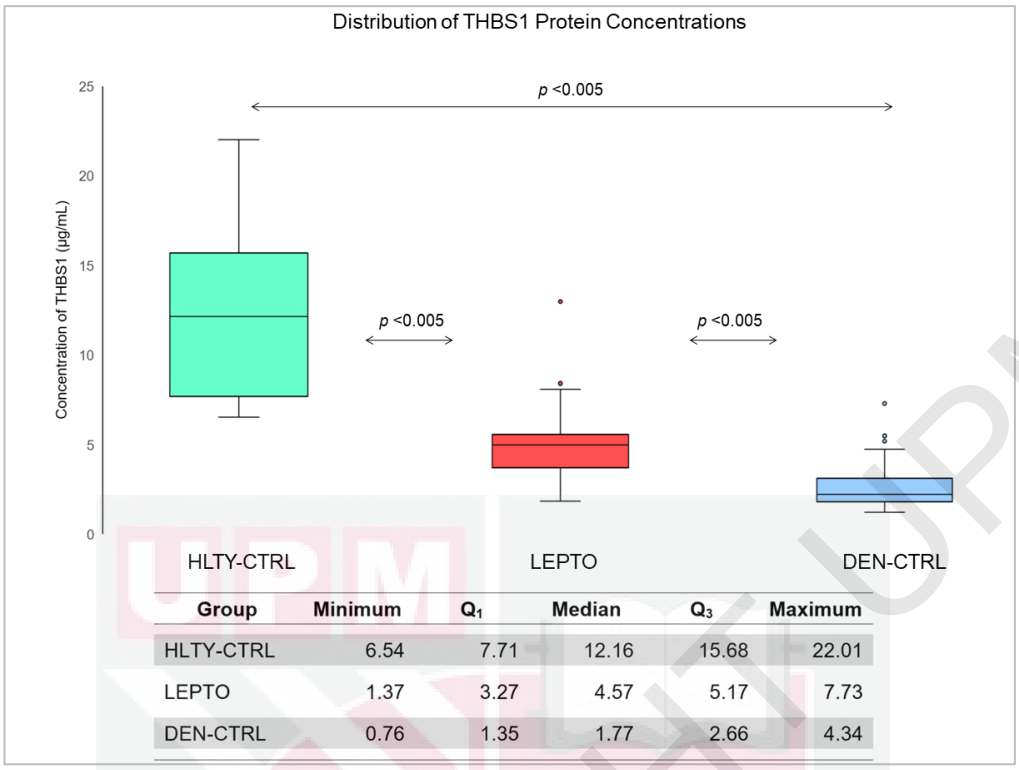
Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	197.02	245.43	351.88	450.90	592.20
LEPTO	140.59	206.47	268.82	381.32	571.32
DEN-CTRL	184.80	235.80	301.86	382.96	464.10

Figure 35: Distribution of PFN1 Protein Concentrations across Study Groups in Verification Cohort



Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	322.67	339.71	365.89	432.47	472.21
LEPTO	2,169.80	2,496.62	2,800.59	3,179.28	3,380.81
DEN-CTRL	350.73	1,419.07	2,543.76	2,902.95	3,382.07

Figure 36: Distribution of SAA1 Protein Concentrations across Study Groups in Verification Cohort



Group	Minimum	Q_1	Median	Q_3	Maximum
HLTY-CTRL	6.54	7.71	12.16	15.68	22.01
LEPTO	1.37	3.27	4.57	5.17	7.73
DEN-CTRL	0.76	1.35	1.77	2.66	4.34

Figure 37: Distribution of THBS1 Protein Concentrations across Study Groups in Verification Cohort

Following verification in the verification cohort, a panel of eight plasma protein biomarkers has been identified for leptospirosis (Table 10).

Table 10: Identified plasma protein biomarkers for leptospirosis

UniProtKB	Protein Name	LEPTO vs. HLTY-CTRL		LEPTO vs. DEN-CTRL	
		Fold Change	*p*-Value	Fold Change	*p*-Value
P02741	C-reactive protein	**+ 35.8**	**<0.005**	**+ 14.43**	**<0.005**
P02750	Leucine-rich alpha-2-glycoprotein	**+ 5.66**	**<0.005**	**+ 2.70**	**<0.005**
P18428	Lipopolysaccharide-binding protein	**+ 7.06**	**<0.005**	**+ 4.06**	**<0.005**
P35579	Myosin-9	- 0.50	**<0.005**	**+ 2.58**	**0.015**
P02775	Platelet basic protein	- 0.31	**<0.005**	**+ 2.45**	**<0.005**
P02776	Platelet factor 4	- 0.73	**<0.005**	**+ 1.90**	**<0.005**
P0DJI8	Serum amyloid A-1 protein	**+ 7.65**	**<0.005**	**+ 1.10**	**<0.005**
P07996	Thrombospondin-1	- 0.38	**<0.005**	**+ 2.58**	**<0.005**

Note:
"**+**" indicates an increase in the direction of protein fold change.
"**-**" indicates a decrease in the direction of protein fold change.

CHAPTER 5

DISCUSSION

Renal involvement in leptospirosis may present across a broad clinical spectrum, ranging from urinary irregularities to serum electrolyte imbalances. In severe cases, it may lead to AKI requiring dialysis. In achieving the first objective of this study, the renal profile variations in individuals with clinical suspicion of leptospirosis were analysed retrospectively. The renal profiles of this retrospective cohort reflected the diverse clinical presentations observed in leptospirosis, with some individuals exhibiting renal dysfunction and others not. Analysis of these renal profiles revealed that individuals with leptospirosis exhibited hypocalcaemia (calcium <2.10 mmol/L) and hypochloraemia (chloride <98 mmol/L) compared to non-leptospirosis cases.

Mild hypocalcaemia can develop without noticeable symptoms or may present as cardiac arrest, confusion, numbness, and seizures (Soar et al., 2010; Fong and Khan, 2012). It can be induced by various factors, including renal failure, rhabdomyolysis, calcium channel blocker overdose, and specific medications (Soar et al., 2010). In the context of leptospirosis, hypocalcaemia has been recognised as a contributor to arrhythmias (Zakout et al., 2013; Soares et al., 2017). The biphasic pattern of calcium levels observed in leptospirosis, as documented by Zakout et al. (2013), parallels the observations in rhabdomyolysis-induced AKI. Hypocalcaemia occurs during the oliguric phase, followed by hypercalcaemia during the diuretic phase of recovery.

Hypochloraemia has been documented in cases of leptospirosis in mammals (Fouché et al., 2020; Anugrah et al., 2023). Although not yet confirmed within the context of leptospirosis, dyschloraemia has been proposed as a potential risk factor for the development of AKI, with hypochloraemic patients showing a higher mortality rate (Shao et al., 2016). Hypokalaemia (potassium <3 mmol/L) is a recurrent finding in leptospirosis (Katz et al., 2001), whereas hyponatremia (sodium <136 mmol/L) typically resolves rapidly once the infection is brought under controlled (Sitprija, 2008). Leptospirosis-induced AKI is characterised by a swift elevation in serum creatinine and blood urea nitrogen levels (Visith and Kearkiat, 2005).

The present study, and several similar investigations comparing serum biochemistry profiles between leptospirosis and non-leptospirosis patients highlights that variations in these profiles may exhibit specific characteristics within different cohorts or regions. For instance, in a cohort-based study conducted in Sri Lanka by Rajapakse et al. (2016), high creatinine levels (>150 μmol/L), elevated bilirubin levels (>30 μmol/L), neutrophil counts exceeding 80%, and low platelet counts (<85,000/mm^3) were positively associated with confirmed diagnoses. Similarly, in a Thai cohort-based study by Sukmark et al. (2018), predictors for leptospirosis included the presence of acute kidney failure, hypokalaemia, hyponatremia, hypotension, jaundice, and low haemoglobin levels (<12 g/dL).

Another factor that could account for the differences in findings among these similar studies is the dissimilarity in inclusion criteria. Although there is no consistent overlap among the predictors identified in these studies, variations

in renal profiles could act as indicators of leptospirosis infection with varying degrees of disease severity.

Leptospirosis diagnosis could not rely solely on MAT and positive blood culture for several reasons. MAT may not be applicable in diagnosing leptospirosis in patients who have not yet undergone seroconversion. Even when the disease has reached the seroconversion stage, MAT may require collecting acute and convalescent serum samples taken two weeks apart for a confirmative diagnosis. It may be impractical to wait for convalescent samples, particularly in the early stages of the disease when prompt intervention is needed. On the other hand, blood culture may not be suitable for patients who have undergone seroconversion. Leptospiraemia, the presence of leptospires in the bloodstream, may cease shortly after the initial onset of symptoms (Adler, 2015). Additionally, leptospires are often fastidious and slow-growing in culture media, which results in a retrospective diagnosis. Given the challenges in leptospirosis diagnosis, exploring plasma proteins as potential biomarkers could improve diagnostic accuracy and timeliness.

An increasing trend in scientific research involves sample pooling in proteomic studies, allowing the discovery of biomarkers differentiating patient groups while minimising individual differences to reveal trends. Protein expression in pooled samples generally aligns with the average expression of the individuals within the pool (Diz et al., 2009). Although the biological variance between pools decreases compared to that between individuals, the reduction is less significant than anticipated (Diz et al., 2009).

The decision of sample pooling strategy was carefully considered and subsequently adopted in the multiplex iTRAQ labelling-MS approach. A gender-matched design was implemented, with a limited number of samples per pool (two, three, or four) to minimise the loss of information on biological variation. Furthermore, the leptospiraemia and seroconversion samples were separately processed in distinct pools to identify shared proteins indicative of leptospirosis irrespective of the disease stage. An additional verification phase designed following the profiling step would verify the significance of the discovered biomarkers.

In this study, the initial iTRAQ analysis identified a total of 450 proteins and then refined them to a list of 290 proteins after a series of exclusion criteria were applied. Among these 290 proteins, a subset was differentially expressed in the Leptospiraemia (85 proteins) and Seroconversion (113 proteins) Groups. These findings suggest that many proteins are dynamically regulated in response to the progression of leptospirosis, with distinct expression patterns between different stages of the disease. The GO annotation data reveals that the later stages of the disease, represented by the Seroconversion Group, involve a broader range of molecular activities and an increase of proteins participating in most categories within Molecular Function, Biological Process, and Cellular Component. The expansion in the number of proteins might be attributed to the immune response, an increased need for tissue repair, metabolic adaptations, and the involvement of various cell types, tissues, and anatomical entities during the later stages of infection.

