UNIVERSITY OF GHANA

UNIVERSITY OF GHANA MEDICAL SCHOOL

COLLEGE OF HEALTH SCIENCES

SOLUBLE PROGRAMMED CELL DEATH LIGAND 1 (sPD-L1) AMONG FEBRILE PATIENTS WITH EXPOSURE TO SELECTED PATHOGENS

BY

JONATHAN KLUTSE

(10525347)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY IN MICROBIOLOGY

DEPARTMENT OF MEDICAL MICROBIOLOGY

JANUARY 2023

DECLARATION

I, Jonathan Klutse, hereby declare that, with the exception of appropriately cited references to literature, this work has not been presented in whole or in part elsewhere. This study was carried out under the guidance and directions of Prof. Patience Borkor Tetteh-Quarcoo and Rev. Prof. Patrick Ferdinand Ayeh-Kumi (Department of Medical Microbiology, Medical School, College of Health Sciences, University of Ghana). There is no part of this thesis that has previously been submitted for a degree or other qualification.

Signed: ..... Date. 08/02/23

Jonathan Klutse

(Student)

Signed: ..... Date: 10/02/2023

Prof. Patience Borkor Tetteh-Quarcoo

(Supervisor)

Signed: ..... Date. 08-02-23

Rev. Prof. Patrick Ferdinand Ayeh-Kumi

(Supervisor)

DEDICATION

I am dedicating this thesis to my entire family (Mrs Judith Alormenu, Rev. Dr. Sampson Dorkunor, and Mrs. Janet Dorkunor), supervisors, study participants, colleagues, and MPhil 2020/22-year group at the University of Ghana Medical School's Department of Medical Microbiology. This study would not have been a success without your guidance, constructive criticism, encouragement, efforts, and support.

ACKNOWLEDGEMENT

I am profoundly grateful to the Almighty God for His guidance, provision, and granting me the strength to complete this work. My sincere appreciation also goes to my supervisors, Prof. Patience Borkor Tetteh-Quarcoo, and Rev. Prof. Patrick Ferdinand Ayeh-Kumi for making time out of your busy schedules to supervise my work, from bench work to thesis write-up. I am immensely grateful to Dr. Christoph Schmidt of Ulm University, Germany for providing me with the soluble PD-L1 ELISA kits used in this work. My family has also been an integral part of this work. I also acknowledge Mrs Joana Twasam and the entire LEKMA hospital for their enormous support during my sample collection. My gratitude also goes to the head and staff of NPHRL, Korle-Bu for allowing me use their ELISA Plate Reader. I am also grateful to the head and faculty members, as well as the staff of the Department of Medical Microbiology at the University of Ghana Medical School, for greatly imparting me and providing me with the solid foundation that I require for my future career. To Mr Emmanuel Afutu, Miss Gloria Gardiner, Miss Dzifa Azumah, Mr Evans Aduhene, Mr Pious Appiah, Miss Ellen Konadu Opoku and Madam Abigail (National Service persons), and all my study participants, I say thank you very much. Finally, I share this happiness with the entire MPhil 2020/22-year group.

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ABBREVIATIONS

AKT-mTOR - Phosphatidylinositol-3-kinase and the mammalian target of rapamycin
BRAF - v-raf murine sarcoma viral oncogene homolog B1
CD274 - Cluster of differentiation 274
CD8+ - Cluster of differentiation 8
CTLA-4 - Cytotoxic T-lymphocyte-associated protein 4
EDTA - Ethylenediaminetetraacetic acid
EGFR - Epidermal growth factor receptor
ELISA - Enzyme-linked immunosorbent assay
ERK - Extracellular signal–regulated kinase
Hb - Haemoglobin
HBV - Hepatitis B virus
HIV - Human immunodeficiency virus
HSV - Herpes simplex virus
ICB - Immune checkpoint blocker
ICMs - Immune checkpoint molecules
IFNs - Interferons
IFN-α - Interferon gamma
IgG - Immunoglobulin-G
IgM - Immunoglobulin-M
IL-10 - Interleukin-10
ITIM - immunoreceptor tyrosine-based inhibition motif
LAG-3 - Lymphocyte-activation gene 3
MAPK - Mitogen-activated protein kinase
MEK - Mitogen-activated protein kinase kinase
mRNA - Messenger RNA
PAMP - Pathogen associated molecular pattern
PD-1 - Programmed cell death protein 1
PD-L1 - Programmed cell death ligand 1
Pf - *Plasmodium falciparum*

pHRP-II	- *Plasmodium falciparum* histidine-rich protein-2
PI3K	- Phosphatidylinositol-3-kinase
pLDH	- Parasite lactate dehydrogenase
PTEN	- Phosphatase and TENsin homolog
RBCs	- Red blood cells
RDT	- Rapid diagnostic test
RSV	- Respiratory syncytial virus
SARS-CoV-2	- severe acute respiratory syndrome coronavirus 2
SHIP	- SH2-containing inositol-5'-phosphatase
sPD-L1	- Soluble programmed cell death ligand 1
STAT-3	- signal transducer and activator of transcription 3
TCR	- T-cell receptor
TGF-β	- Transforming growth factor beta
TIM-3	- T-cell immunoglobulin and mucin domain-containing-3
TLR-3	- Toll-like receptor 3
TLR-7	- Toll-like receptor 7
TLR-8	- Toll-like receptor 8
TLR-9	- Toll-like receptor 9
Tregs	- Regulatory T-cells
WBCs	- White blood cells
ZAP70	- Zeta-chain associated protein kinase 70

ABSTRACT

Introduction: Soluble programmed cell death ligand -1(sPD-L1) is an immune inhibitory checkpoint protein shown to play a pivotal role in maintaining immune homeostasis. However, disrupting the interaction between sPD-L1 and its receptor can result in the evasion of immune surveillance by many cancers and poor prognosis in various T-cell-mediated infectious diseases. Therefore, understanding the role of sPD-L1 is critical especially in infectious diseases. However, knowledge in this area is lacking in most endemic infections, which may impede the development of new diagnostic methods, therapeutics, and vaccines for these infections. Suggesting the need to investigate the possible association and potential role of sPD-L1 levels in common endemic infections. Also, most studies on PD-L1 involved the use of whole blood samples in investigating membrane-bound PD-L1, with the use of flow cytometry. This technique may be challenging in some Sub-Saharan African countries, as it is expensive, requires trained professionals, and is laborious. Therefore, having a study which uses a simpler technique such ELISA in comparing sPD-L1 levels in both blood and urine to see if urine samples alone will be sufficient for assessing sPD-L1 would be beneficial, as urine is an easy clinical sample to obtain, and collection is less invasive than blood. The present study investigated the expression of sPD-L1 in the blood and urine of febrile patients with proof of exposure to selected pathogens.

Methodology: This was a longitudinal study involving baseline febrile patients with exposure to selected pathogens and post-treatment on cohorts three weeks (21 days) after treatment. Both whole blood and urine samples were collected from consented patients with febrile illness who presented to Ledzokuku-Krowor Municipal Assembly (LEKMA) hospital. A total of 5 ml of whole blood was collected into EDTA tubes, out of which 2 ml was used in the screening of patients for exposure to selected pathogens and full blood count analysis. Plasma was separated from the remaining 3 ml by centrifugation. The concentration of plasma and urine sPD-L1 was measured using a commercial ELISA following the manufacturer´s instructions. The optical density (OD) was measured spectrophotometrically and a four (4) parameter logistic (4-PL) standard curve was generated using an online platform, and R^2-values above 0.9 were considered acceptable.

Results: Exposure to *Plasmodium* species, *Salmonella Typhi*, HSV-1, HSV-2, *Toxoplasma gondii*, HIV, hepatitis B virus, and syphilis was 7.08%, 1.5%, 52.8%, 46.17 %, 11%, 1.1%, 1.1%, and 2.2%, respectively. From this study, the plasma and urine concentrations of sPD-L1

were significantly higher in patients than the controls prior to treatment (all $p < .001$); also, significantly higher sPD-L1 was seen in patients before treatment compared to patients after treatment (all $p < .001$), suggesting that sPD-L1 levels are upregulated during infection. In addition, no statistical difference was observed in sPD-L1 concentrations between malaria patients and patients with malaria and typhoid immunoglobulins, nor between controls and patients with typhoid immunoglobulins only (All $p > .05$), suggesting that typhoid immunoglobins may not necessarily contribute to elevated levels of sPD-L1. Also, there is no statistical difference in sPD-L1 levels in plasma and urine of patients with malaria only and patients with malaria and HSV immunoglobulins ($p = .818$ and $p = 426$, respectively, all $p < .05$), suggesting that HSV antibodies may also not contribute to further upregulated expression of sPD-L1 in the presence of *Plasmodium* infection.

A positive correlation was established between *Plasmodium* parasite density, plasma sPD-L1 (*rho* =.554, p =.001) and urine sPD-L1 (*rho* =.412, p =.005). In addition, the present study found a correlation between sPD-L1 and hemoglobin levels, WBCs, platelets, lymphocytes, neutrophils, and eosinophils.

Conclusion: This study demonstrated that plasma and urine sPD-L1 are detectable during systemic infections such as *Plasmodium spp.* infection, with patients having higher concentration at pre-treatment than post-treatment and controls. Our findings have also shown that upregulated expression of sPD-L1 in malaria patients is associated with increased *Plasmodium* parasite density. The present study has also shown for the first time that urine samples alone are enough to assess sPD-L1 expression in malaria patients.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The immunosuppressive immune checkpoint protein, soluble PD-L1 (sPD-L1), is a glycoprotein belonging to the B7 ligand family of immunomodulatory checkpoint molecules (Ceeraz *et al*., 2013). Programmed cell death ligand 1 (PD-L1) is one of the two ligands for programmed cell death protein (PD-1). A second ligand is the protein PD-L2 (programmed cell death ligand-2). (Hotchkiss *et al*., 2013).

Immune checkpoint molecules (ICMs) generally are regulatory receptors/ligands that operate as co-stimulatory or co-inhibitory molecules to modulate immune responses to defend the body against infections and maintain tolerance to self-antigens under normal physiological function, thereby preventing immune mediated-cellular damage or autoimmunity (Farrukh *et al*., 2021; Wang *et al*., 2015; Chaudhry and Rudensky., 2013; Waterhouse *et al*.,1995). On the other hand, these molecules have been demonstrated to play a role in immune modulation by impairing T-lymphocyte function in many diseases, including malignant tumors and infectious diseases (Cai *et al*., 2020), by sending the turn-off signals to T-cells during infections. This is accomplished partly by impairing T cell function through a phenomenon known as T cell exhaustion (Salmaninejad *et al*., 2019; Crespo *et al*., 2013) by inducing regulatory T cells (Tregs).

Despite their role in immune homeostasis, immune checkpoint proteins, particularly the PD-1/PD-L1 signaling pathway, have been shown to impair immune response in many infections and cancers by modulating T cell activity. It has been shown that various tumours and infectious pathogens can avoid the host's immune surveillance by exploiting the impaired T cell activity. (Yokoyama *et al*., 2016; Butte *et al*., 2007). The receptor-ligand interaction

between PD-1 and PD-L1 inhibits T-cell activation and cytotoxicity (Schönrich and Raftery., 2019; Okazaki *et al*., 2001; Tseng *et al*., 2001), leading to the thwarting of autoimmunity by repressing the expansion of auto-reactive T cells (Yearley *et al*., 2017). This interaction and overexpression of PD-L1 have led to poor prognosis of many cancers (Kula *et al*., 2020; Chakrabarti *et al*., 2019; Ding *et al*., 2017) and infectious diseases (Kauffman *et al*., 2021; Correa-Rocha *et al*., 2018; Wykes and Lewin., 2018; Crawford & Wherry, 2009; Kaufmann and Walker., 2009; Sharpe *et al*., 2007).