PPI analysis following proteome profiling may help uncover functional relationships and novel interactions of key proteins. It also aids in validating biomarkers by linking them to disease-related proteins and pathways (Boja et al., 2014).

The PPI network identifies three main clusters. Cluster 1 exhibits the most extensive interactions (93 edges), underscoring the central role of reverse cholesterol transport in the network. Research on leptospirosis has shown that the infection profoundly disrupts lipid metabolism and alters lipoprotein profiles, potentially impairing reverse cholesterol transport. Gazi et al. (2011) demonstrated that leptospirosis leads to significant reductions in high-density lipoprotein cholesterol, a crucial component of reverse cholesterol transport, while simultaneously increasing triglyceride levels and shifting low-density lipoprotein cholesterol towards more atherogenic small dense low-density lipoprotein particles. Moreover, evidence of reduced lecithin-cholesterol acyltransferase activity, as reported by Silva et al. (2020), further suggests that reverse cholesterol transport is likely compromised during leptospirosis.

Cluster 2 and Cluster 3 primarily consist of proteins involved in platelet aggregation. Fang et al. (2018) discovered that von Willebrand factor A proteins from *L. interrogans* inhibit von Willebrand factor-mediated platelet aggregation, which can lead to haemorrhage. Isogai et al. (1997) demonstrated that leptospiral LPS can stimulate platelet aggregation via activation of platelet-activating factor.

Overall, PPI analysis enriches our understanding of the molecular basis of diseases and enhances the applicability of identified biomarkers (Boja et al., 2014).

In LEPTO cases compared to the control groups, 11 proteins showed significant expression variations, which are APOA2, CRP, FERMT3, LRG1, LBP, MYH9, PPBP, PF4, PFN1, SAA1, and THBS1. The robust positive correlations observed in all groups between the iTRAQ data and ELISA validation data suggest a strong and consistent relationship between the protein fold changes measured by iTRAQ and ELISA.

Each of these 11 proteins is associated with one or more of the main biological processes mentioned above in the PPI clusters, including APOA2 in reverse cholesterol transport, PPBP, PF4, and THBS1 in platelet aggregation, FERMT3, MYH-9, PFN1, and THBS1 in cell-matrix adhesion, LRG1, MYH-9, and THBS1 in fibroblast migration, and CRP, LRG1, LBP, and SAA1 in acute-phase response. Given the biological relevance of these identified proteins to the core biological processes in the PPI network, their potential as biomarkers for leptospirosis has been enhanced.

Upon verification in the verification cohort, the directional changes in protein expression for 8 (CRP, LRG1, LBP, MYH9, PPBP, PF4, SAA1, and THBS1) out of the initial 11 proteins were consistent with those of the derivative cohort. Several of these verified biomarkers have been previously associated with leptospirosis. For instance, Maillard et al. (2023) and Le Turnier et al. (2019)

proposed the utility of elevated CRP levels in distinguishing between leptospirosis and dengue fever. Furthermore, a comparison of whole blood transcriptional profiles in fatal cases and survivors of leptospirosis revealed a two- to five-fold decrease in the gene expression levels of PPBP and PF4 (Lindow et al., 2016).

Instead of using host proteome profiling, the majority of previous leptospirosis biomarker studies have focused on specific aspects, such as cytokine expression levels (Wagenaar et al., 2009; Mikulski et al., 2015; Chirathaworn et al., 2016), predetermined proteins associated with infectious disease pathology (Conroy et al., 2014), oxidative stress markers (Fernando et al., 2016), selected indicators of endothelial activation and systematic inflammation (Lukasz et al., 2014), and predefined markers related to endotheliopathy (Libório et al., 2015).

In contrast to other host proteome-based studies on human leptospirosis, consistent protein biomarkers overlaps are absent. Differences in protein profiles could be attributed to the variations in the type of specimen, control group choice, and differences in study designs. For instance, Srivastava et al. (2012) compared serum proteome profiles in leptospirosis patients against both malaria patients and healthy volunteers, while Tan et al. (2017) examined serum profiles among mild and severe leptospirosis patients compared to healthy individuals. Moreover, the use of immunodepletion, the utilisation of two-dimensional gel electrophoresis (2-DE) for protein separation before MS analysis, and the application of label-free MS in the studies conducted by

Srivastava et al. (2012) and Tan et al. (2017) may explain the variations observed in their findings in contrast to the current study. In this research, we analysed the global plasma proteome of leptospiraemia and seroconversion leptospirosis patients compared to two control groups (dengue patients and healthy subjects) using the iTRAQ labelling-MS approach.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

Diagnosing leptospirosis solely based on MAT and positive blood cultures, which can often lead to retrospective diagnoses, is inadequate for this disease. Timely and accurate diagnosis is crucial for effective clinical management. This research aims to contribute to diagnostic tool development by identifying plasma protein biomarkers and evaluating renal profile variations in leptospirosis-infected individuals. The study achieved the main objectives: comparing renal electrolyte and waste product levels, analysing plasma proteome profiles, and verifying the identified plasma protein biomarkers. The comparative analysis of renal profiles revealed significant differences in renal calcium and chloride levels between leptospirosis and non-leptospirosis patients. Subsequently, the comparative analysis of proteome profiles has identified eight plasma proteins with differential expressions in leptospirosis compared to the control groups. Given the diverse clinical presentations in leptospirosis, this study suggests using renal profile variations as preliminary criteria to distinguish leptospirosis patients, followed by measuring the concentrations of the eight plasma protein biomarkers. Instead of relying on the concept of "one disease, one biomarker," a panel of eight protein biomarkers can significantly improve diagnosis accuracy.

However, it is essential to acknowledge the limitations of this research. The verified biomarkers require further validation in more extensive and diverse patient populations to ensure their reliability and specificity for leptospirosis

diagnosis. Additionally, the observed renal profile variations should be correlated with disease severity and clinical outcomes to assess their clinical significance. Given the diverse manifestations of leptospirosis, the findings of this study may have limitations in their broader applicability.

Moving forward, it is recommended that the research findings on Clusters 1, 2, and 3 from the PPI network analysis be expanded upon. A deeper investigation of these protein clusters may reveal previously undiscovered relationships and interactions among proteins, potentially shedding light on critical pathways involved in leptospirosis. The unique differentially expressed proteins in the Leptospiraemia and Seroconversion Groups may be explored further to uncover specific molecular mechanisms that underlie the disease progression. Furthermore, it is recommended that comprehensive validation studies involving diverse cohorts from multiple geographic regions be conducted to confirm the specificity and sensitivity of the identified biomarkers. Continued proteomic profiling by recruiting different control groups may uncover additional biomarkers and expand our understanding of leptospirosis.

REFERENCES

AB SCIEX Pte. Ltd. (2010). iTRAQ® Reagents – 8plex Amine-Modifying Labeling Reagents for Multiplexed Relative and Absolute Protein Quantitation Protocol. .4375249 Rev. C. Foster City, CA, USA, AB SCIEX Pte. Ltd.

AB SCIEX Pte. Ltd. (2017). Tips and Tricks for Cation-Exchange Chromatography for the iTRAQ® Protocol. Foster City, CA, USA, AB SCIEX Pte. Ltd.

Adler, B. (2015). History of leptospirosis and *Leptospira*. Leptospira and Leptospirosis. B. Adler. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 1-9.

Adler, B. and Faine, S. (2006). The genus *Leptospira*. The Prokaryotes: Volume 7: Proteobacteria: Delta, Epsilon Subclass. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt. New York, NY, Springer New York**:** 294-317.

Agampodi, S. B., Matthias, M. A., Moreno, A. C. and Vinetz, J. M. (2012). Utility of quantitative polymerase chain reaction in leptospirosis diagnosis: association of level of leptospiremia and clinical manifestations in Sri Lanka. *Clinical Infectious Diseases* **54**(9): 1249-1255.

Agresti, A. (2013). Categorical data analysis. New York, Wiley.

Anderson, N. L. and Anderson, N. G. (2002). The Human Plasma Proteome: History, Character, and Diagnostic Prospects. *Molecular & Cellular Proteomics* **1**(11): 845-867.

Anugrah, D., Apada, A. M. S., Rell, F., Suharto, R. H. and Mursalim, M. F. (2023). Treatment Leptospira Infection In Dog At Pet Vet Clinic, Center of Jakarta. *Media Kedokteran Hewan* **34**(3): 189-197.

Ashford, D. A., Kaiser, R. M., Spiegel, R. A., Perkins, B. A., Weyant, R. S., Bragg, S. L., Plikaytis, B., Jarquin, C., De Lose Reyes, J. O. and Amador, J. J. (2000). Asymptomatic infection and risk factors for leptospirosis in Nicaragua. *The American Journal of Tropical Medicine and Hygiene* **63**(5): 249-254.

Athanazio, D. A., Silva, E. F., Santos, C. S., Rocha, G. M., Vannier-Santos, M. A., McBride, A. J. A., Ko, A. I. and Reis, M. G. (2008). Rattus norvegicus as a model for persistent renal colonization by pathogenic Leptospira interrogans. *Acta Tropica* **105**(2): 176-180.

Barrett, K. E., Barman, S. M., Brooks, H. L. and Yuan, J. X. J. (2019). Ganong's Review of Medical Physiology. New York, NY, McGraw-Hill Education.

Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A., Levett, P. N., Gilman, R. H., Willig, M. R., Gotuzzo, E. and Vinetz, J.

M. (2003). Leptospirosis: a zoonotic disease of global importance. *The Lancet Infectious Diseases* **3**(12): 757-771.

Bio-Rad Laboratories (2011). Mini-PROTEAN® Precast Gels Instruction Manual and Application Guide.1658100 Rev F. Hercules, CA, Bio-Rad Laboratories.

Bio-Rad Laboratories (n.d.). *RC DC* Protein Assay Instruction Manual.4110107 Rev A. Hercules, CA, Bio-Rad Laboratories.

Bio-Techne Corporation (2018). ELISA Guide. .BR_ELISA Guide_ASDFY20-10071. Minnesota, USA, Bio-Techne Corporation.

Boja, E. S., Fehniger, T. E., Baker, M. S., Marko-Varga, G. and Rodriguez, H. (2014). Analytical validation considerations of multiplex mass-spectrometry-based proteomic platforms for measuring protein biomarkers. *Journal of Proteome Research* **13**(12): 5325-5332.