Recently, elevated expression of sPD-L1 was detected in COVID-19 patients. Findings from this study showed that increased concentrations of sPD-L1 led to low lymphocyte counts and, subsequently, poor prognosis in the patients (Sabbatino *et al*., 2021). It was also shown that the COVID-19 patient who died had higher levels of PD-L1(Sabbatino *et al*., 2021). Another study reported significantly higher sPD-L1 in Ugandan males with prostate cancer than in healthy controls (Katongole *et al*., 2022). Patients with upregulated PD-L1 levels have more severe disease and a poorer prognosis than patients with low PD-L1 levels.

Expression of programmed cell death ligand 1 (PD-L1) has been linked with various biological and immunological changes, including cytokines (Raftery *et al*., 2018; Garcia-Diaz *et al*., 2017) and activation of the complement pathway in chronic or acute infections. In addition, inflammatory diseases (autoimmune diseases) may also affect PD-L1 expression (Hoan *et al*., 2022).

Despite the significant contribution of the complement system as an effective immune surveillance system and its essential role in maintaining homeostasis (Ricklin *et al*., 2010), recent studies have shed new light on the immunosuppressive functions of some complement components. Over the past decade, research has shown that these complement components help

regulate the function of diseased cells, such as tumors, and suppress immune responses (Zhang *et al*., 2019).

1.2 Problem Statement and Justification

The discovery of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Krummel and Allison., 1995) and programmed death-1 (PD-1) protein (Ishida *et al*., 1992) has revolutionized cancer immunotherapy. However, there are only a few studies of these molecules in infectious diseases (primarily HIV and hepatitis B and C virus) (Okuma *et al*., 2017; Finkelmeier *et al*., 2016; Frigola *et al*.,2012). This has led to paucity of scientific evidence of sPD-L1 in other common endemic infectious diseases in Ghana and other Sub-Saharan African countries.

Studies investigating infectious diseases have demonstrated that some pathogens exploit elevated levels of PD-L1 to evade immune response (Wykes & Lewin, 2018). Therefore, understanding the function of sPD-L1 is critical but, unfortunately, not known in most endemic infections. Suggesting the need for more studies to explore and understand the possible association between the PD-1/PD-L1 pathway and other immune checkpoint molecules in common endemic infections in our part of the world.

Also, despite reports of a correlation between upregulated PD-L1 levels and poor clinical response, these studies fail to show any association between the levels of the circulating form of PD-L1 (sPD-L1) in bodily fluids like blood and urine for these diseases. Again, most immune checkpoint molecules studies used flow cytometry to investigate mPD-L1. This technique may be challenging in some Sub-Saharan African countries as it is expensive, requires trained professionals, and is laborious to use.

This study, therefore, seeks to determine the association (if any) between the expression of sPD-L1 in patients' blood and urine for the diseases caused by the selected pathogens using enzyme-linked immunosorbent assay (ELISA). Firstly, using ELISA has become an essential

technique in investigating sPD-L1. Due to economic hardship, using flow cytometry for routine laboratory diagnoses seems complicated and unrealistic many other parts of Sub-Saharan Africa. Moreover, the use of ELISA is easy and inexpensive.

Secondly, using urine as a predictive and diagnostic marker for pathogenic infections in our part of the world will require less technical support and is less invasive than blood. Hence, having a study comparing sPD-L1 in blood and urine to see if urine samples alone will be sufficient for assessing sPD-L1 would be beneficial, as urine is an easy clinical sample to obtain.

As a result, this research aims to provide an understanding of the possible involvement of exposure to the selected pathogens in the upregulation of sPD-L1 and also to determine haematological parameters associated with exposure to selected pathogens and sPD-L1 expression, as these will then provide extensive knowledge to inform decisions about the development of vaccines and immune checkpoint blockers for these infections.

1.3 Aims and Objectives

1.3.1 Aim

To investigate the expression of sPD-L1 among febrile patients exposed to selected pathogens.

1.4.2 Specific objectives

The specific objectives are:

A. To investigate the proof of exposure to common selected pathogens in the blood of patients with febrile illness (*Plasmodium* spp., *Toxoplasma gondii*, *Salmonella Typhi*, *Treponema pallidum*, HIV, hepatitis B virus, and herpes simplex virus).
B. To determine the levels of sPD-L1 in the blood and urine of patients exposed to the selected pathogens before and after treatment
C. To determine haematological parameters associated with exposure to selected pathogens and sPD-L1 expression.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Immune Checkpoint Molecules

Immune checkpoint molecules (ICMs) are negative regulatory proteins that are inducible expressed on the surface of immune cells as receptors (Cai *et al*., 2020). Under normal physiological conditions, ICMs function as co-stimulatory and co-inhibitory proteins to prevent immune response against self-antigens and prevent unnecessary immune activation and autoimmunity (Farrukh *et al*., 2021 Wang *et al*., 2015; Chaudhry and Rudensky., 2013; Waterhouse *et al*., 1995; Pardoll., 2012), by repressing expansion of auto-reactive T lymphocytes (Yearley *et al*., 2017). Signal transduction via immune checkpoint pathways can ameliorate or dampen immune response (Sharma & Allison, 2015). Signalling via the stimulatory pathway promotes, cell cycle progression, cell survival, and expansion into effector and memory cells, whereas signalling through the inhibitory pathway terminates these activities (Foks and Kuiper., 2017)

Despite the associated role of immune checkpoint molecules in maintaining immune haemostasis, they can participate in immunoregulatory mechanisms by impeding T cell function in many infectious diseases, including acute and chronic infections, as well as many malignancies under the influence of antigen-induced persistent T cell stimulation. (Cai *et al*., 2020). This leads to a phenomenon referred to as T-cell exhaustion (depletion), which is marked by elevated expression of ICMs (such as CTLA-4 and PD-1,), loss of T-cell function such as proliferation, apoptosis of the T cell, cytokine release, and cytolytic factor secretion (Muller *et al*., 2021 Salmaninejad *et al*., 2019; Wherry., 2011). Eventually, the host cannot eliminate such diseases and infections (Cai *et al*., 2020).

Several ICMs have been identified and studied for their role in infectious diseases and a variety of cancers over the past decades, including but not limited to Allison's 1995 discovery of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Krummel and Allison., 1995) and Honjo's 1992 discovery of programmed death-1 (PD-1) protein (Ishida *et al*., 1992). T-cell immunoglobulin and mucin domain-containing-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3) are two other examples of ICMs (Tang *et al*., 2019; Andrews *et al*., 2017).

There have been reports on the correlation between disease burden and upregulation of these immune inhibitory molecules such as PD-1/PD-L1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on immune cells of many diseases ranging from cancers, acute infections such as malaria, as well as during persistent viral infections, including HIV and hepatitis B virus (HBV) (Cai *et al*., 2020; Wykes and Lewin., 2018). These signalling pathways are essential for the prevention of immune-related diseases. However, they may also limit immune-mediated defence against infection (Wykes and Lewin., 2018).

The consistently shown to be upregulated levels of programmed cell death ligand 1 (PD-L1) and other immune checkpoint molecules in a variety of acute, chronic, or persistent viral infections and oncogenic viruses, raises the possibility that anti-PD-1/PD-L1 ICBs could be used to treat viral infections. Anti-PD-1/PD-L1 ICBs, on the other hand, have been tested for use in treating individuals with various viral infections in order to boost their immune system by enhancing T cell effector function (Rao *et al*., 2017).

Immune checkpoint blockade, particularly anti-PD-1/PD-L1 therapy, has become a powerful weapon in the fight against several cancers in recent years (He and Xu, 2020). However, there is still limited knowledge of these therapeutics as a treatment option for some of the selected pathogens studied in this research, including *Plasmodium* spp. and *Salmonella Typhi*, which are endemic to Ghana. Butler *et al*. (2012) demonstrated that administration of anti-PD-L1 and

lymphocyte activation gene-3 (LAG-3) rapidly clears established blood-stage *Plasmodium* infection, suggesting that checkpoint blockade during malaria could improve protective immunity.

2.2 The PD-1/PD-L1 pathway and its role in cancers

Programmed cell death protein 1 (PD-1), a transmembrane immunosuppressive receptor, possesses an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (tyrosine residues found in the cytoplasmic domain). (Salmaninejad *et al*., 2019; Chen *et al*., 2016; Zamani *et al*., 2016; Wherry & Kurachi, 2015). The PD-L1 ligand has an affinity to programmed cell death protein 1 (PD-1). Ligating PD-L1 to its receptor, PD-1 causes dephosphorylation of Zeta-chain-associated protein kinase 70 (ZAP70), which primarily functions in TCR activation via tyrosine kinase activity (Chen *et al*., 2020), resulting in the inhibition of downstream TCR signalling, which aids in the inhibition of T-cell function (Farrukh *et al*., 2021 Salmaninejad *et al*., 2019; Chen *et al*., 2016; Zamani *et al*., 2016; Wherry and Kurachi, 2015).

Under normal physiological conditions of the body, interaction of PD-L1 with its receptor (PD-1) functions in regulation, prevention of cell-mediated cellular damage and T-cell cytotoxicity, thereby playing an essential function in maintaining tolerance to auto-antigen, all of which, if out of balance, can result in autoimmunity (Farrukh *et al*., 2021). However, many cancers leverage this interaction to enhance their growth while evading the effector activity T-cells.

The discovery of the PD-1/PD-L1 signalling pathway prompted extensive research that resulted in the development of many immunotherapies such as immune checkpoint blocker (ICB) therapies with therapeutic potential in a variety of cancers. In cancer, PD-L1 can be inducibly expressed as a result of genetic amplification or activation of oncogenic pathways to inhibit anti-tumour T-cell responses. (Chen *et al*., 2019). Increased PD-L1 expression on the surface

of cancer cells is associated with shorter overall and disease-free survival in human malignancies (Pyo *et al*., 2017).

In cancers, upregulated PD-L1 expression has been associated with pro-oncogenic signal transduction pathways such as, MEK-ERK, EGFR, MAPK, and AKT-mTOR which are linked to cancer progression and immune evasion (Escors *et al*., 2018). For instance, Jiang *et al*. (2013) reported that the mitogen-activated protein kinase (MAPK) pathway was activated in v-raf murine sarcoma viral oncogene homolog B1- resistant (BRAF) melanoma cells via genetic alterations (mutations) and growth factors, leading to elevated PD-L1 levels. The AKT/PI3K signalling pathway is also involved in cancer cell PD-L1 upregulation. First, this signaling pathway is activated by genetic mutations in its negative regulators, PTEN or SHIP. (Li *et al*., 2018; Papa *et al*., 2014). Following that, AKT activates the transcription factor NF-κB, which can upregulate PD-L1 by binding to its promoter and increasing its transcription (Chen *et al*., 2016). Similar to NF-κB, increase PD-L1 expression can be induce directly by the signal transducer and activator of transcription 3 (STAT3). (Abdelhamed *et al*., 2016).

2.3 Immune checkpoint molecules in viral infections

Infections caused by viruses, particularly chronic viral infections, seriously threaten global health. Viruses use a variety of arsenal mechanisms to overcome the formidable obstacles posed by hosts to replicate (Virgin *et al*., 2009). Among these are host inhibitory receptor signalling pathways, among many others (Ong *et al*., 2016). Several studies have found that multiple immunosuppressive checkpoint proteins, including CTLA4, PD-1, TIM3, and LAG3, are upregulated on CD8+ T and CD4+ T cells in untreated HIV patients (Pardons *et al*., 2019; Tian *et al*., 2015; Day *et al*., 2006; Trautmann *et al*., 2006). Furthermore, during acute viral infection, activation of the PD-1/PD-L1 signaling pathway has been shown to regulate the level of cytotoxic CD8+ T cell activity, ensuring a successful viral clearance while preventing tissue damage (Schönrich and Raftery., 2019). T cell exhaustion during viral infection has also been

linked to the upregulation of immune checkpoint proteins such as increased expression of PD-L1 and PD-1 on T-cells. T-cells are driven into an exhausted state by repeated stimulation by antigens or, in this case, by the tight ligation of PD-L1 to its receptor (PD-1) (Salmaninejad *et al*., 2019; Pauken and Wherry., 2015). As mentioned earlier, loss of effector functions, such as less expansion and apoptosis of T-lymphocytes, a normal immune response to prevent excessive tissue damage caused by inflammation, is the hallmark of this phenotype (Salmaninejad *et al*., 2019).