Bruce, M. G., Sanders, E. J., Leake, J. A. D., Zaidel, O., Bragg, S. L., Aye, T., Shutt, K. A., Deseda, C. C., Rigau-Perez, J. G., Tappero, J. W., Perkins, B. A., Spiegel, R. A. and Ashford, D. A. (2005). Leptospirosis among patients presenting with dengue-like illness in Puerto Rico. *Acta Tropica* **96**(1): 36-46.

Bubis, J. A., Levitsky, L. I., Ivanov, M. V., Tarasova, I. A. and Gorshkov, M. V. (2017). Comparative evaluation of label-free quantification methods for shotgun proteomics. *Rapid Communications in Mass Spectrometry* **31**(7): 606-612.

Bulach, D. M., Kalambaheti, T., Pena-Moctezuma, A. and Adler, B. (2000). Lipopolysaccharide biosynthesis in *Leptospira*. *Journal of Molecular Microbiology and Biotechnology* **2**.

Bursac, Z., Gauss, C. H., Williams, D. K. and Hosmer, D. W. (2008). Purposeful selection of variables in logistic regression. *Source Code for Biology and Medicine* **3**(1): 17.

Carey, C., Purohit, S. and She, J.-X. (2010). Advances and challenges in biomarker development for type 1 diabetes prediction and prevention using 'omic' technologies. *Expert opinion on medical diagnostics* **4**: 397-410.

Chirathaworn, C., Supputtamongkol, Y., Lertmaharit, S. and Poovorawan, Y. (2016). Cytokine levels as biomarkers for leptospirosis patients. *Cytokine* **85**: 80-82.

Conroy, A. L., Gélvez, M., Hawkes, M., Rajwans, N., Liles, W. C., Villar-Centeno, L. A. and Kain, K. C. (2014). Host biomarkers distinguish dengue from leptospirosis in Colombia: a case–control study. *BMC Infectious Diseases* **14**: 35-35.

Cook, E. A. J., de Glanville, W. A., Thomas, L. F., Kariuki, S., Bronsvoort, B. M. d. C. and Fèvre, E. M. (2017). Risk factors for leptospirosis seropositivity in slaughterhouse workers in western Kenya. *Occupational and Environmental Medicine* **74**(5): 357.

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., Stein, C., Abela-Ridder, B. and Ko, A. I. (2015). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Neglected Tropical Diseases* **9**(9): e0003898.

Croda, J., Neto, A. N. D., Brasil, R. A., Pagliari, C., Nicodemo, A. C. and Duarte, M. I. S. (2010). Leptospirosis pulmonary haemorrhage syndrome is associated with linear deposition of immunoglobulin and complement on the alveolar surface. *Clinical Microbiology and Infection* **16**(6): 593-599.

Cross, C. L. and Daniel, W. W. (2018). Biostatistics: A foundation for analysis in the health sciences. New York, Wiley.

Deutsch, E. W., Omenn, G. S., Sun, Z., Maes, M., Pernemalm, M., Palaniappan, K. K., Letunica, N., Vandenbrouck, Y., Brun, V., Tao, S. C., Yu, X., Geyer, P. E., Ignjatovic, V., Moritz, R. L. and Schwenk, J. M. (2021). Advances and Utility of the Human Plasma Proteome. *Journal of Proteome Research* **20**(12): 5241-5263.

Diz, A. P., Truebano, M. and Skibinski, D. O. F. (2009). The consequences of sample pooling in proteomics: an empirical study. *Electrophoresis* **30**(17): 2967-2975.

Doudier, B., Laban, D., Cazorla, C., Fournier, P.-E., Parola, P. and Brouqui, P. (2010). Leptospirosis diagnosed by molecular DNA detection on skin biopsy. *BMJ Case Reports* **2010**.

Drabik, A. and Silberring, J. (2016). Chapter 8 - Quantitative Measurements in Proteomics: Mass Spectrometry. Proteomic Profiling and Analytical Chemistry (Second Edition). Boston, Elsevier**:** 145-160.

Effler, P. V., Bogard, A. K., Domen, H. Y., Katz, A. R., Higa, H. Y. and Sasaki, D. M. (2002). Evaluation of eight rapid screening tests for acute leptospirosis in Hawaii. *Journal of Clinical Microbiology* **40**(4): 1464-1469.

Fang, J. Q., Imran, M., Hu, W. L., Ojcius, D. M., Li, Y., Ge, Y. M., Li, K. X., Lin, X. and Yan, J. (2018). vWA proteins of *Leptospira interrogans* induce hemorrhage in leptospirosis by competitive inhibition of vWF/GPIb-mediated platelet aggregation. *eBioMedicine* **37**: 428-441.

Feist, P. and Hummon, A. (2015). Proteomic challenges: Sample preparation techniques for microgram-quantity protein analysis from biological samples. *International Journal of Molecular Sciences* **16**(2): 3537.

Fernando, N., Wickremesinghe, S., Niloofa, R., Rodrigo, C., Karunanayake, L., de Silva, H. J., Wickremesinghe, A. R., Premawansa, S., Rajapakse, S. and Handunnetti, S. M. (2016). Protein carbonyl as a biomarker of oxidative stress in severe leptospirosis, and its usefulness in differentiating leptospirosis from dengue infections. *PLoS ONE* **11**(6): e0156085.

Flannery, B., Pereira, M. M., Velloso L de, F., Carvalho C de, C., De Codes, L. G., Orrico G de, S., Dourado, C. M., Riley, L. W., Reis, M. G. and Ko, A. I. (2001). Referral pattern of leptospirosis cases during a large urban epidemic of dengue. *The American Journal of Tropical Medicine and Hygiene* **65**(5): 657-663.

Fong, J. and Khan, A. (2012). Hypocalcemia: updates in diagnosis and management for primary care. *Canadian Family Physician • Le Médecin de famille canadien* **58**(2): 158-162.

Fontes, R. M., Cavalcanti, L. P. d. G., Oliveira, A. C. A., Bezerra, L. F. d. M., Gomes, A. M. M., Colares, J. K. B. and Lima, D. M. (2015). A new possibility for surveillance: do we identify all cases of leptospirosis? *Revista do Instituto de Medicina Tropical de São Paulo* **57**(5): 443-446.

Fouché, N., Graubner, C., Lanz, S., Schweighauser, A., Francey, T. and Gerber, V. (2020). Acute kidney injury due to Leptospira interrogans in 4 foals and use of renal replacement therapy with intermittent hemodiafiltration in 1 foal. *Journal of Veterinary Internal Medicine* **34**(2): 1007-1012.

Fowler, J., Cohen, L. and Jarvis, P. (2013). Practical Statistics for Field Biology, John Wiley & Sons.

Fraga, T. R., Barbosa, A. S. and Isaac, L. (2011). Leptospirosis: aspects of innate immunity, immunopathogenesis and immune evasion from the complement system. *Scandinavian Journal of Immunology* **73**(5): 408-419.

Fraga, T. R., Carvalho, E., Isaac, L. and Barbosa, A. S. (2015). *Leptospira* and Leptospirosis. Molecular Medical Microbiology (Second Edition). M. Sussman, D. Liu, I. Poxton and J. Schwartzman. Boston, Academic Press**:** 1973-1990.

Gan, C. S., Chong, P. K., Pham, T. K. and Wright, P. C. (2007). Technical, experimental, and biological variations in isobaric tags for relative and absolute quantitation (iTRAQ). *Journal of Proteome Research* **6**(2): 821-827.

Ganoza, C. A., Matthias, M. A., Saito, M., Cespedes, M., Gotuzzo, E. and Vinetz, J. M. (2010). Asymptomatic renal colonization of humans in the Peruvian Amazon by *Leptospira*. *PLoS Neglected Tropical Diseases* **4**(2): e612.

Garba, B., Bahaman, A. R., Khairani-Bejo, S., Zakaria, Z. and Mutalib, A. R. (2017). Retrospective study of leptospirosis in Malaysia. *EcoHealth* **14**(2): 389-398.

Gasem, M. H., Wagenaar, J. F. P., Goris, M. G. A., Adi, M. S., Isbandrio, B. B., Hartskeerl, R. A., Rolain, J.-M., Raoult, D. and van Gorp, E. C. M. (2009). Murine typhus and leptospirosis as causes of acute undifferentiated fever, Indonesia. *Emerging Infectious Diseases* **15**(6): 975-977.

Gazi, I. F., Apostolou, F. A., Liberopoulos, E. N., Filippatos, T. D., Tellis, C. C., Elisaf, M. S. and Tselepis, A. D. (2011). Leptospirosis is associated with markedly increased triglycerides and small dense low-density lipoprotein and decreased high-density lipoprotein. *Lipids* **46**(10): 953-960.

Geyer, P. E., Holdt, L. M., Teupser, D. and Mann, M. (2017). Revisiting biomarker discovery by plasma proteomics. *Molecular Systems Biology* **13**(9).

Ghasemian, R., Shokri, M., Makhlough, A. and Suraki-Azad, M. A. (2016). The course and outcome of renal failure due to human leptospirosis referred to a hospital in North of Iran; A follow-up study. *Caspian Journal of Internal Medicine* **7**(1): 7-12.

Haake, D. A. and Levett, P. N. (2015). Leptospirosis in humans. *Current Topics in Microbiology and Immunology* **387**: 65-97.

Iragorri, S. and Tullus, K. (2009). Leptospirosis mimicking hemolytic uremic syndrome: a case report. *American Journal of Kidney Diseases* **54**(4): 721-725.

Ismail, T. F., Wasfy, M. O., Abdul-Rahman, B., Murray, C. K., Hospenthal, D. R., Abdel-Fadeel, M., Abdel-Maksoud, M., Samir, A., Hatem, M. E., Klena, J., Pimentel, G., El-Sayed, N. and Hajjeh, R. (2006). Retrospective serosurvey of leptospirosis among patients with acute febrile illness and hepatitis in Egypt. *The American Journal of Tropical Medicine and Hygiene* **75**(6): 1085-1089.

Isogai, E., Hirose, K., Kimura, K., Hayashi, S., Kubota, T., Fujii, N. and Isogai, H. (1997). Role of platelet-actlvatlng-factor (PAF) on cellular responses after stimulation with leptospire lipopolysaccharide. *Microbiology and Immunology* **41**(3): 271-275.

Izurieta, R., Galwankar, S. and Clem, A. (2008). Leptospirosis: the "mysterious" mimic. *Journal of Emergencies, Trauma, and Shock* **1**(1): 21-33.

Katz, A. R., Ansdell, V. E., Effler, P. V., Middleton, C. R. and Sasaki, D. M. (2001). Assessment of the clinical presentation and treatment of 353 cases of laboratory-confirmed leptospirosis in Hawaii, 1974-1998. *Clinical Infectious Diseases* **33**(11): 1834-1841.