2.3.1 PD-L1 regulation during viral infection

During an infection, T cells play an essential role in adaptive immunity. To prevent immune-mediated cellular damage during viral infections, activated T cells, which are integral component of the adaptive immunity, must be "turned off" once the infection has been successfully cleared. As a result, expression of PD-1 is upregulated on the surface of T-cells, allowing the immune system to suppress T-cells when needed (Schönrich and Raftery., 2019). During acute infection caused by viruses, the PD-1/PD-L1 signalling pathway regulates the extent of cellular activity of cytotoxic CD8+ T lymphocytes to make certain that it is effective enough to successfully clear viral particles but not so toxic that it causes cell-mediated tissue damage due to excessive inflammation (Schönrich and Raftery., 2019). In viral infections that lead to chronic or persistent infections, repeated stimulation of the T-cell receptor (TCR) by viral antigens promotes NFATc1-mediated increased levels of PD-1 on T-cells (Pauken and Wherry., 2015). In addition to elevated PD-1 levels, many viral infections also induce spontaneous upregulation of PD-L1.

Upregulation of programmed cell death ligand 1 (PD-L1) during viral infection allows viruses to evade immune surveillance, although it is also required to prevent an overly aggressive immune response that could cause host tissue damage (Farrukh *et al*., 2021). Viruses have evolved to evade the host's immune response and cause infection. This is achieved through

several mechanisms, including the inhibition of interferon production, the degradation of specific receptors required for immune system signalling, and inhibition of host protein synthesis (Schulz *et al*., 2016). Viruses can also regulate PD-L1 production by being recognized by Toll-like receptors (TLRs), which are pattern recognition receptors (PRRs) that include toll-like receptor 3 (TLR-3), toll-like receptor 7 (TLR7), toll-like receptor 8 (TLR8), and toll-like receptor 9 (TLR9). They all have pathogen-associated molecular patterns (PAMP) for recognizing viral particles and bacteria cells (Takeuchi & Akira, 2010). These recognitions activate the type I interferons (IFN) pathway, which includes the proteins interferon alpha (IFN-α) and interferon beta (IFN-β), both of which regulate PD-L1 (Takeuchi & Akira, 2010). Furthermore, during viral replication, viruses can promote upregulated PD-L1 production, which results in the production of anti-inflammatory cytokines like interleukin 10 (IL-10), which increases PD-L1 expression (Schönrich and Raftery., 2019).

In order to evade the immune response, studies have shown that viruses induce the production of viral-specific proteins that upregulates immune checkpoint proteins, including PD-L1, known to partake in T-cell deactivation (Schonrich and Raftery., 2019). Elevated PD-1 levels on the surface of CD8+ T-cells, as a result of PD-L1 ligation to PD-1, leads to loss of T-cell function. (Schönrich & Raftery, 2019; Wherry and Kurachi., 2015).

Viruses that cause acute infection have been shown to induce high levels of PD-L1 in immune cells and other host cells (Farrukh *et al*., 2021). In the H9N2 subtype of avian influenza viruses, upregulated mRNA PD-L1 and protein levels was observed in pulmonary microvascular endothelial cells while inhibiting immune responses mediated by T-cells and T-cell expansion (Zhang *et al*., 2020). Furthermore, increased PD-L1 levels in macrophages have been observed in viral infections having influenza A and respiratory syncytial virus (RSV) (Staples *et al*., 2015). A recent study showed that severe acute respiratory syndrome coronavirus 2 (SARS-

CoV-2) infected individuals had higher expression of PD-L1 in basophils and eosinophils, and this was linked with the severity of COVID-19 disease. (Vitte *et al.*, 2020).

In chronic or persistent infections, viral antigens constantly stimulate T-cell receptors, resulting in PD-1 upregulation on T cells (Pauken and Wherry., 2015). Upregulation of PD-1, like other co-inhibitory receptors such as CTLA-4, is linked with increased numbers of depleted T-cells (exhausted T-cells) in various viral infections (Pauken and Wherry., 2015). Recently, PD-1 expression levels on NK cells and T-cells were found to be highly upregulated in COVID-19 patients (Chiappelli *et al*., 2020; Demaria *et al*., 2020; Diao *et al*., 2020). Analysis of peripheral blood mononuclear cells from 14 COVID-19 patients and three healthy controls found that COVID-19 patients have increased levels of both PD-1 and TIM-3, as well as significantly raised serum levels of IL-6, IL-10, and TNF-α. These data imply that immunological checkpoint-mediated T-cell depletion plays a role in the severe COVID-19 symptoms (Diao *et al*., 2020).

Chronic viral infections that upregulate PD-L1 expression include the hepatitis C virus (HCV), which promotes the expression of PD-L1 in monocytes (Zhai *et al*., 2017). Also, it has been found that hepatocytes infected with HBV cause higher expression of PD-L1 in monocytes (Kakizaki *et al*., 2018). In addition, individuals with HIV express high levels of soluble PD-L1 in their plasma, which correlates with membrane-bound PD-L1 (mPD-L1) in dendritic cells (Correa-Rocha *et al*., 2018; León-Flores *et al*., 2018). In all the aforementioned examples, upregulated levels of programmed cell death ligand 1 (PD-L1) was linked with more advanced disease and worst clinical outcome compared to patients with low PD-L1 expression.

2.4 Immune checkpoint molecules in bacterial infections

Like other infectious diseases, immune checkpoint proteins have been investigated in many bacterial infections. T cell dysfunction and immune checkpoint protein such as PD-1 has been

observed in an individual with *Helicobacter pylori* infection (Wu *et al*., 2010; Das *et al*., 2006). It has been reported that *H. pylori*-induced PD-L1 expression promotes persistent infection and significantly contributes to immune evasion and cancer progression on gastric epithelial cells (Jubel *et al*., 2020). Also, upregulation of PD-1 expression in tissues and peripheral blood has been reported in humans and animals with *Mycobacterium tuberculosis* (MTB) infection (Pan *et al*., 2016). In addition, Pan et al. (2022) demonstrated that active TB patients had an increased expression CD4+ T cells and PD-L1 on CD14+ monocytes. The higher expression of PD-L1 on CD14+ monocytes was associated with higher bacterial burden and worse treatment responses. Furthermore, PD-1 and its ligand PD-L1 were found significantly upregulated on CD4+ T cells and antigen presenting cell subsets, respectively in Lyme disease caused by *Borrelia burgdorferi* (Helble *et al*., 2022).

2.5 Immune checkpoint molecules in parasitic infections

T-cell dysfunction has also been reported during parasitic protozoan infections. This is attributed to the parasite's ability to evade immune surveillance by promoting the production of anti-inflammatory molecules such as transforming growth factor beta (TGF-β) and IL-10 during chronic parasite infection. Furthermore, studies have demonstrated that infections caused by parasites are linked with higher expression of co-inhibitory receptors and their ligands (Dyck and Mills., 2017). Studies have reported PD-1 expression during chronic infections caused by parasites including *Toxoplasma gondii*, *Leishmania major*, and *Plasmodium falciparum* (Habib *et al*., 2018; Fonseca *et al*., 2018). The study of immune checkpoint proteins has become a new focus in developing vaccines to control parasitic infections such as malaria over the past few years (Wykes and Lewin., 2018). Several field studies in Mali and Kenya have found that individuals recently infected with *Plasmodium* falciparum expressed PD-1 on CD4+ (Illingworth *et al*., 2013; Butler *et al*., 2012) and CD8+ T lymphocytes, implying that PD-1 is involved in the parasite's immune evasion. Also, in an

experimental study using mice infected with *T. gondii*, the rodents developed persistent infections and CD8+ T cell exhaustion (Bhadra *et al*., 2011).

2.6 Programmed cell death ligand 1(PD-L1)

Programmed Cell Death Ligand-1 (PD-L1) (also known as CD274 or B7-H1) is a transmembrane immunoinhibitory checkpoint ligand belonging to the B7 family of immune regulatory molecules (Ceeraz *et al*., 2013). Programmed cell death ligand 1 (PD-L1) is one among the two ligands of programmed cell death protein 1(PD-1); the second ligand is programmed cell death ligand-2 (PD-L2) (Hotchkiss *et al*., 2013).

Although PD-L1 is expressed on cancer cells, it is also constitutively expressed on all hematopoietic cells, such as activated T-cells and B-cells, dendritic cells (DCs), monocytes and macrophages, occasionally on some non-hematopoietic cell types such as endothelial and parenchyma/epithelial cells (Bailly *et al*., 2021; Sharpe & Pauken, 2018; Keir *et al*., 2007), as well as pathogenic neoplastic cells (Niedźwiedzka-Rystwej *et al*., 2022). Contrary, PD-L2 is only found on the surface of non-hematopoietic cells and professional antigen-presenting cells. (APCs) (Schönrich and Raftery, 2019). The expression of PD-L1 is generally low under normal physiological conditions and upregulated during inflammatory stimulation (Yearley *et al*., 2017).

The gene encoding for PD-L1 expression is the PDCDL1 (also known as CD274) gene (gene ID: 29,126) located at p24.1on the human chromosome 9 (Gao & Chen, 2021). Amplification of the PDCDL1 gene has been linked to increased PD-L1 levels in various cancers (Wang *et al*., 2016). Furthermore, increased PD-L1 levels in various cancers have also been associated with higher transcriptional factors such as s STAT3 and HIF-1α (Noman *et al*., 2014; Marzec *et al*., 2008). In addition, various biological and immunological alterations that occur during acute and chronic pathologies also influence the expression of PD-L1(Hoan *et al*., 2022).

Human type I interferons (IFNs), including several IFN- α subtypes and IFN- β, have also been shown in studies to partake in increased PD-L1 expression (Garcia-Diaz *et al*., 2017). In addition, other cytokines such as IFN-γ, TNF-α, IL-17, IL-27, IL-10, IL-4, IL-2, and IL-10 have also been linked to PD-L1 induction (Raftery *et al*., 2018; Garcia-Diaz *et al*., 2017). T-cell secretion of these cytokines and interferons activates the JAK-STAT pathway, resulting in transcription factor IRF1 binding to the PD-L1 promoter (Escors *et al*., 2018).

2.7 Soluble PD-L1 (sPD-L1)

PD-L1 exists in various forms in the body, such as membrane-bound (mPD-L1), exosomes (exoPD-L1), cell nuclei (nPD-L1), and as a circulating soluble protein in plasma (sPD-L1) (Bailly *et al*., 2021). Tumours and mature haematopoietic cells, such as activated mature dendritic cells, can produce and release soluble PD-L1, but not immature dendritic cells, macrophages, monocytes, or T cells (Vikerfors *et al*., 2021). Membrane-bound PD-L1 (mPD-L1) is inducible on activated T and B lymphocytes, macrophages, professional antigen-presenting dendritic cells (APCs), monocytes, and on the surface of many cancer cells (Bailly *et al*., 2021). Exosomal PD-L1, on the other hand, can be expressed on the surface exosomes released as extracellular vesicles from many malignant tumours. (Bailly *et al*., 2021).

The circulating soluble PD-L1 (sPD-L1) is a product resulting from cleavage of portion of membrane-bound PD-L1 (mPD-L1) on the surface of haematopoietic and non-haematopoietic cells by metalloproteinase enzymes, including metalloproteinases (MMP) and A disintegrin, as well as metalloproteases (ADAM) (Bailly *et al*., 2021; Chen *et al*., 2011), or by translation of alternate splicing of membrane-bound PD-L1 (messenger RNAs) (Bailly *et al*., 2021; Orme *et al*., 2020; Zhou *et al*., 2017; Chen *et al*., 2011), or through the tumour-derived extracellular vehicles (EVs) bearing mPD-L1 (Daassi *et al*., 2020; Poggio *et al*., 2019; Chen *et al*., 2018). In addition, soluble PD-L1 can bind to PD-1 after being released into blood plasma and thus

could suppress T cell function by inhibiting T cell activation and proliferation (Frigola *et al*., 2012).

Soluble PD-L1 was first detected in human serum or plasma using an enzyme-linked immunosorbent assay (ELISA), a less invasive and more convenient clinical specimen than biopsy tissue (Ding *et al*., 2017). Furthermore, it was detected in cancer patients' urine samples, urine is an easy clinical sample to obtain, and collection is less invasive than blood. Studies have shown that sPD-L1 is detectable and quantifiable in patients' plasma, serum, and urine during various diseases (Vikerfors *et al*., 2021).

2.8 Haematological parameters associated with febrile illnesses.

Disease conditions, including endemic infections, can cause changes in haematological parameters (Kotepui *et al*., 2014; Maina *et al*., 2010). Changes in total blood cell count are one of the most frequently encountered complications in febrile illnesses such as malaria, and they play an essential role in the pathogenesis of the disease (Kotepui *et al*., 2014). These changes affect RBCs, leucocytes, and thrombocytes (Bakhubaira, 2013; van Wolfswinkel *et al*., 2013; Warimwe *et al*., 2013). In malaria patients, platelets, WBCs, lymphocytes, eosinophils, RBCs, and Hb levels appear significantly lower, whereas monocyte and neutrophil counts are significantly higher compared to non-malaria infected patients (Bakhubaira, 2013; van Wolfswinkel *et al*., 2013). However, these alterations depend on the degree of malaria endemicity, background haemoglobinopathy, nutritional status, demographic factors, and malaria immunity (Erhart *et al*., 2004; Price *et al*., 2001; Wickramasinghe & Abdalla, 2000). Thrombocytopenia is the most common complication of malaria infection (Khan *et al*., 2012; Erhart *et al*., 2004). According to Srivastava *et al*. (2011), thrombocytopenia may occur as a result of platelet sequestration, splenic platelet pooling, antibody-mediated platelet destruction, and adenosine diphosphate (ADP) release after parasitized RBC haemolysis, dysmegakaryopoiesis, platelet aggregation and activation, platelet invasion by parasites,

platelet phagocytosis, platelet adhesion to erythrocytes, and oxidative stress. In Ghana, patients with malaria are also found to have thrombocytopenia and other haematological changes, such as leukopenia and anaemia. (Anabire *et al*., 2018; Awoke and Arota, 2019).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

This was a longitudinal study involving baseline patients with febrile illness, and post-treatment follow-up on cohorts after 28 days post-treatment, as well as healthy donors who served as controls. Individuals reporting to the Ledzokuku-Krowor Municipal Assembly (LEKMA) hospital were among those who took part in the study. Each patient's blood and urine samples were collected using standard ethical procedure before and after treatment. The blood samples were screened for exposure to *Plasmodium* infection, hepatitis B virus infection (HBV), HIV-1&2, herpes simplex virus (1 and 2), *Salmonella Typhi*, *Treponema pallidum*, and *Toxoplasma gondii*. Patients were followed up after four weeks (28 days) after treatment, and their blood and urine samples were collected. The blood samples were again screened to ensure baseline pathogen specific antigens and immunoglobulins were cleared. Plasma was separated from the blood samples, aliquoted and stored at ≤ -20 °C until sPD-L1 detection. The urine samples were also centrifuged to remove particulates, aliquoted and stored at ≤ -20 °C for sPD-L1 detection.

3.2 Study site and population

The study included consented patients with febrile illness from the Ledzokuku-Krowor Municipal Assembly (LEKMA) hospital in Teshie, Accra. The LEKMA hospital is a 100-bed capacity government health facility built in 2010. It is district hospital located at the Ledzokuku Municipal and serves as the main referral hospital stretching from Lashibi in the Tema West Municipality to La in the La Dade-Kotopon Municipality. Individuals accessing the facility are from different ethnic, geographical, and socio-economic background. LEKMA hospital was chosen because previous records shows that the hospital receives a high number of febrile

illnesses, on which the study will concentrated, and it is a research center for malaria and other diseases.

KEY: ⊕

Study site

Figure 3. 1: Map of Teshie, Accra-Ghana, showing LEKMA hospital

Adapted from:

(https://www.google.com/search?q=map+of+teshie&oq=map&aqs=chrome.1.69i57j69i59l2j69i60l2j69i65j69i60l2.5135j0j7&sourceid=chrome&ie=UTF-8#)

3.2.1 Case definition

The case definition for febrile illness is a patient (with no age restriction) who presented with a fever hof temperature ≥38°C without a localizing etiology (i.e. otitis media, meningitis)

3.2.2 Inclusion criteria

The inclusion criteria are:

- Patients having a documented fever of ≥38°C at the time of visiting the hospital

- Febrile patients must test positive for either malaria (RDT positive for *Plasmodium* histidine-rich protein 2 (pHRP-II) and *Plasmodium* lactate dehydrogenase (pLDH), and presence of *Plasmodium* parasites determined my microscopy) or typhoid immunoglobulin at the time of pre-treatment screening
- The controls were be healthy donors, as well as patient that have documented fever with body temperature of ≥38°C who had tested negative for all the selected screened pathogens.
- Who has agreed to participate to the study

3.2.3 Exclusion criteria

- Patients who had been on antimicrobial therapy for two weeks prior to clinical sampling.
- Patients with a clearly defined localized source of infection.

Participants who met the febrile illness case definition provided informed consent (or parental consent and child assent if under the age of 18 years). Afterwards, questionnaires were used to capture pertinent data such as social and demographic information. Subsequently, their laboratory specimens including peripheral blood and urine was collected from each patient.

3.2.4 Sample size

Sample size is 400, calculated from $n = Z^2 P (1-P) / d^2$ (Cochran, 1977), based on the individual prevalence of the selected pathogens to be investigated.

Where n is the sample size, Z is the statistic corresponding to the level of confidence 95% =1.96, and d is precision =5%

A prevalence of 1.6% for HIV (Ali *et al*., 2019), 20.63% for *Plasmodium* infection (Ejigu and Wencheko., 2021), 74.37% for *Toxoplasma* infection (Pappoe *et al*., 2017), 3.2% for *Treponema* infection (Banong-le *et al*., 2019), and 99.2% seroprevalence for herpes simplex

infection (Debrah *et al*., 2018) was used for the calculation of each pathogen. Based on this individual prevalence, the minimum sample size for HIV is 24, that of *Plasmodium* infection is 251, *Toxoplasma* infection is 292, 48 for *Treponema* infection, and 12 for herpes simplex infection. To ensure that the minimum size of each selected pathogen was covered, the highest minimum sample size (292 for *Toxoplasma* infection) of the calculated individual minimum sample size was adapted and further increased to 400.

Overall, a total of 50 participants were enrolled out of the 94 positive cases from the screening of 1200 patients.

3.3 Overview of study procedure

A total of 1200 patients reporting to the out-patient department (OPD), paediatric and adult emergency unit of LEKMA hospital with febrile illness were illegible for participation in the study and were screened for exposure to the selected pathogens. Individuals were given permission to participate in the study after reporting to the hospital. Consenting individuals were given consent forms to fill out with the help of the researcher. A child assent form with additional parental/guardian consent was administered to participants under the age of eighteen. Following that, participants were given questionnaires to collect basic demographic information. To ensure confidentiality, participants were assigned unique study numbers. The questionnaire contained no names or identifiers that could be used to link participants to data. Blood and urine samples were aseptically collected before treatment and four weeks (28 days) after treatment and processed within an hour. In this study, exposure to selected pathogens were assessed by screening using antigen-based and antibody-based point of care rapid diagnostic tests (POC-RDTs) to detect antigens or immunoglobulins selected pathogens. Plasma was separated from the blood samples, aliquoted and stored at ≤ -20 °C until sPD-L1 detection. The urine samples were centrifuged to remove particulates, aliquoted and stored at

≤ -20 °C for detection of sPD-L1. Expression of sPD-L1 was evaluated using the PD-L1/B7-H1 Quantikine PD-L1 immunoassay ELISA kits.

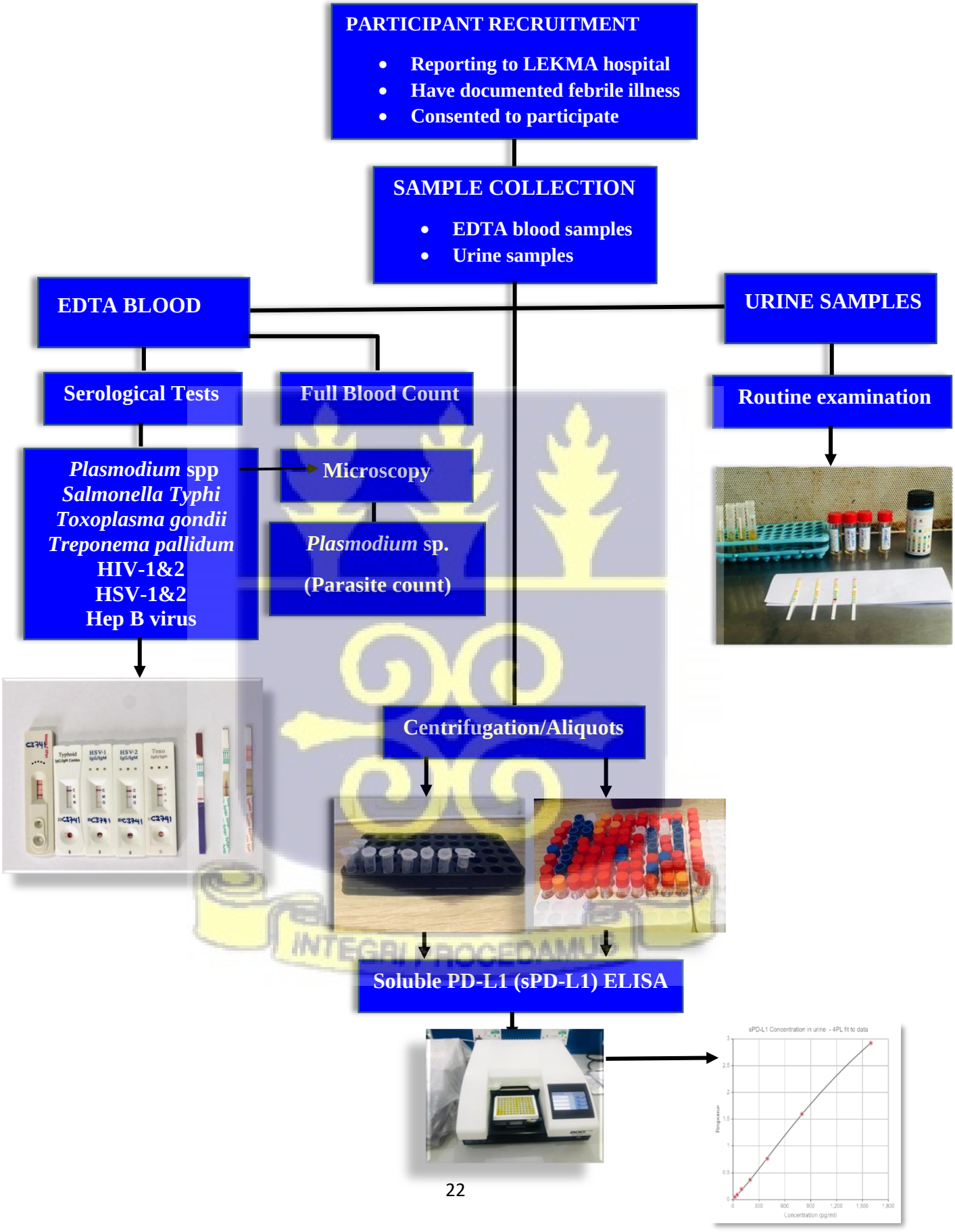


Figure 3. 2: Flowchart outlining research methodology

3.4 Sample collection and storage

3.4.1 Blood sample collection

Following informed consent from study participants/guardians (for children), approximately 5ml of whole blood sample was collected aseptically from the participants into ethylenediaminetetraacetic acid (EDTA) tubes via venipuncture sampling. Following that, 2ml of the collected blood was transferred into another EDTA tube for laboratory analysis. The remaining 3ml were centrifuged at 1000 x g for 15 minutes within 30 minutes to separate the plasma from the sample. Each plasma sample will be aliquot into a sterile 1.8ml polypropylene tube and stored at ≤ -20 °C.