Kishimoto, M., Brown, J. D., Chung, H. H. and Howman, S. (2004). Leptospirosis misdiagnosed as pulmonary-renal syndrome. *The American Journal of the Medical Sciences* **328**(2): 116-120.

Le Turnier, P., Bonifay, T., Mosnier, E., Schaub, R., Jolivet, A., Demar, M., Bourhy, P., Nacher, M., Djossou, F. and Epelboin, L. (2019). Usefulness of C-Reactive Protein in Differentiating Acute Leptospirosis and Dengue Fever in French Guiana. *Open Forum Infectious Diseases* **6**(9): ofz323.

Lee, Y. M., Li, C., Lam, S. H., Gong, Z. and Lin, Q. (2018). Proteomic analysis of zebrafish (*Danio rerio*) after chemical exposure. Teratogenicity Testing: Methods and Protocols. L. Félix. New York, NY, Springer New York**:** 443-459.

Levett, P. N. (2001). Leptospirosis. *Clinical Microbiology Reviews* **14**(2): 296-326.

Levett, P. N. (2003). Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. *Clinical Infectious Diseases* **36**(4): 447-452.

Libório, A. B., Braz, M. B. M., Seguro, A. C., Meneses, G. C., Neves, F. M. d. O., Pedrosa, D. C., Cavalcanti, L. P. d. G., Martins, A. M. C. and Daher, E. d. F. (2015). Endothelial glycocalyx damage is associated with leptospirosis acute kidney injury. *The American Journal of Tropical Medicine and Hygiene* **92**(3): 611-616.

Lindow, J. C., Wunder, E. A., Jr., Popper, S. J., Min, J. N., Mannam, P., Srivastava, A., Yao, Y., Hacker, K. P., Raddassi, K., Lee, P. J., Montgomery, R. R., Shaw, A. C., Hagan, J. E., Araújo, G. C., Nery, N., Jr., Relman, D. A., Kim, C. C., Reis, M. G. and Ko, A. I. (2016). Cathelicidin Insufficiency in Patients with Fatal Leptospirosis. *PLoS Pathogens* **12**(11): e1005943.

Liu, Y., Hüttenhain, R., Collins, B. and Aebersold, R. (2013). Mass spectrometric protein maps for biomarker discovery and clinical research. *Expert Review of Molecular Diagnostics* **13**(8): 811-825.

Lo, Y.-C., Kintziger, K. W., Carson, H. J., Patrick, S. L., Turabelidze, G., Stanek, D., Blackmore, C., Lingamfelter, D., Dudley, M. H., Shadomy, S. V., Shieh, W.-J., Drew, C. P., Batten, B. C. and Zaki, S. R. (2011). Severe leptospirosis similar to pandemic (H1N1) 2009, Florida and Missouri, USA. *Emerging Infectious Diseases* **17**(6): 1145-1146.

Lukasz, A., Hoffmeister, B., Graf, B., Wölk, B., Noeckler, K., Bode-Böger, S. M., Hadem, J., Pischke, S. and Kielstein, J. T. (2014). Association of angiopoietin-2 and dimethylarginines with complicated course in patients with leptospirosis. *PLoS ONE* **9**(1): e87490.

Maillard, O., Hirschinger, D., Bénéteau, S., Koumar, Y., Vague, A., Girerd, R., DiAscia, L., Jabot, J., Cousty, J., Randrianjohany, A., Bertolotti, A. and Raffray, L. (2023). C-reactive protein: An easy marker for early

differentiation between leptospirosis and dengue fever in endemic area. *PLOS ONE* **18**(5): e0285900.

Małecki, M., Gołembiewska, E., Muraszko, A. M., Fiecek, B., Lewandowska, M., Urasińska, E., Stępniewska, J., Fijałkowski, M. and Ciechanowski, K. (2019). Atypical renal presentation in severe leptospirosis. *Kidney International Reports* **4**(1): 168-170.

Maroun, E., Kushawaha, A., El-Charabaty, E., Mobarakai, N. and El-Sayegh, S. (2011). Fulminant leptospirosis (Weil's disease) in an urban setting as an overlooked cause of multiorgan failure: a case report. *Journal of Medical Case Reports* **5**(1): 7.

Mikulski, M., Boisier, P., Lacassin, F., Soupé-Gilbert, M.-E., Mauron, C., Bruyere-Ostells, L., Bonte, D., Barguil, Y., Gourinat, A.-C., Matsui, M., Vernel-Pauillac, F. and Goarant, C. (2015). Severity markers in severe leptospirosis: a cohort study. *European Journal of Clinical Microbiology & Infectious Diseases* **34**(4): 687-695.

Ministry of Health Malaysia. (2011). Guidelines for the diagnosis, management, prevention and control of leptospirosis in Malaysia. Retrieved 18 February 2022, from http://www.moh.gov.my.

Ministry of Health Malaysia. (2023). Main MOH Publications. Retrieved 1 October 2023, from http://www.moh.gov.my.

Monahan, A. M., Callanan, J. J. and Nally, J. E. (2008). Proteomic analysis of Leptospira interrogans shed in urine of chronically infected hosts. *Infection and Immunity* **76**(11): 4952-4958.

Morey, R. E., Galloway, R. L., Bragg, S. L., Steigerwalt, A. G., Mayer, L. W. and Levett, P. N. (2006). Species-specific identification of *Leptospiraceae* by 16S rRNA gene sequencing. *Journal of Clinical Microbiology* **44**(10): 3510-3516.

Naing, L., Winn, T. and Rusli, B. N. (2006). Practical issues in calculating the sample size for prevalence studies. *Archives of Orofacial Sciences* **1**(1): 9-14.

Nhan, T.-X., Bonnieux, E., Rovery, C., De Pina, J.-J. and Musso, D. (2016). Fatal leptospirosis and chikungunya co-infection: do not forget leptospirosis during chikungunya outbreaks. *IDCases* **5**: 12-14.

Noor Rafizah, A. A., Aziah, B. D., Azwany, Y. N., Kamarul Imran, M., Mohamed Rusli, A., Mohd Nazri, S., Nabilah, I., Siti Asma, H., Zahiruddin, W. M. and Zaliha, I. (2012). Leptospirosis in northeastern Malaysia: misdiagnosed or coinfection? *International Journal of Collaborative Research on Internal Medicine & Public Health* **4**(7): 1419-1427.

Omenn, G. S., Lane, L., Overall, C. M., Pineau, C., Packer, N. H., Cristea, I. M., Lindskog, C., Weintraub, S. T., Orchard, S., Roehrl, M. H. A., Nice, E., Liu, S., Bandeira, N., Chen, Y. J., Guo, T., Aebersold, R., Moritz, R.

L. and Deutsch, E. W. (2023). The 2022 Report on the Human Proteome from the HUPO Human Proteome Project. *Journal of Proteome Research* **22**(4): 1024-1042.

Othman, N., Intan, H. I., Yip, C. W., Alias, M. and Amran, F. (2007). Severe leptospirosis with unusual manifestation. *Journal of Tropical Pediatrics* **53**(1): 55-58.

Özdemir, D., Sencan, İ., Yıldırım, M., Güçlü, E., Yavuz, T. and Karabay, O. (2008). Andiscriminated aseptic meningitis case between rickettsia and leptospiral meningitis. *European Journal of General Medicine* **5**(1): 51-53.

Parker, C. E. and Borchers, C. H. (2014). Mass spectrometry based biomarker discovery, verification, and validation – Quality assurance and control of protein biomarker assays. *Molecular Oncology* **8**(4): 840-858.

Paulo, J. A., Kadiyala, V., Banks, P. A., Steen, H. and Conwell, D. L. (2012). Mass spectrometry-based proteomics for translational research: A technical overview. *Yale Journal of Biology and Medicine* **85**(1): 59-73.

Pierce, A., Unwin, R. D., Evans, C. A., Griffiths, S., Carney, L., Zhang, L., Jaworska, E., Lee, C. F., Blinco, D., Okoniewski, M. J., Miller, C. J., Bitton, D. A., Spooncer, E. and Whetton, A. D. (2008). Eight-channel iTRAQ enables comparison of the activity of six leukemogenic tyrosine kinases. *Molecular & Cellular Proteomics* **7**(5): 853-863.

Polanski, M. and Anderson, N. L. (2007). A list of candidate cancer biomarkers for targeted proteomics. *Biomarker Insights* **1**: 1-48.

Pothuri, P., Ahuja, K., Kumar, V., Lal, S., Tumarinson, T. and Mahmood, K. (2016). Leptospirosis presenting with rapidly progressing acute renal failure and conjugated hyperbilirubinemia: a case report. *The American Journal of Case Reports* **17**: 567-569.

Rafizah, A. A. N., Aziah, B. D., Azwany, Y. N., Imran, M. K., Rusli, A. M., Nazri, S. M., Nikman, A. M., Nabilah, I., Asma', H. S., Zahiruddin, W. M. and Zaliha, I. (2013). A hospital-based study on seroprevalence of leptospirosis among febrile cases in northeastern Malaysia. *International Journal of Infectious Diseases* **17**(6): e394-e397.

Rai, A. J., Gelfand, C. A., Haywood, B. C., Warunek, D. J., Yi, J., Schuchard, M. D., Mehigh, R. J., Cockrill, S. L., Scott, G. B. I., Tammen, H., Schulz-Knappe, P., Speicher, D. W., Vitzthum, F., Haab, B. B., Siest, G. and Chan, D. W. (2005). HUPO plasma proteome project specimen collection and handling: Towards the standardization of parameters for plasma proteome samples. *Proteomics* **5**(13): 3262-3277.

Rajapakse, S., Rodrigo, C., Handunnetti, S. M. and Fernando, S. D. (2015). Current immunological and molecular tools for leptospirosis: diagnostics, vaccine design, and biomarkers for predicting severity. *Annals of Clinical Microbiology and Antimicrobials* **14**: 2.

Rajapakse, S., Weeratunga, P., Niloofa, R., Fernando, N., de Silva, N. L., Rodrigo, C., Maduranga, S., Nandasiri, N., Premawansa, S., Karunanayake, L., de Silva, H. J. and Handunnetti, S. (2016). A diagnostic scoring model for leptospirosis in resource limited settings. *PLoS Neglected Tropical Diseases* **10**(6): e0004513.

Rifai, N., Gillette, M. A. and Carr, S. A. (2006). Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nature Biotechnology* **24**(8): 971-983.