3.4.2 Urine sample collection

Each study participant's first urine of the day (mid-stream) was also aseptically collected into sterile containers. The urine samples were centrifuged to remove particulate matter before being assayed immediately or aliquoted and stored at ≤ -20 °C for analysis.

3.5 Laboratory procedures

3.5.1 Full blood count analysis

A sample roller was used to mix two to three millilitres (2-3 ml) of whole blood collected into an EDTA tube with the anticoagulant for one minute. The blood sample was then analyzed using a Sysmex Hematological analyzer, which uses the flow cytometry principle to provide vital information about the types and numbers of blood cells (white blood cells, red blood cells, and platelets) to aid in the detection of any abnormalities. The blood was aspirated and run through the hematological analyzer within few minutes of collecting the sample. The full blood

count printed data was used to extract the white blood cells, red blood cells, platelets count, lymphocytes, neutrophils, eosinophils, basophils, and monocytes data.

3.5.2 Confirmation of exposure to selected pathogens

Blood samples were screened for human immunodeficiency virus (HIV-1&2), herpes simplex virus 1&2 (HSV-1&2), *Salmonella Typhi*, *Treponema pallidum* (for syphilis), and *Toxoplasma gondii* and *Plasmodium spp* exposure. The screening was accomplished using antigen-based and antibody-based point of care rapid diagnostic test (POC-RDTs) to detect pathogen-specific antigens/antibodies in both patients and healthy donors' blood.

3.5.3 Malaria Rapid Diagnostic Test

Aria malaria (Pf & PAN) (CTK Biotech, Inc., USA) immunological-based RDT was used for screening malaria parasites according to the manufacturer's protocol. The test was performed by adding 5µl of EDTA blood from the patients into the sample well of the kit, followed by two drops (50-100µl) of blood lysis buffer to the buffer well. The kit was then incubated at room temperature for 15 minutes, and the results were read immediately. Two or three purple-coloured bands identified the positive samples. One on the control region and either one or two on the test region (either for Pf or PAN or both). Only the PAN line on the test kit indicates the presence of pLDH, and the result is *Plasmodium falciparum* negative. If only the Pf line develops, the test indicates the presence of pHRP-II, and the result is *P. falciparum* positive. If both PAN and Pf develop, the test indicates the presence of both pLDH and pHRP-II. The results are *P. falciparum* positive and may also be positive for *P falciparum* and any of the three Plasmodium species (*P. ovale*, *P. vivax*, and *P. malariae*) the negative samples had one purple band, which was in the control region.

3.5.3.1 Malaria Blood Thick Film

A drop of 6µl of the patient's blood was placed onto a pre-cleaned and labelled glass slide, and the corner of another glass slide was used to prepare the blood smear in a circular pattern, which was allowed to dry at room temperature. The blood smear was stained with a 10% Giemsa stain and left to dry for 10 minutes before being washed. The slide was allowed to dry, followed by adding one drop of immersion oil onto the smear and immediately examining it under a light microscope with ×100 objective lens to look for any *Plasmodium* parasites.

The entire smear was first examined under low magnification (×10 and ×40 objective lens) to detect suitable fields before being examined under ×100 objective lens (oil immersion). Before a smear was declared positive or negative, at least 100 fields were examined at high magnification.

The density of *Plasmodium* parasites was determined for each malaria-positive slide by counting 200 white blood cells (including neutrophils, eosinophils, lymphocytes, and basophils) with their corresponding *Plasmodium* parasites from each of the 100 fields using tally counters. *Plasmodium* parasites were counted per 200 leucocytes to calculate the parasite density.

3.5.4 *Toxoplasma* Rapid Diagnostic Test

Toxoplasma gondii was detected using an immunologically based kit Aria *Toxoplasma* IgG/IgM (CTK Biotech, USA), as directed by the manufacturer. In brief, 10µl of plasma was placed in the sample well, followed by two drops (60-80 µL) of the buffer. It was incubated at room temperature for 10 minutes before the results were read. Only IgM or IgG-positive samples had two purple bands on the test (IgM or IgG) and the control region. Positive IgM and IgG samples had three purple bands on tests (IgM and IgG) and control regions. In the control region, the negative samples had only one band.

3.5.5 HIV and Herpes Simplex Virus Rapid Diagnostic Test

The Aria HIV -1 & 2 kits (CTK Biotech, USA) was used for HIV testing. It is an immunoassay-based kit. The procedure was carried out per the instructions provided by the kit's manufacturer. The screening was accomplished by placing 20 µl of blood sample in the sample well and adding two drops (60-80 µl) of the buffer. The result was read immediately after 15 minutes of incubation at room temperature. The positive sample was expected to have two or three purple bands, one in the control region and another(s) in the test region (one each for HIV-1, HIV-2, or both). In the control region, negative samples had only one band.

The Aria HSV-1 IgM/IgG and Aria HSV-2 IgM/IgG test kits were used for immunological herpes virus screening (CTK Biotech, Inc., USA). The testing was carried out following the manufacturer's specifications. During the test, 10µl of blood was placed in the sample well, and two drops (60-80 µl) of buffer were immediately added. Then, it was incubated at room temperature for 10 minutes before being read immediately. The positive sample showed two or three purple bands, one in the control region and the other(s) in the test region (one each for IgG, IgM, or both). In the control region, negative samples had only one band.

3.5.6 Typhoid Rapid Diagnostic Test

The Aria Typhoid IgG/IgM Combo Rapid Test (CTK Biotech, USA) was used to screen for exposure to *S. Typhi.* The test was carried out by adding a drop of EDTA blood (40-50µl) from the patients into the sample well, followed by a drop (about 35-50 µl) of sample diluent to the center of the well. This was followed by ten minutes of incubation at room temperature before being read immediately. The positive sample showed two or three purple bands, one in the control region and the other(s) in the test region (one each for IgG, IgM, or both). In the control region, negative samples had only one band.

3.5.6 Hepatitis B virus Rapid Diagnostic test

The Wondfo One Step HBsAg immunochromatographic test trip was used to screen for hepatitis B virus exposure (HBV). The test was performed by adding approximately 60µl to 80µl of plasma from whole blood onto the test trip's sample pad, which absorbs the plasma into the test trip via capillary action. After incubating the kit at room temperature for 15 minutes, the results were read immediately. Rose-pink bands appear in the control and test regions for positive results. Positive results indicate that the concentration of HBsAg is equal to or greater than the test's detection limit. A negative result is indicated by a rose-pink band in only the control region and no coloured band in the test region. A negative result indicates that the HBsAg level is zero or lower than the test's detection limit.

3.5.7 Syphilis Rapid Diagnostic test

The Wondfo One Step Syphilis double antigen sandwich immunoassay-based test trip was used to screen for Treponema pallidum exposure, by determination of *T. pallidum* antibodies in plasma samples. The test was performed by adding approximately 10µl of plasma from whole blood onto the test trip's sample pad, which absorbs the plasma into the test trip via capillary action. After incubating the kit at room temperature for 15 minutes, the results were read immediately. Visible coloured bands in the control and test regions indicate a positive result for *T. pallidum* antibodies. A coloured band visible only in the control region indicates a negative outcome. A negative result indicates that the concentration of *T. pallidum* antibodies in the test sample is zero or less than the test's detection limit.

3.6 Detection of soluble sPD-L1 (sPD-L1)

Plasma was separated from the remaining 3 ml of whole blood by centrifugation at 1000 rmp for 15 min at 4°C and stored at -20°C until analysis. In addition, urine samples were also centrifuged at 1000 rpm for 15 minutes (min) at 4°C and immediately aliquoted and stored at −20°C until analysis. The concentration of plasma and urine sPD-L1 was measured using a commercial ELISA kit following the manufacturer´s instructions (USA R&D Systems, Inc.).

The optical density (OD) of each well was measured spectrophotometrically using a plate reader at a wavelength of 450 nm. Every sample was tested in duplicates, and a standard curve is used to determine sPD-L1 levels. The average (mean) was found for each well's duplicate OD rea. In addition, means of absorbance versus concentration were plotted, and a four-parameter nonlinear logistic (4PL) regression model was fitted using an online platform, and R2 values greater than 0.9 were considered acceptable.

3.6.1 Principle of PD-L1/B7-H1 Quantikine ELISA

The PD-L1/B7-H1 Quantikine ELISA employs that quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human B7-H1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any B7-H1 present bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for human B7-H1is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of B7-H1 bound in the initial step. The colour development is stopped and the intensity of the colour is measured.

3.*6.2* Procedure for PD-L1/B7-H1 Quantikine ELISA assay

The stock reagents and working standards were reconstituted according to the manufacturer's guidelines. Afterwards, human plasma and urine samples stored at ≤ -20 °C were removed and partially thawed by briefly placing them in a water bath at 37 °C while gently mixing them. After partially thawing the samples, they were placed in an ice bath until they were completely thawed. All reagents and samples were brought to room temperature before use. The procedure of assay was performed according to the manufacturer's instructions.

3.6.2.1 Dilution of test samples (blood and urine), positive control, and negative control

50µL of Assay Diluent RD1-41 was added to each well containing serum sample, and Assay Diluent RD1-54 was added to wells containing urine samples based on the manufacturers' instructions.

3.6.2.2 Running and incubation of samples

After appropriately labelling the test kit, 100µL of standard, control, and test samples were added to each well and covered with the adhesive strip. The addition of standards, control, and test samples was followed by incubation for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. The manufacturer provided a plate layout to record standards and samples assayed.

3.6.2.3 Washing wells after incubation

The wells were emptied after incubation and washed four times with 400 µL Wash Buffer by filling and emptying the wells at each step. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. Afterwards, the plate was invented and blotted against clean paper towels.

3.62.4 Addition of conjugate, washing, and addition of substrate

To each well, 200 µL of Human/Cynomolgus Monkey B7-H1 Conjugate was added and covered with a new adhesive strip. Followed by incubation for 2 hours at room temperature on a shaker. After incubation, the wells were washed following the steps described in step C. Then 200 µL of Substrate Solution was added to each well and incubated for 30 minutes at room temperature. Finally, 50 µL of Stop Solution was added to each well to bring the reaction to an end.

Figure 3. 3: A picture showing the sPD-L1 ELISA kit.

From A to K

- A (B1-H1 sPD-L1 kit)
- B (B1-H1 Conjugate)
- C (Assay Diluent RD1-54, for urine samples)
- D (Assay Diluent RD1-54, for plasma samples)
- E (Calibrator Diluent RD5-33)
- F (Colour Reagent A)
- G (Colour Reagent B)
- H (Stop Solution)
- I (Wash Buffer)
- J and K (B1-H1 Standards

3.7 Data analysis

This study's data was collected into data sheets and a notebook and later entered, stored and managed in Microsoft Excel, 2016, and analyzed with the Statistical Package for Social Science (SPSS) version 25 (SPSS Inc., Chicago, IL, USA). The primary tool used to analyze the data collected was descriptive statistics. Tables and graphical displays were used to summarize data, including frequency and mean. A test for normality using the Shapiro-Wilk test showed non-normal distributions, so non-parametric analyses were done for all datasets. Mann-Whitney U Test, Wilcoxson Signed Rank test, and Kruskal Wallis Test with Dunn's post-hoc test was used to determine the significant differences in the median of different groups. Spearman's correlation was employed to determine the association between continuous variables and categorical variables, as well as continuous variables versus continuous variables. The significance level for all statistical analyses was set at a 95% confidence interval ($p < .05$).

3.8 Ethical consideration

Ethical approval was obtained from the Ethical and Protocol Review Committee at the University of Ghana's College of Health Sciences, with protocol identification number: ***CHS-ET/M.9-P 5.6/2021-2022*** **(APPENDIX 6)**. Permission was also obtained from the hospital from which the clinical samples were collected. All patients provided written informed consent to participate in the study. The decision to participate or not to participate was entirely voluntary, and there was no penalty for refusing to participate. Each study participant was free to leave at any time. The study participants' rights and confidentiality were treated with the utmost care during the data collection and handling process. All study-related data was saved on a secure, password-protected computer. Records and data were kept secure in password-protected files to protect the privacy of those who voluntarily agreed to participate. Study participants' identities were not associated with results or findings in any published format.