Ristow, P., Bourhy, P., Kerneis, S., Schmitt, C., Prevost, M.-C., Lilenbaum, W. and Picardeau, M. (2008). Biofilm formation by saprophytic and pathogenic leptospires. *Microbiology* **154**(5): 1309-1317.

Rita, M. T., Laura, S. C., Robert, C. H., Claudia, S. and Marta, A. G. (2014). Leptospirosis-associated hospitalizations, United States, 1998-2009. *Emerging Infectious Disease Journal* **20**(8): 1273.

Saengjaruk, P., Chaicumpa, W., Watt, G., Bunyaraksyotin, G., Wuthiekanun, V., Tapchaisri, P., Sittinont, C., Panaphut, T., Tomanakan, K., Sakolvaree, Y., Chongsa-Nguan, M., Mahakunkijcharoen, Y., Kalambaheti, T., Naigowit, P., Wambangco, M. A. L., Kurazono, H. and Hayashi, H. (2002). Diagnosis of human leptospirosis by monoclonal antibody-based antigen detection in urine. *Journal of Clinical Microbiology* **40**(2): 480-489.

Schiess, R., Wollscheid, B. and Aebersold, R. (2009). Targeted proteomic strategy for clinical biomarker discovery. *Molecular Oncology* **3**(1): 33-44.

Shah, K., Amonkar, G. P., Kamat, R. N. and Deshpande, J. R. (2010). Cardiac findings in leptospirosis. *Journal of Clinical Pathology* **63**(2): 119-123.

Shao, M., Li, G., Sarvottam, K., Wang, S., Thongprayoon, C., Dong, Y., Gajic, O. and Kashani, K. (2016). Dyschloremia is a risk factor for the development of acute kidney injury in critically ill patients. *PLoS ONE* **11**(8): e0160322.

Shivakumar, S. (2013). Indian guidelines for the diagnosis and management of human leptospirosis. *Medicine Update* **23**: 23-29.

Shivakumar, S. and Shareek, P. S. (2004). Diagnosis of leptospirosis utilizing modified Faine's criteria. *The Journal of the Association of Physicians of India* **52**: 678-679.

Silva, J. A. X., Albertini, A. V. P., Fonseca, C. S. M., Silva, D. C. N., Carvalho, V. C. O., Lima, V. L. M., Fontes, A., Costa, E. V. L. and Nogueira, R. A. (2020). Biomechanical and biochemical investigation of erythrocytes in late stage human leptospirosis. *Brazilian Journal of Medical and Biological Research* **53**(8): e9268.

Sitprija, V. (2008). Altered fluid, electrolyte and mineral status in tropical disease, with an emphasis on malaria and leptospirosis. *Nature Clinical Practice Nephrology* **4**: 91.

Sivasankari, K., Shanmughapriya, S. and Natarajaseenivasan, K. (2016). Leptospiral renal colonization status in asymptomatic rural population of Tiruchirapalli district, Tamilnadu, India. *Pathogens and Global Health* **110**(4-5): 209-215.

Smith, G. S., Walter, G. L. and Walker, R. M. (2013). Chapter 18 - Clinical Pathology in Non-Clinical Toxicology Testing. Haschek and Rousseaux's Handbook of Toxicologic Pathology (Third Edition). W. M. Haschek, C. G. Rousseaux and M. A. Wallig. Boston, Academic Press**:** 565-594.

Smythe, L. D., Wuthiekanun, V., Chierakul, W., Suputtamongkol, Y., Tiengrim, S., Dohnt, M. F., Symonds, M. L., Slack, A. T., Apiwattanaporn, A., Chueasuwanchai, S., Day, N. P. and Peacock, S. J. (2009). The microscopic agglutination test (MAT) is an unreliable predictor of infecting *Leptospira* serovar in Thailand. *The American Journal of Tropical Medicine and Hygiene* **81**(4): 695-697.

Soar, J., Perkins, G. D., Abbas, G., Alfonzo, A., Barelli, A., Bierens, J. J. L. M., Brugger, H., Deakin, C. D., Dunning, J., Georgiou, M., Handley, A. J., Lockey, D. J., Paal, P., Sandroni, C., Thies, K.-C., Zideman, D. A. and Nolan, J. P. (2010). European Resuscitation Council Guidelines for Resuscitation 2010 Section 8. Cardiac arrest in special circumstances: electrolyte abnormalities, poisoning, drowning, accidental hypothermia, hyperthermia, asthma, anaphylaxis, cardiac surgery, trauma, pregnancy, electrocution. *Resuscitation* **81**(10): 1400-1433.

Soares, D. d. S., Galdino, G. S., Rodrigues, B. C., Silva Junior, G. B. d. and Daher, E. D. F. (2017). Arrhythmias in leptospirosis-associated acute kidney injury: a case series. *The Brazilian Journal of Infectious Diseases* **21**(2): 209-210.

Spichler, A., Moock, M., Chapola, E. G. and Vinetz, J. (2005). Weil's disease: an unusually fulminant presentation characterized by pulmonary hemorrhage and shock. *Brazilian Journal of Infectious Diseases* **9**: 336-340.

Srivastava, R., Ray, S., Vaibhav, V., Gollapalli, K., Jhaveri, T., Taur, S., Dhali, S., Gogtay, N., Thatte, U., Srikanth, R. and Srivastava, S. (2012). Serum profiling of leptospirosis patients to investigate proteomic alterations. *Journal of Proteomics* **76**: 56-68.

States, D. J., Omenn, G. S., Blackwell, T. W., Fermin, D., Eng, J., Speicher, D. W. and Hanash, S. M. (2006). Challenges in deriving high-confidence protein identifications from data gathered by a HUPO plasma proteome collaborative study. *Nature Biotechnology* **24**(3): 333-338.

Sukmark, T., Lumlertgul, N., Peerapornratana, S., Khositrangsikun, K., Tungsanga, K., Sitprija, V., Srisawat, N. and Thai-Lepto AKI study group (2018). Thai-Lepto-on-admission probability (THAI-LEPTO) score as an early tool for initial diagnosis of leptospirosis: result from Thai-Lepto AKI study group. *PLoS Neglected Tropical Diseases* **12**(3): e0006319.

Suputtamongkol, Y., Pongtavornpinyo, W., Lubell, Y., Suttinont, C., Hoontrakul, S., Phimda, K., Losuwanaluk, K., Suwancharoen, D., Silpasakorn, S., Chierakul, W. and Day, N. (2010). Strategies for diagnosis and treatment of suspected leptospirosis: a cost-benefit analysis. *PLoS Neglected Tropical Diseases* **4**(2): e610.

Tan, X. T., Amran, F. b., Thayan, R., Ahmad, N., Jaafar, R., Haron, R., Abdullah, R., bin Shamsuddin, S. R., Md. Riffin, N. S. b. and Abdul-Rahman, P. S. (2017). Potential serum biomarkers associated with mild and severe leptospirosis infection: A cohort study in the Malaysian population. *Electrophoresis* **38**(17): 2141-2149.

Taylor, A. J., Paris, D. H. and Newton, P. N. (2015). A systematic review of the mortality from untreated leptospirosis. *PLoS Neglected Tropical Diseases* **9**(6): e0003866.

Thermo Fisher Scientific Inc. (2011). Instructions: GelCode® Blue Stain Reagent Rockford, lL, Thermo Fisher Scientific Inc.

Torgerson, P. R., Hagan, J. E., Costa, F., Calcagno, J., Kane, M., Martinez-Silveira, M. S., Goris, M. G. A., Stein, C., Ko, A. I. and Abela-Ridder, B. (2015). Global burden of leptospirosis: estimated in terms of disability adjusted life years. *PLoS Neglected Tropical Diseases* **9**(10): e0004122.

Uhlén, M., Karlsson, M. J., Hober, A., Svensson, A. S., Scheffel, J., Kotol, D., Zhong, W., Tebani, A., Strandberg, L., Edfors, F., Sjöstedt, E., Mulder, J., Mardinoglu, A., Berling, A., Ekblad, S., Dannemeyer, M., Kanje, S., Rockberg, J., Lundqvist, M., Malm, M., Volk, A. L., Nilsson, P., Månberg, A., Dodig-Crnkovic, T., Pin, E., Zwahlen, M., Oksvold, P., von Feilitzen, K., Häussler, R. S., Hong, M. G., Lindskog, C., Ponten, F., Katona, B., Vuu, J., Lindström, E., Nielsen, J., Robinson, J., Ayoglu, B., Mahdessian, D., Sullivan, D., Thul, P., Danielsson, F., Stadler, C., Lundberg, E., Bergström, G., Gummesson, A., Voldborg, B. G., Tegel, H., Hober, S., Forsström, B., Schwenk, J. M., Fagerberg, L. and Sivertsson, Å. (2019). The human secretome. *Science Signaling* **12**(609).

Victoriano, A. F. B., Smythe, L. D., Gloriani-Barzaga, N., Cavinta, L. L., Kasai, T., Limpakarnjanarat, K., Ong, B. L., Gongal, G., Hall, J., Coulombe, C. A., Yanagihara, Y., Yoshida, S.-i. and Adler, B. (2009). Leptospirosis in the Asia Pacific region. *BMC Infectious Diseases* **9**(1): 147.

Vijayachari, P., Sugunan, A. P., Murhekar, M. V., Sharma, S. and Sehgal, S. C. (2004). Leptospirosis among schoolchildren of the Andaman & Nicobar Islands, India: low levels of morbidity and mortality among pre-exposed children during an epidemic. *Epidemiology and Infection* **132**(6): 1115-1120.

Vijayachari, P., Sugunan, A. P., Umapathi, T. and Sehgal, S. C. (2001). Evaluation of darkground microscopy as a rapid diagnostic procedure in leptospirosis. *The Indian Journal of Medical Research* **114**: 54-58.

Visith, S. and Kearkiat, P. (2005). Nephropathy in leptospirosis. *Journal of Postgrad Medicine* **51**(3): 184-188.

Wagenaar, J. F. P., Gasem, M. H., Goris, M. G. A., Leeflang, M., Hartskeerl, R. A., van der Poll, T., van 't Veer, C. and van Gorp, E. C. M. (2009). Soluble ST2 levels are associated with bleeding in patients with severe leptospirosis. *PLoS Neglected Tropical Diseases* **3**(6): e453.

Waggoner, J. J., Soda, E. A., Seibert, R., Grant, P. and Pinsky, B. A. (2015). Molecular detection of *Leptospira* in two returned travelers: higher bacterial load in cerebrospinal fluid versus serum or plasma. *The American Journal of Tropical Medicine and Hygiene* **93**(2): 238-240.

Wang, N., Han, Y.-H., Sung, J.-Y., Lee, W.-S. and Ou, T.-Y. (2016). Atypical leptospirosis: an overlooked cause of aseptic meningitis. *BMC Research Notes* **9**: 154.

World Health Organization (2003). Human leptospirosis: guidance for diagnosis, surveillance and control, World Health Organization. **45**.

Wysocki, J., Liu, Y. and Shores, N. (2014). Leptospirosis with acute liver injury. *Baylor University Medical Center Proceedings* **27**(3): 257-258.

Yang, C. W., Wu, M. S. and Pan, M. J. (2001). Leptospirosis renal disease. *Nephrology, Dialysis and Transplantation* **16 Supplement 5**: 73-77.

Yersin, C., Bovet, P., Mérien, F., Clément, J., Laille, M., Van Ranst, M. and Perolat, P. (2000). Pulmonary haemorrhage as a predominant cause of death in leptospirosis in Seychelles. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94**(1): 71-76.

Yong, L. S. and Koh, K. C. (2013). A case of mixed infections in a patient presenting with acute febrile illness in the tropics. *Case Reports in Infectious Diseases* **2013**: 3.

Yuhana, Y., Hanboonkunupakarn, B., Tanganuchitcharnchai, A., Sujariyakul, P., Sonthayanon, P., Chotivanich, K., Pukrittayakamee, S., Blacksell, S. and Paris, D. (2022). Rickettsial infections are neglected causes of acute febrile illness in Teluk Intan, Peninsular Malaysia. *Tropical Medicine and Infectious Disease* **7**: 77.

Zakout, R., Carmo, M. d. and Freitas, P. (2013). Reversible myocardial calcification following severe leptospirosis complicated with rhabdomyolysis-induced acute kidney injury and magnesium-wasting nephropathy. *Journal of Medical Cases* **4**(2): 92-98.

Zhang, Y., Fonslow, B. R., Shan, B., Baek, M.-C. and Yates, J. R. (2013). Protein analysis by shotgun/bottom-up proteomics. *Chemical Reviews* **113**(4): 2343-2394.

Zhang, Z., Wu, S., Stenoien, D. L. and Paša-Tolić, L. (2014). High-throughput proteomics. *Annual Review of Analytical Chemistry* **7**(1): 427-454.

Zhou, C., Simpson, K. L., Lancashire, L. J., Walker, M. J., Dawson, M. J., Unwin, R. D., Rembielak, A., Price, P., West, C., Dive, C. and Whetton, A. D. (2012). Statistical considerations of optimal study design for human plasma proteomics and biomarker discovery. *Journal of Proteome Research* **11**(4): 2103-2113.

Zuerner, R. L. (2006). Laboratory maintenance of pathogenic *Leptospira*. *Current Protocols in Microbiology* **00**(1): 12E.11.11-12E.11.13.

APPENDICES

Appendix A

Ethics Approval Letter (Page 1 of 4)

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 KUALA LUMPUR

Tel.: 03-2287 4032/2282 0491/2282 9085
03-2282 9082/2282 1402/2282 1449
Faks: 03-2282 0015

Ruj. Kami : (I)KKM/NIHSEC/P16-231
Tarikh : 14hb Mac 2016

PROF. DR ZAMBERI SEKAWI
UNIVERSITY PUTRA MALAYSIA (UPM)

Tuan/ Puan,

NMRR- 15-2148-27536 (IIR)
Identification of early diagnostic and prognostic biomarkers for human leptospirosis through systems biology approaches encompassing clinical, biochemical, proteomics, transcriptomics, metabolomics and immunological aspects.

Dengan hormatnya perkara di atas adalah dirujuk.

2. Bersama dengan surat ini dilampirkan surat kelulusan saintifik dan etika bagi projek ini. Segala rekod dan data subjek adalah SULIT dan hanya digunakan untuk tujuan kajian dan semua isu serta prosedur mengenai ***data confidentiality*** mesti dipatuhi.

3. Pegawai penyelidik bersama yang terlibat di dalam kajian ialah:

- Dr Anim md shah
- Dr Leslie Than Thian Lung
- Dr Maha Abdullah
- Dr Mithra A/P C.Seganathirajah
- Dr Niazlin Mohd Taib

4. Kebenaran daripada Pegawai Kesihatan Daerah/Pengarah Hospital dan Ketua-Ketua Jabatan atau pegawai yang bertanggung jawab disetiap lokasi kajian di mana kajian akan dijalankan mesti diperolehi sebelum kajian dijalankan. Tuan/Puan perlu akur dan mematuhi keputusan tersebut. Sila rujuk kepada garispanduan Institut Kesihatan Negara mengenai penyelidikan di Institusi dan fasiliti Kementerian Kesihatan Malaysia (Pindaan 01/2015) serta lampiran *Appendix 5* untuk templet surat memohon kebenaran tersebut.

5. Adalah dimaklumkan bahawa kelulusan ini adalah sah sehingga **13hb Mac 2017**.Dato'/Dr./ Tuan/ Puan perlu menghantar perkara-perkara berikut kepada JEPP

Appendix A

Ethics Approval Letter (Page 3 of 4)

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 KUALA LUMPUR

Tel.: 03-2287 4032/2282 0491/2282 9085
03-2282 9082/2282 1402/2282 1449
Faks: 03-2282 0015

Ruj. Kami : (15)KKM/NIHSEC/P16-231
Date : 14hb Mac 2016

Protocol Title:
NMRR-15-2148-27536 (IIR)
Identification of early diagnostic and prognostic biomarkers for human leptospirosis through systems biology approaches encompassing clinical, biochemical, proteomics, transcriptomics, metabolomics and immunological aspects.

PRINCIPLE INVESTIGATOR:
PROF. DR ZAMBERI SEKAWI
UNIVERSITY PUTRA MALAYSIA (UPM)

Documents received and reviewed with reference to the above study:

1. Study Protocol version 3 dated 10-03-2016
2. Patient Information Sheet (English) & Informed Consent Form (English) version 2 dated 10-03-2016
3. Patient Information Sheet (BM) & Informed Consent Form (BM) version 2 dated 10-03-2016
4. Study Clinical Report Form (CRF) version 1 dated 19-01-2016
5. Curriculum vitae of :
   Prof. Dr Zamberi Sekawi
   Dr Anim Md Shah
   Dr Leslie Than Thian Lung
   Dr Maha Abdullah
   Dr Mithra A/P C.Seganathirajah
   Dr Niazlin Mohd Taib

Please note that the approval is valid until **13th March2017**. The following items are to be submitted tothe Medical Research and Ethics Committee (MREC) as appropriate.The required forms can be obtained from the MREC website (**http://www.nih.gov.my/mrec**).

Appendix C

List of Antibodies and Protein Standards Used in ELISAs

Target Protein	Primary Antibody, Secondary Antibody, Protein Standard	Brand	Product No.
Apolipoprotein A-II	Human Apolipoprotein A-II/ApoA2 monoclonal Antibody	R&D Systems	MAB4215
	Rat IgG HRP-conjugated Antibody	R&D Systems	HAF005
	Apolipoprotein A2 (APOA2) Recombinant Protein	MyBioSource	MBS2009181
C-reactive protein	Human C-Reactive Protein/CRP Antibody	R&D Systems	AF1707
	HRP-conjugated Anti-Sheep IgG Secondary Antibody	R&D Systems	HAF016
	Recombinant Human C-Reactive Protein/CRP Protein	R&D Systems	1707-CR-200
Fermitin family homolog 3	Sheep Anti-Human FERMT3 Polyclonal IgG	R&D Systems	AF7004
	HRP-conjugated Anti-Sheep IgG Secondary Antibody	R&D Systems	HAF016
	Fermitin family homolog 3 (FERMT3) Recombinant Protein	MyBioSource	MBS1352843
Leucine-rich alpha-2-glycoprotein 1	Sheep Anti-Human LRG1 Polyclonal IgG	R&D Systems	AF7890
	HRP-conjugated Anti-Sheep IgG Secondary Antibody	R&D Systems	HAF016
	Recombinant Human LRG1 Protein	R&D Systems	7890-LR-025
Lipopolysaccharide-binding protein	Human LBP Antibody	R&D Systems	AF870
	HRP-conjugated Anti-Goat IgG Secondary Antibody	R&D Systems	HAF019
	Recombinant Human LBP Protein	R&D Systems	870-LP-025
Myosin-9	Rabbit anti-Human Myh9 Polyclonal Antibody IgG	MyBioSource	MBS7601713
	Goat anti-Rabbit IgG Secondary Antibody, HRP	MyBioSource	MBS674746
	MYH9 recombinant protein	MyBioSource	MBS5304175
Platelet basic protein	Goat Anti-Human CXCL7/NAP?2 Antigen Polyclonal IgG	R&D Systems	AF393
	HRP-conjugated Anti-Goat IgG Secondary Antibody	R&D Systems	HAF019
	Recombinant Human CXCL7/NAP-2 Protein	R&D Systems	393-NP-050
Platelet factor 4	Goat Anti-Human CXCL4/PF4 Polyclonal IgG	R&D Systems	AF795
	HRP-conjugated Anti-Goat IgG Secondary Antibody	R&D Systems	HAF019
	Recombinant Human CXCL4/PF4 Protein	R&D Systems	795-P4-025
Profilin-1	Mouse Anti-Human Profilin 1 Monoclonal Antibody	R&D Systems	MAB7779
	HRP-conjugated Anti-Mouse IgG Secondary Antibody	R&D Systems	HAF018
	Profilin 1 (PFN1) Recombinant Protein	MyBioSource	MBS2009178
Serum amyloid A-1 protein	Human Serum Amyloid A1 Antibody	R&D Systems	MAB3019
	HRP-conjugated Anti-Rat IgG Secondary Antibody	R&D Systems	HAF005
	Serum Amyloid A (SAA) Recombinant Protein	MyBioSource	MBS5304469
Thrombospondin-1	Goat Anti-Human Thrombospondin?1 Polyclonal IgG	R&D Systems	AF3074
	HRP-conjugated Anti-Goat IgG Secondary Antibody	R&D Systems	HAF019
	Recombinant Human Thrombospondin-1 Protein	R&D Systems	3074-TH-050

Appendix D

Protein Concentrations Estimated using ELISAs in Verification Cohort (Page 1 of 3)