CHAPTER FOUR

4.0 RESULTS

4.1 Screening result of Study Population

Out of a total of 1200 patients screened, 91 tested positive for either malaria only (n=73), typhoid immunoglobulins (n=6), or both malaria and typhoid immunoglobulins (n=12) (**Table 4.1 and Table 4.2**). Forty-five (45) out of the 91 positive participants were eventually enrolled in the following proportions; 32 being malaria positives patients, eight (8) malaria with typhoid immunoglobulin positive patients, and five typhoid immunoglobulin positive cases. In addition, five (5) controls (3 healthy donors and two febrile cases which were negative for the selected pathogens) were added, making a total of 50 enrolled participants (**Fig 4.1A**).

4.1.1 Malaria status among the study population

In general, 85 out of the 1200 screened patients tested positive for malaria, and a 7.1% (85/1200) prevalence rate was estimated by the malaria RDT test.

4.1.2 Seroprevalence of *S. Typhi* among the study population

In total, 18 out of the 1200 patients tested seropositive to *S. Typhi* immunoglobulins (IgM & IgM) by rapid diagnostic kit (RDT). Seropositive cases were identified by the presence of IgM, IgG or both IgM & IgG antibodies. Therefore, a 1.5% (18/1200) prevalence rate was estimated by Typhoid IgG/IgM Combo Rapid Test. Twelve of the 18 seropositive patients had typhoid IgM, three had IgG, and three had both IgG and IgM (**Table 4.2**).

4.1.3 Prevalence of syphilis among the study population

Generally, only two out of the 91 patients who tested positive of the 1200, tested positive for *T. pallidum* (**Table 4.1**), yielding a prevalence rate of 2.2% (2/91) using syphilis RDT.

4.1.4 Prevalence of hepatitis B viral infection among the study population

Only one out of the 91 patients who tested positive of the 1200 febrile patients tested positive for hepatitis B viral infection by hepatitis b RDT (**Table 4.1**), yielding a prevalence rate of 0.01% (1/91).

4.1.5 Prevalence of HIV-1&2 among the study population

A prevalence rate of 0.01% (1/91) was recorded for HIV-1 by RDT among the 91 positive patients.

4.1.6 Seroprevalence of HSV-1&2 among the study population

A total of 48 patients out of the 91 patients who tested positive of the 1200 screened tested positive for HSV-1 immunoglobins by HSV-1 rapid diagnostic kit (RDT). These included 13 patients who were positive for IgM, 21 patients who were positive for IgG and 14 patients who were positive for both IgM and IgG (**Table 4.2**). A prevalence rate of 52.75% (48/91) was calculated.

Forty-one (41) out of the 91 patients who tested positive of the 1200 febrile patients who were screened tested positive for HSV-2 immunoglobins by HSV-2 rapid diagnostic kit (RDT). Out of these, 26 patients were positive for IgM, 12 patients were positive for IgG, and the remaining three positive patients tested positive for both IgG and IgM (**Table 4.2**). In general, a prevalence rate of 45.05% (41/91) was estimated by HSV-2 RDT.

4.1.7 Seroprevalence of *T. gondii* among the study population

Ten (10) out of the 91 patients who tested positive of the 1200 febrile patients who were screened tested seropositive for *T. gondii* IgG by rapid diagnostic kit (**Table4.2**), yielding a prevalence rate of 11% (10/91).

Table 4. 1Table 4.1: Results on Exposure to Selected Pathogens by antigen testing

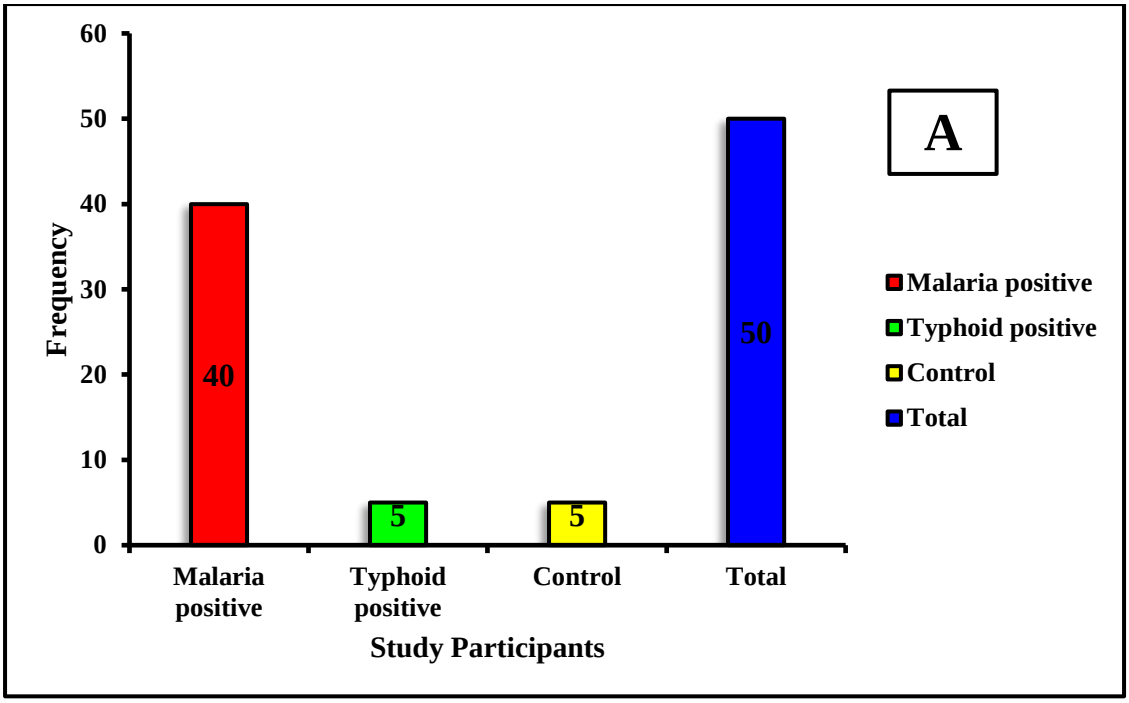
Test	Results (N)
***Plasmodium* sp**	85
Hepatitis B virus	1
T. pallidum	2

N= number of positives.

Table 4. 2: Results on Exposure to Selected Pathogens by antibody testing

Test	Results N (%)		IgM (%)	IgG (%)	IgG & IgM (%)
	Positives	Negatives			
S. Typhi	18 (19.8)	73 (80.2)	12 (13.2)	3 (3.3)	3 (3.3)
HIV-1&2	1 (1.1)	90 (98.9)	NA	NA	NA
HSV-1	48 (52.7)	43 (47.3)	13 (14.3)	21 (23.1)	14 (15.4)
HSV-2	41 (45.1)	50 (54.9	26 (28.6)	12 (13.2)	3 (3.3)
T. gondii	10 (11)	81 (89)	NA	10 (89)	NA

NA= Not applicable; **N**= number of positives.



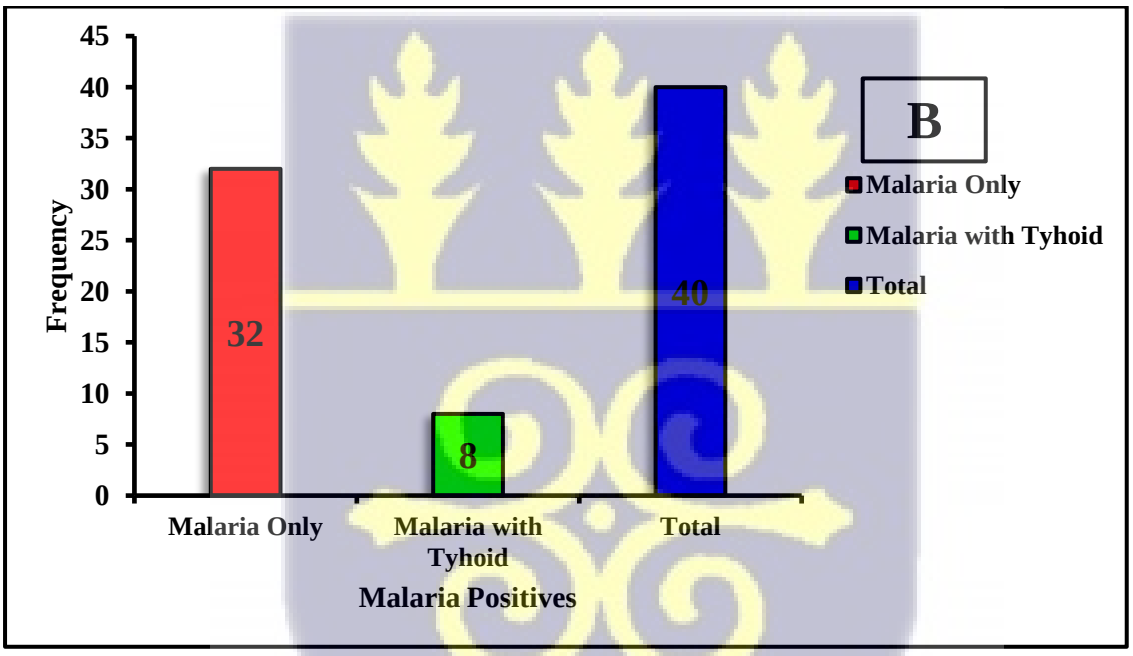


Figure 4. 1: Types of exposure among malaria positive patients.

4.2 Socio-demographic characteristics of positive participants and enrolled participants

There were males 51/91 and females 40/91 among the positive patients, representing 56% and 44% respectively. Majority of the positive patients fall within age range of 9 years and below (n = 25/91; 27.5%), followed by 10-19 (n = 18/91; 19.8%) and 20-29 (n= 18/91; 19.8%). Most of the positive patients were Akan (n=36; 39.6), followed by Ga/Dangme (n=30; 33%). Most had up to Senior High School education (n=25; 27.5%). Majority were students (n=35; 38.5%), followed by traders (n=16; 17.6%)

The detailed socio-demographic characteristics of the enrolled participants is summarized in **Table 4.3**. There were 31 (62%) males and 19 (38%) females among the enrolled participants. Ages of enrolled participants ranged from <1 year to >70 years, with the highest enrollment age group being 20-29 years (24%). Most of the study participants [n=19 (38%)] were students, followed by traders [n=10 (20%)], with government workers [n=1 (2%)] been the least. Among the students, 16 (32%) had at least up to Senior High School education. Akans [n=20 (40%)] were the most common ethnic group among study participants, when compared to other ethnic groups, Akans have a higher population distribution in Southern Ghana.

The clinical symptoms reported by study participants (enrolled febrile patients) are summarized in **Figure 4.2**. Fever is the most prevalent clinical symptom in the study group [n=45 (100%)], followed by chills [n=30 (66.7%)], headache, and vomiting (55.6% and 46.7%, respectively).

Table 4. 3: Demographic Characteristics of Enrolled Participants

Variable		Frequency	Percentage (%)
Gender			
	Male	31	62.0
	Female	19	38.0
Age Group			
	≤ 9	8	16.0
	10-19	10	20.0
	20-29	12	24.0
	30-39	7	14.0
	40-49	7	14.0
	≥ 50	6	12.0
Ethnic Group			
	Akan	20	40.0
	Ga/Dangme	14	28.0
	Ewe	8	16.0
	Northerner	8	16.0
Education Level			
	Primary	6	12.0
	JHS	11	22.0
	SHS	16	32.0
	Tertiary	11	22.0
	No Education	2	4.0
	Other	4	8.0
Occupation			
	Student	19	38.0
	Trader	10	20.0
	Government Worker	1	2
	Private Company Worker	8	16
	Unemployed	2	4
	Other	10	20

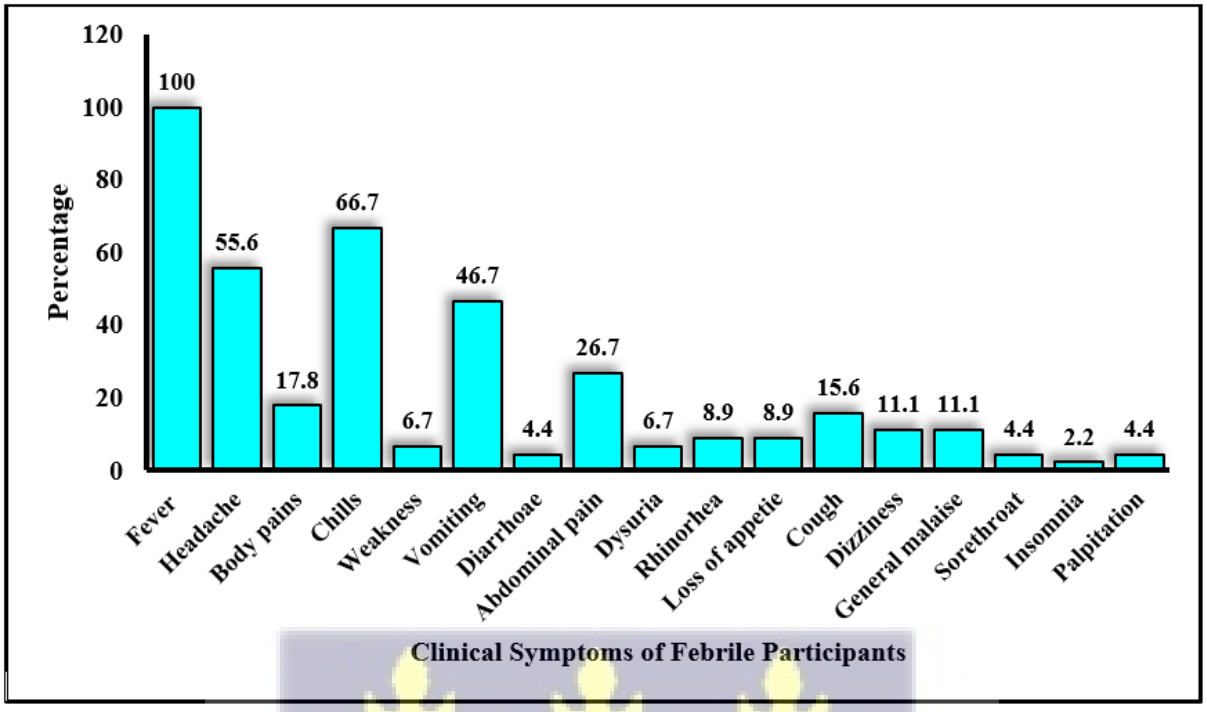


Figure 4. 2: Symptoms reported by study participants

4.3 Proof of exposure to *S. Typhi* among enrolled participants

Figure 4.3; Table 4.4 summarizes the result of seroprevalence of *S. Typhi* among the enrolled participants. Thirteen (26%) out of the 50 study participants were seropositive to *S. Typhi* immunoglobulins (IgM & IgM) by rapid diagnostic kit (RDT). Seropositive cases were identified by the presence of IgM, IgG or both IgM & IgG antibodies. Among the study participants with malaria and typhoid, there were 7 (14%) IgM and 1(2%) IgG positive. On the other hand, study participants who had only typhoid recorded only 3 (6%) IgM positives and 2 (4%) IgM & IgG positives with no IgG positive.

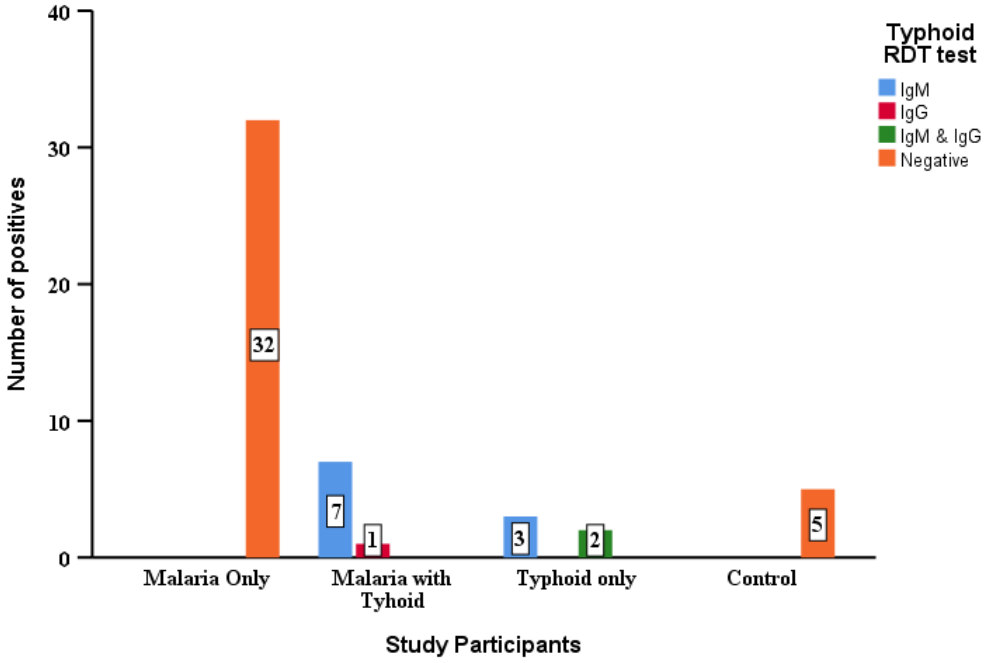


Figure 4. 3: Seroprevalence of *S. Typhi* among study participants.

Table 4. 4: Overall exposure of *S. Typhi* among enrolled participants.

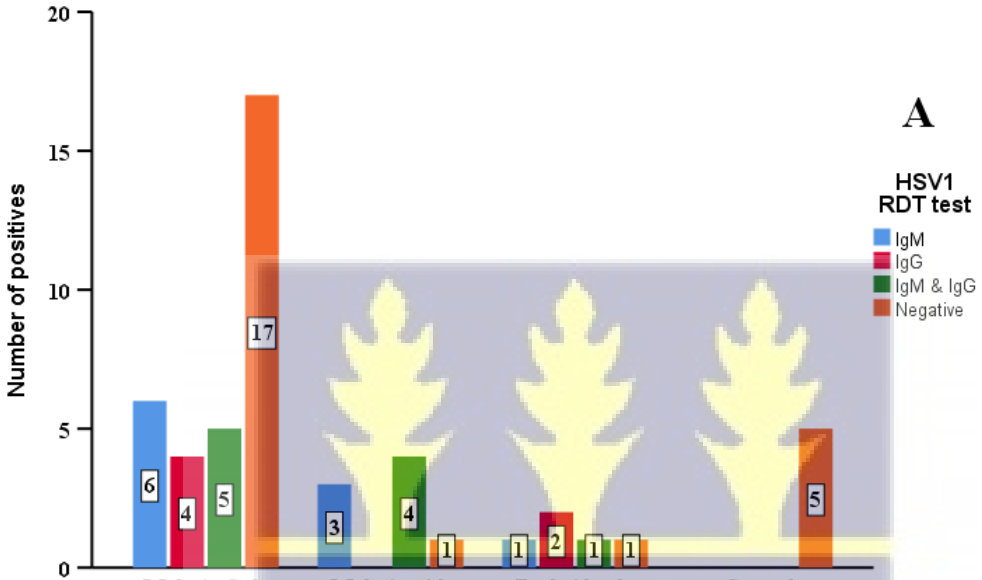
	IgM (%)	IgG (%)	IgM & IgG (%)	Negative (%)
Malaria only	0 (0.00)	0 (0.00)	0 (0.00)	32 (64.00)
Malaria with typhoid	7 (14.00)	1 (2.00)	0 (0.00)	0 (0.00)
Typhoid only	3 (6.00)	0 (0.00)	2 (4.00)	0 (0.00)
Control	0 (0.00)	0 (0.00)	0 (0.00)	5 (10.00)

4.4 Exposure to HSV-1&2 among enrolled participants

Fifteen out (30% of enrolled participants) of 32 malaria positives only were seronegative for HSV-1 antibodies. Six (12%) were positive for IgM, 4 (8%) IgG positives and 5 (10%) IgM & IgG positives. Out of the 8 enrolled participants who had malaria with typhoid, 3 (6%) were IgM positive for HSV-1, while 4 (8%) were positive for both IgM and IgG with no recorded IgG positive (**Figure 4.4A**).

The exposure to HSV-2 among the enrolled participants is summarized in **Figure 4.4B**. Out of the malaria-positive cases, there were 8 (16%) patients who were positive for IgM, 2 (4%)

patients who were positive for IgG, and 1(2%) patient who was positive for both IgM and IgG. Four (8%) out of the eight malaria-positives with typhoid patients were IgM positive for HSV-2, while 2 (4%) were positive for both IgM & IgG and 1(2%) positive for IgG. Only 1 (2%) patient out of the five typhoid only cases tested seropositive for HSV-2 IgG.



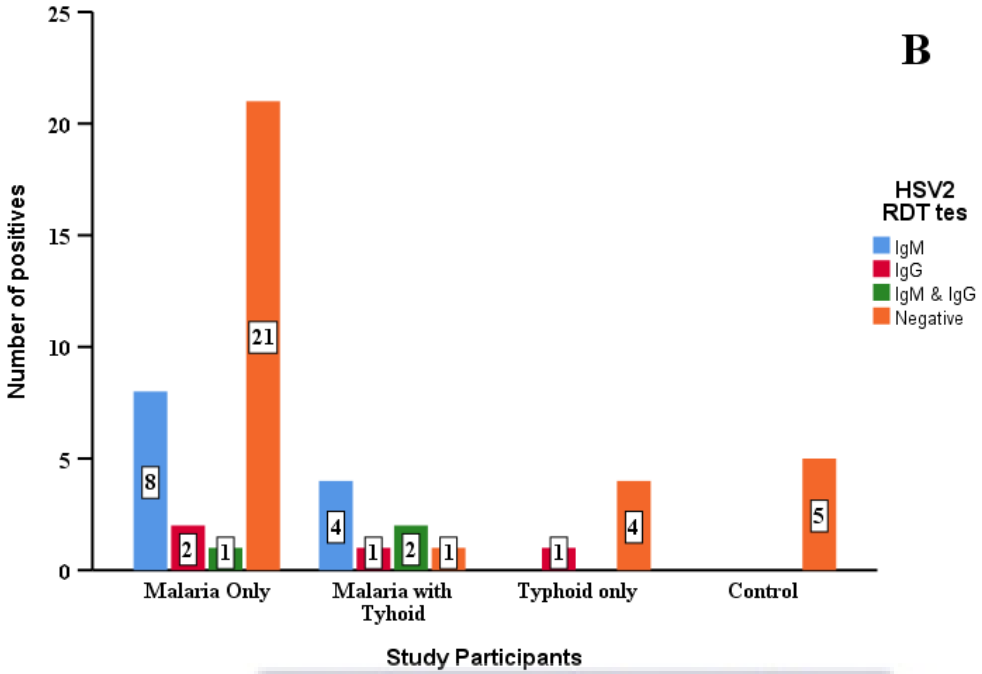


Figure 4. 4: Seroprevalence of HSV-1 & 2 among the study participants.

4.5 Overall seroprevalence of *T. gondii* among enrolled participants.

Only 2 (4%) out of the 32 malaria positives only were IgG seropositive for *T. gondii*. Three (6%) patients with Malaria and typhoid also were IgG seropositive for *T. gondii*, whereas, only 1 (2%) typhoid patient tested seropositive for *T. gondii* IgG (**Figure 4.5**).