Subject	Protein										
	APOA2	CRP	FERMT3	LRG1	LBP	MYH9	PPBP	PF4	PFN1	SAA1	THBS1
	µg/mL	µg/mL	ng/mL	µg/mL	µg/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	µg/mL
HLTY-CTRL 1	99.0	4.7	224.0	24.9	4.5	470.3	29.7	6.3	199.8	419.2	20.1
HLTY-CTRL 2	115.4	4.4	73.1	24.7	8.6	411.0	34.6	4.8	339.2	340.1	22.0
HLTY-CTRL 3	104.8	3.3	153.0	20.7	5.0	414.8	35.4	8.1	467.3	338.5	12.5
HLTY-CTRL 4	104.2	4.3	157.3	21.5	4.8	421.0	23.0	8.0	432.6	322.7	9.1
HLTY-CTRL 5	115.5	5.6	97.7	23.6	5.1	360.4	17.5	5.9	365.9	974.8	13.8
HLTY-CTRL 6	136.3	5.5	226.0	25.3	7.9	401.5	22.6	8.0	197.0	376.8	7.8
HLTY-CTRL 7	110.0	6.1	78.8	25.8	4.6	452.5	19.1	7.1	314.6	397.7	7.4
HLTY-CTRL 8	102.9	3.2	179.3	22.0	5.5	497.3	17.1	6.8	226.7	472.2	13.2
HLTY-CTRL 9	155.7	3.1	155.6	21.2	4.2	498.9	30.4	4.6	445.4	331.6	11.9
HLTY-CTRL 10	118.6	8.0	198.5	39.8	6.2	473.5	22.7	8.1	251.7	364.7	6.9
HLTY-CTRL 11	112.5	8.4	133.0	36.6	3.7	477.0	18.2	6.4	478.8	367.1	14.5
HLTY-CTRL 12	104.5	5.9	88.0	23.7	4.6	312.6	20.8	7.6	364.6	356.8	19.1
HLTY-CTRL 13	124.7	6.1	140.8	38.2	4.1	388.7	21.5	8.7	592.2	1,179.0	6.5
HLTY-CTRL 14	109.6	3.8	76.0	25.3	3.1	343.6	18.9	8.0	326.1	361.7	9.5
LEPTO 1	156.5	117.0	87.9	145.1	45.9	63.3	12.6	5.7	253.7	2,467.5	2.5
LEPTO 2	106.7	198.7	71.4	124.3	28.2	254.6	4.8	6.3	347.1	3,239.0	2.6
LEPTO 3	129.7	90.3	137.6	157.6	37.4	278.7	7.4	5.4	546.0	3,271.4	6.0
LEPTO 4	110.2	126.0	102.0	120.5	31.9	208.3	8.4	5.8	242.9	2,182.4	4.9
LEPTO 5	136.2	36.9	107.6	136.3	29.0	208.3	8.6	7.4	207.3	2,169.8	4.3
LEPTO 6	128.2	133.4	160.7	122.2	22.5	198.4	3.8	4.6	408.2	2,775.7	6.4
LEPTO 7	114.7	157.5	115.6	153.2	35.6	279.8	15.3	6.5	214.3	2,581.4	7.4
LEPTO 8	101.5	269.8	88.8	127.8	23.8	208.4	6.5	4.8	279.1	2,600.2	2.3
LEPTO 9	92.0	290.8	164.0	146.7	34.7	321.4	10.4	6.2	151.1	2,867.5	4.2
LEPTO 10	101.3	82.0	136.7	117.4	37.7	173.5	7.6	5.9	533.3	3,020.4	1.6
LEPTO 11	99.2	162.1	126.8	145.3	39.2	241.2	8.0	4.9	195.4	3,330.8	3.2
LEPTO 12	155.4	224.0	84.1	164.2	26.0	166.5	8.5	4.7	206.2	3,147.2	6.3
LEPTO 13	91.4	280.3	130.5	137.3	35.0	319.6	3.9	5.3	189.9	2,494.4	3.8
LEPTO 14	119.0	340.0	145.0	159.0	37.2	233.3	2.3	6.0	654.0	2,935.6	3.6
LEPTO 15	92.3	185.1	101.2	140.3	42.9	185.3	5.9	4.4	489.8	3,141.1	5.1
LEPTO 16	106.6	153.5	88.3	157.7	35.4	235.8	12.3	6.7	280.7	3,269.8	4.6
LEPTO 17	119.3	267.5	113.4	162.8	24.3	62.2	3.0	6.6	430.6	2,804.0	4.9
LEPTO 18	112.0	202.6	110.7	132.3	32.9	238.7	7.6	5.3	223.8	3,354.6	5.0
LEPTO 19	107.5	132.0	139.3	127.9	33.5	217.2	4.0	3.5	571.3	2,597.6	3.7
LEPTO 20	160.7	243.6	80.9	136.3	44.2	287.7	20.7	4.1	306.1	3,226.4	12.7

Appendix D

Protein Concentrations Estimated using ELISAs in Verification Cohort (Page 2 of 3)

Subject	Protein										
	APOA2	CRP	FERMT3	LRG1	LBP	MYH9	PPBP	PF4	PFN1	SAA1	THBS1
	µg/mL	µg/mL	ng/mL	µg/mL	µg/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	µg/mL
LEPTO 21	123.6	289.0	148.7	159.8	43.5	111.0	23.6	4.3	508.9	3,183.3	8.1
LEPTO 22	145.4	96.5	122.4	164.6	27.4	76.1	2.9	5.2	202.4	858.7	1.5
LEPTO 23	121.3	222.8	116.2	143.4	36.3	155.5	12.9	5.0	377.3	2,685.3	7.7
LEPTO 24	142.4	180.1	176.3	135.4	23.3	265.2	2.8	4.2	140.6	2,210.3	4.4
LEPTO 25	104.5	279.9	164.8	123.0	41.0	135.9	3.0	5.8	382.7	2,436.1	4.7
LEPTO 26	123.3	240.0	192.6	138.6	33.0	65.6	4.3	5.1	267.7	3,200.8	3.7
LEPTO 27	109.7	74.0	121.4	144.4	30.0	49.9	4.9	4.7	204.1	2,503.4	5.1
LEPTO 28	101.5	175.6	163.2	142.7	37.2	53.1	3.6	5.8	392.2	3,205.6	2.9
LEPTO 29	120.0	134.3	168.3	133.8	32.2	243.8	25.6	6.2	147.3	3,075.1	4.0
LEPTO 30	101.5	93.9	121.4	154.4	30.1	57.8	2.1	6.5	204.1	3,380.8	4.7
LEPTO 31	122.6	277.7	122.2	164.6	29.3	272.5	13.6	5.2	364.9	3,241.2	5.0
LEPTO 32	111.4	317.1	109.1	131.9	32.2	166.6	5.2	4.4	227.2	3,016.3	3.6
LEPTO 33	104.4	90.2	150.3	140.2	31.8	283.0	6.3	5.6	164.8	2,663.5	4.8
LEPTO 34	110.3	245.5	97.8	129.7	29.8	62.3	4.1	6.8	253.4	3,032.5	2.7
LEPTO 35	107.2	219.1	116.2	153.3	38.2	244.5	15.2	4.9	213.3	2,436.1	5.2
LEPTO 36	129.4	62.2	78.5	145.8	34.2	50.0	6.7	5.4	315.8	2,710.0	2.6
LEPTO 37	100.2	162.3	71.1	124.3	43.3	238.0	11.0	4.8	348.7	2,554.6	2.6
LEPTO 38	96.9	302.2	98.3	119.8	29.5	343.1	6.1	6.2	252.1	3,035.6	6.1
LEPTO 39	110.8	195.5	150.1	140.1	30.0	209.2	13.6	4.6	494.4	3,167.1	5.3
LEPTO 40	95.8	166.4	91.8	158.5	39.2	62.3	17.0	5.3	269.9	2,682.2	3.9
LEPTO 41	90.2	188.6	168.0	163.7	28.4	223.0	6.9	6.6	364.3	2,797.1	5.2
LEPTO 42	101.1	225.9	167.7	143.6	23.5	86.1	6.9	6.5	365.9	2,484.6	5.2
LEPTO 43	105.4	72.4	145.4	139.2	33.6	236.5	3.6	5.5	170.4	2,392.8	1.4
LEPTO 44	108.9	122.2	109.3	122.0	34.5	55.1	9.1	4.4	226.7	2,475.3	4.6
DEN-CTRL 1	101.8	6.0	82.1	36.5	11.1	272.2	2.9	2.3	301.9	1,135.7	1.1
DEN-CTRL 2	106.5	21.8	126.1	55.2	10.0	218.0	3.0	3.0	379.3	2,798.6	1.8
DEN-CTRL 3	119.4	11.4	154.5	41.0	10.0	221.9	10.3	4.3	454.5	1,523.2	4.8
DEN-CTRL 4	98.9	59.2	97.0	39.5	8.3	228.8	3.1	3.8	255.6	2,718.9	3.8
DEN-CTRL 5	126.6	8.5	164.1	42.9	9.7	257.8	4.6	5.5	386.6	2,451.3	2.9
DEN-CTRL 6	106.6	12.4	174.3	54.9	12.0	242.8	2.8	3.2	333.3	3,143.7	1.4
DEN-CTRL 7	115.9	26.9	168.1	63.7	9.8	82.3	2.2	6.2	364.1	2,415.6	3.6
DEN-CTRL 8	131.6	16.2	122.7	54.7	7.4	389.7	3.0	2.5	202.0	1,152.3	2.2
DEN-CTRL 9	119.8	12.5	81.7	46.4	8.5	315.2	2.3	3.2	303.4	3,205.0	0.9
DEN-CTRL 10	103.7	14.7	94.9	39.1	10.7	226.2	5.5	4.0	261.1	2,543.8	1.5

Appendix D

Protein Concentrations Estimated using ELISAs in Verification Cohort (Page 3 of 3)

Subject	Protein										
	APOA2	CRP	FERMT3	LRG1	LBP	MYH9	PPBP	PF4	PFN1	SAA1	THBS1
	µg/mL	µg/mL	ng/mL	µg/mL	µg/mL	ng/mL	ng/mL	ng/mL	ng/mL	ng/mL	µg/mL
DEN-CTRL 11	122.7	11.5	120.2	44.2	11.7	287.6	2.5	2.9	206.2	3,003.6	1.4
DEN-CTRL 12	128.1	11.3	157.0	51.5	5.9	213.9	2.5	3.8	434.8	2,806.0	2.7
DEN-CTRL 13	97.7	7.6	88.1	57.7	6.6	248.3	3.0	2.5	281.2	1,116.5	1.3
DEN-CTRL 14	114.0	11.3	160.5	62.2	9.4	270.3	2.2	2.7	409.3	2,052.8	4.3
DEN-CTRL 15	111.6	17.5	90.2	58.1	6.7	65.2	2.6	3.3	274.7	2,710.9	2.3
DEN-CTRL 16	139.6	22.7	102.0	40.5	10.2	49.1	3.6	3.7	242.9	3,116.9	0.8
DEN-CTRL 17	103.2	12.7	114.8	43.1	9.5	72.0	2.2	2.8	215.8	2,753.6	1.4
DEN-CTRL 18	105.6	12.6	159.6	52.0	7.8	91.5	3.4	2.5	415.7	1,417.0	2.2
DEN-CTRL 19	113.1	49.6	95.0	59.1	6.4	59.2	4.8	2.3	260.9	3,080.4	2.4
DEN-CTRL 20	110.2	12.8	172.7	64.6	6.9	54.5	8.6	1.5	340.9	1,504.0	1.8
DEN-CTRL 21	105.1	23.3	84.3	46.9	5.4	49.9	2.4	2.1	294.1	2,660.3	1.4
DEN-CTRL 22	108.0	26.7	134.1	66.9	9.2	93.3	2.5	1.9	184.8	2,779.4	0.8
DEN-CTRL 23	98.9	6.5	158.6	51.8	7.8	50.4	2.2	8.0	422.5	2,550.9	1.3
DEN-CTRL 24	117.0	12.6	126.6	55.4	9.1	68.9	2.6	3.3	195.7	3,015.2	1.6
DEN-CTRL 25	115.5	7.2	87.2	57.5	8.2	63.7	3.3	2.4	284.1	1,421.1	1.2
DEN-CTRL 26	108.8	12.9	103.7	60.8	8.0	57.0	2.9	3.2	239.0	1,882.2	6.9
DEN-CTRL 27	111.0	10.1	168.8	43.8	9.3	64.9	3.0	2.3	360.1	1,893.5	2.0
DEN-CTRL 28	103.0	11.2	108.2	31.7	5.1	61.1	4.8	2.0	229.1	661.8	1.5
DEN-CTRL 29	110.8	13.5	82.2	46.5	5.3	58.1	2.7	2.8	301.6	922.7	1.6
DEN-CTRL 30	108.5	15.7	78.1	45.7	9.3	71.7	2.8	3.2	317.5	2,977.0	2.1
DEN-CTRL 31	102.4	11.8	131.9	66.4	7.2	197.4	2.5	2.2	464.1	3,382.1	1.7
DEN-CTRL 32	99.8	12.5	167.7	53.6	9.3	67.9	2.7	2.1	365.9	3,115.2	1.6
DEN-CTRL 33	109.0	4.4	155.9	41.2	7.8	80.9	2.8	3.1	443.7	1,447.6	3.9
DEN-CTRL 34	101.7	9.4	102.0	60.5	8.0	49.5	14.2	2.4	243.0	1,152.0	4.9
DEN-CTRL 35	111.7	6.8	114.0	52.9	7.8	58.0	2.7	2.1	217.4	2,697.4	1.1
DEN-CTRL 36	165.9	7.4	145.0	49.0	8.2	191.6	3.3	1.7	654.0	1,149.5	2.0
DEN-CTRL 37	107.8	21.4	124.7	65.1	8.4	59.1	2.8	2.1	198.7	1,238.0	2.1
DEN-CTRL 38	117.4	10.4	143.2	38.7	6.8	115.2	2.7	2.3	627.8	350.7	1.2
DEN-CTRL 39	127.4	13.3	109.0	41.9	8.2	172.4	2.2	3.5	227.3	2,828.9	4.1
DEN-CTRL 40	128.3	18.6	109.4	62.0	6.5	69.7	4.2	2.9	226.6	3,112.4	1.4
DEN-CTRL 41	109.9	13.1	71.0	54.3	8.3	79.4	2.0	3.5	348.9	2,204.8	1.2
DEN-CTRL 42	102.6	19.5	76.8	45.4	5.4	55.8	2.3	2.6	322.6	2,252.6	1.8
DEN-CTRL 43	110.1	33.1	106.6	61.4	10.0	110.0	2.9	3.3	232.6	2,671.0	2.6
DEN-CTRL 44	141.5	22.5	74.6	45.0	6.0	57.2	9.6	2.8	332.0	3,082.2	5.1
DEN-CTRL 45	121.2	11.2	163.6	62.8	5.6	86.6	2.5	2.7	389.4	1,004.4	1.2

Appendix E

Protein abbreviation list for uniquely expressed proteins in the Leptospiraemia Group

Display Name (Abbreviation)	Protein Name
APOC2	Proapolipoprotein C-II
APOC3	Apolipoprotein C-III
CD5L	CD5 antigen-like
CFHR2	Complement factor H-related protein 2
CFHR5	Complement factor H-related protein 5
CKM	Creatine kinase M-type
FCN3	Ficolin-3
FGA	Fibrinogen alpha chain
FGB	Fibrinogen beta chain
FGG	Fibrinogen gamma chain
H4C6	Histone H4
HBA2	Haemoglobin subunit alpha
HBB	Haemoglobin subunit beta
LYZ	Lysozyme C
SAA2	Serum amyloid A-2 protein

Appendix F

Protein abbreviation list for uniquely expressed proteins in the Seroconversion Group (Page 1 of 2)

Display Name (Abbreviation)	Protein Name
ALDOA	Fructose-bisphosphate aldolase A
APMAP	Adipocyte plasma membrane-associated protein
APOD	Apolipoprotein D
APOE	Apolipoprotein E
APOM	Apolipoprotein M
AZGP1	Zinc-alpha-2-glycoprotein
B2M	Beta-2-microglobulin form pI 5.3
C4BPB	C4b-binding protein beta chain
C5	Complement C5 alpha' chain
CACNA1H	Voltage-dependent T-type calcium channel subunit alpha-1H
CFD	Complement factor D
CLU	Clusterin alpha chain
CNDP1	Carnosine dipeptidase 1
CNN2	Calponin-2
COMP	Cartilage oligomeric matrix protein
F12	Coagulation factor XIIa heavy chain
F13A1	Coagulation factor XIII A chain
F5	Coagulation factor V heavy chain
F9	Coagulation factor IXa heavy chain
FBLN1	Fibulin-1
FN1	Fibronectin
GPLD1	Phosphatidylinositol-glycan-specific phospholipase D

Appendix F

Protein abbreviation list for uniquely expressed proteins in the Seroconversion Group (Page 2 of 2)

Display Name (Abbreviation)	Protein Name
GPX3	Glutathione peroxidase 3
GSN	Gelsolin
HSP90AA1	Heat shock protein HSP 90-alpha
HSP90B1	Endoplasmin
HSPA8	Heat shock cognate 71 kDa protein
ITGB1	Integrin beta-1
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2
ITIH3	Inter-alpha-trypsin inhibitor heavy chain H3
ITIH4	35 kDa inter-alpha-trypsin inhibitor heavy chain H4
LYVE1	Lymphatic vessel endothelial hyaluronic acid receptor 1
MMRN1	155 kDa platelet multimerin
ORM2	Alpha-1-acid glycoprotein 2
PCYOX1	Prenylcysteine oxidase 1
PGLYRP2	N-acetylmuramoyl-L-alanine amidase
PIGR	Polymeric immunoglobulin receptor
SERPINA1	Short peptide from AAT
SERPINA4	Kallistatin
SERPINA7	Thyroxine-binding globulin
TUBA4A	Tubulin alpha-4A chain
TUBB1	Tubulin beta-1 chain
VCAM1	Vascular cell adhesion protein 1

Appendix G

Protein abbreviation list for Cluster 1

Display Name (Abbreviation)	Protein Name
APOA1	Truncated apolipoprotein A-I
APOA2	Truncated apolipoprotein A-II
APOA4	Apolipoprotein A-IV
APOC1	Truncated apolipoprotein C-I
APOC2	Proapolipoprotein C-II
APOC3	Apolipoprotein C-III
APOD	Apolipoprotein D
APOE	Apolipoprotein E
APOH	Beta-2-glycoprotein 1
APOM	Apolipoprotein M
CLU	Clusterin alpha chain
PCYOX1	Prenylcysteine oxidase 1
SAA1	Serum amyloid protein A (2-102)
SAA2	Serum amyloid A-2 protein
SERPINA1	Short peptide from AAT, inhibitor of serine

Appendix H

Protein abbreviation list for Cluster 2

Display Name (Abbreviation)	Protein Name
CFL1	Cofilin-1
FERMT3	Fermitin family homolog 3
FGA	Fibrinogen alpha chain
FGG	Fibrinogen gamma chain
FN1	Fibronectin
HP	Haptoglobin alpha chain
ILK	Integrin-linked protein kinase
ITGB1	Integrin beta-1
ITGB3	Integrin beta-3
MSN	Moesin
ORM1	Alpha-1-acid glycoprotein 1
ORM2	Alpha-1-acid glycoprotein 2
PFN1	Profilin-1
THBS1	Thrombospondin-1
TLN1	Talin-1
ZYX	Zyxin

Protein abbreviation list for Cluster 3

Display Name (Abbreviation)	Protein Name
ACTG1	Actin, cytoplasmic 2, N-terminally processed
ACTN1	Alpha-actinin-1
ALDOA	Fructose-bisphosphate aldolase A
FLNA	Filamin-A
GSN	Gelsolin
ITGA2B	Integrin alpha-IIb light chain, form 1
LDHA	Lactate dehydrogenase A
MYH9	Myosin-9
PKM	Pyruvate kinase PKM
VCL	Vinculin

LIST OF PUBLICATIONS

Fish-Low, C.-Y., Balami, A. D., Than, L. T. L., Ling, K.-H., Mohd Taib, N., Md. Shah, A. and Sekawi, Z. (2020). Hypocalcemia, hypochloremia, and eosinopenia as clinical predictors of leptospirosis: A retrospective study. *Journal of Infection and Public Health* **13**(2): 216-220.

Fish-Low, C.-Y., Than, L. T. L., Ling, K.-H., Lin, Q. and Sekawi, Z. (2020). Plasma proteome profiling reveals differentially expressed lipopolysaccharide-binding protein among leptospirosis patients. *Journal of Microbiology, Immunology and Infection* **53**(1): 157-162.

Fish-Low, C.-Y., Than, L. T. L., Ling, K.-H. and Sekawi, Z. (2024). The potential of eight plasma proteins as biomarkers in redefining leptospirosis diagnosis. *Journal of Proteome Research*.