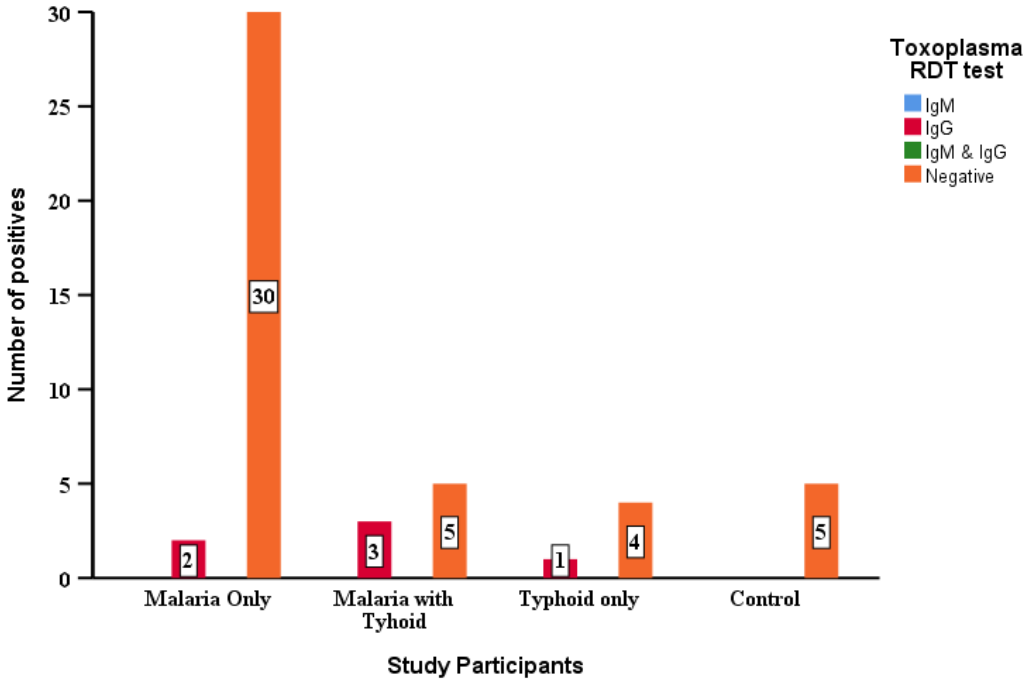


Figure 4. 5: Seroprevalence of *T. gondii* among enrolled participants.

4.6 HIV-1&2 Status Among Enrolled Participants

Only 1 (2%) malaria patient tested positive for HIV-1 **(Figure 4.6)**. The patient also tested seropositive for HSV-2 IgG, but negative for other screened pathogens.

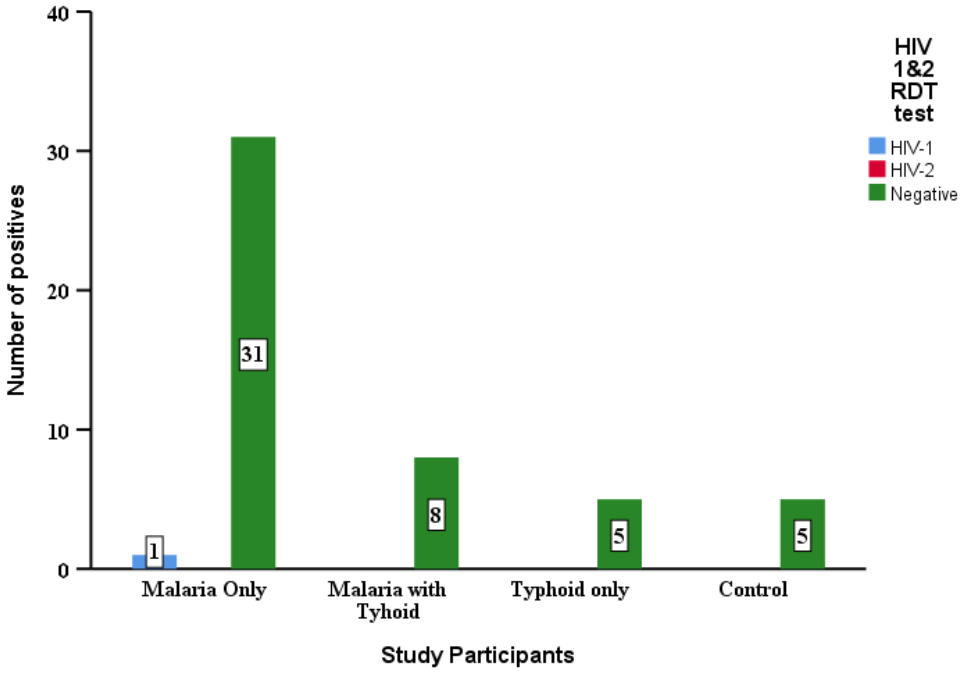


Figure 4. 6: HIV-1 & 2 screening among study participants.

4.7 Hematological parameters of study participants

Results of participants full blood count analyses is summarized in **Table 4.5** below. Patients with Hb less than 12.00 g/dl were frequently encountered among our study participants. The majority of patients 54.95% had Hb less than 12.00 g/dl. Leucopenia was recorded in 19.78 % of our study participants, it was defined as participants with total WBCs count less than 4.00×10^9/L. Also, leucocytosis was recorded in 16.48% of the study participants, this was also defined as participants with total WBCs count greater than 10.00 $\times10^9$/L. In addition to these, thrombocytopenia (platelets count less than 150×10^9/L) was encountered in 70.33% of the study participants. Furthermore, some low lymphocytes (<1.00 $\times10^3$/µL) and neutrophils (<1.50 $\times10^3$/µL**)** count was seen in the study participants, recording 38.46% and 12.09%, respectively. Also, neutrophils count greater than 7.00 $\times10^3$/µL was recorded in 16.48% of the participants, as well as 26.37% of monocytes greater than0.70 $\times10^3$/µL among the participants.

Table 4. 5: Full blood count analyses among study participants prior to treatment

Parameter	Reference range		N (%)
HB	Low	<12.00 g/dl	50 (54.95)
	Normal	**12.00-18.00 g/dl**	41 (45.05)
	High	>18.00 g/dl	0 (0)
WBC	Low	<4.00×10^9/L	18 (19.78)
	Normal	**4.00-10.00 ×10^9/L**	58 (63.74)
	High	>10.00 ×10^9/L	15 (16.48)
RBC	Low	<2.50 ×10^6	5 (5.49)
	Normal	**2.50-5.50 ×10^6**	86 (94.51)
	High	>5.50 ×10^6	3 (3.30)
Platelets	Low	<150×10^9/L	64 (70.33)
	Normal	**150-450×10^9/L**	26 (28.57)
	High	>450×10^9/L	1 (1.10)
Lymphocytes	Low	<1.00 ×10^3/µL	35 (38.46)
	Normal	**1.00-3.7 ×10^3/µL**	48 (52.75)
	High	>3.7 ×10^3/µL	8 (8.79)
Neutrophils	**Low**	<1.50 ×10^3/µL	**11 (12.09)**
	Normal	**1.50-7.00 ×10^3/µL**	65 (71.43)
	High	>7.00 ×10^3/µL	15 (16.48)
Monocytes	Low	<0.00×10^3/µL	0 (0)
	Normal	**0.00-0.70 ×10^3/µL**	67 (73.63)
	High	>0.70 ×10^3/µL	24 (26.37)
Basophils	Low	<0.00×10^3/µL	0 (0)
	Normal	**0.00-0.1×10^3/µL**	91(100)
	High	>0.1×10^3/µL	0 (0)
Eosinophils	Low	<0.00×10^3/µL	0 (0)
	Normal	**0.00-0.4×10^3/µL**	91(100)
	High	>0.4×10^3/µL	0 (0)

4.8 Urine routine examination among study participants before treatment

Urine routine examination of 91 patients at pre-treatment was analyzed and summarized in **Table 4.6**. The majority of the patients, 68.1% (n= 62/91), had straw-colored urine in the analyzed samples, amber colour accounted for 22% (n=20/91), and light-amber accounted for the remaining 9.9% (n=9/91). From the 91 urine samples analyzed, 96.7% had pH values ranging from 5.0 to 6.5, with 3.3% having values ranging from 6.5 to 8.0. In 38 of the 91 (41.8%) patients, the specific gravity (SG) was increased (1.025-1.030). Nitrite was not detected in any of the samples tested. Leukocytes were found in 13.2% of the samples tested. Bilirubin was found in 11% of the patients (n=10/91). 8.8% of the patients tested positive for urobilinogen. Blood was found in 20.9% (n=19/91) of the urine samples tested. Ketone levels

were elevated in 19.8% (n18/91) of the samples analyzed. Proteins were found in 40.7% (n=37/91) of the samples analyzed.

Table 4. 6: Urinalysis test variables in 91 patients before treatment.

Specific gravity (n=91)	**1.010** 9 (9.9%)	**1.015** 34 (37.4%)	**1.020** 10 (10.9%)	**1.025** 22 (24.2%)		**1.030** 16 (17.6%)
pH (n=91)	**5.0** 29 (31.9%)	**6.0** 51 (56%)	**6.5** 8 (8.8%)	**7.5** 1 (1.1%)		**8.0** 2 (2.2%)
Leukocytes (cells/μL) (n=91)	**Negative** 79 (86.8%)		**15 (trace)** 7 (7.7%)	**70** 1 (1.1%)	**125** 4 (4.4%)	**500** 0 (0%)
Nitrites	**Positive** 0 (0%)				**Negative** 91 (100%)	
Glucose (mg/dL) (n=91)	**Negative** 84 (92.3%)		**100** 6 (6.6%)	**250** 0 (0%)	**500** 0 (0%)	**1000** 1 (1.1%)
Ketones (mg/dL) (n=91)	Negative 73 (80.2%)		5 (trace) 3 (3.3%)	15 5 (5.5%)	40 4 (4.4%)	≥80 6 (6.6%)
Blood (n=91) (n=91)	**Positive** 19 (20.9%)				**Negative** 72 (79.1%)	
Bilirubin (mg/dL) (n=91)	**Negative** 81 (89%)		**0.5** 8 (8.8%)	**2** 0 (0%)		**6** 2 (2.2%)
Proteins (mg/dL) (n=91)	**Negative** 53 (58.2%)	**Traces** 16 (17.6%)	**30** 14 (15.4%)	**100** 7 (7.7%)	**300** 1 (1.1%)	**≥500** 0 (0%)
Urobilinogen (mg/dL) (n=91)	**Negative** 83 (91.2%)		**2** 1 (1.1%)	**4** 6 (6.6%)		**≥8** 1 (1.1%)

4.9 Hematological parameters among study participants with exposure to different

An analysis of the haematological parameters performed between the groups of study participants using Kruskal-Wallis one-way between-groups ANOVA for nonparametric data, with types of infection as the independent variable and haematological parameters as the dependent variables showed varied results. The means plus their standard deviations with their associated *p*-values calculated at a 95% confidence interval for each haematological parameter are shown in (**Table 4.7**). Results of the Kruskal-Wallis nonparametric one-way ANOVA showed statistical significance between platelets in study participants (p = .001) and also between neutrophils in the study population (p = .005), as well as between eosinophiles in study participants (p = .004). Furthermore, Dunn's post-hoc analysis revealed a significant difference between the platelet count of patients who had malaria with typhoid on the controls (p = .003) and patients with malaria only and the controls (p = .003). Also, there was a significant

difference between the neutrophils of patients with malaria on patients with typhoid only (p = .023). In addition, a significant difference was observed between the eosinophiles of patients with malaria only and the controls (p = .015).

Table 4. 7: Baseline characteristics of hematological parameters among enrolled participants

Parameters	Reference range	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	*p*-value
		Malaria only (N=32)	Malaria with typhoid (N=8)	Typhoid (N=5)	Controls (N=5)	
Hb (g/dL)	12.00 – 18.00	11.51 ± 2.17	12.52 ± 2.28	13.38±3.87	13.42±1.56	.195
Platelets (10^9/L)	150.00 – 450.00	95.4±71.67	61.50±44.39	188.80±120.40	254.60±40.94	**.001***
WBC (10^9/L)	4.00 – 10.00	6.61±4.00	6.62±4.30	3.53±1.47	4.41±0.79	.051
RBC (10^6/hpf)	2.50 – 5.50	4.50±0.97	4.55±0.93	5.00±1.34	4.81±0.42	.585
Lymphocytes (10^3/µL)	1.00 – 3.70	1.51±1.17	1.36±0.74	1.75±0.67	2.19±0.17	.108
Neutrophiles (10^3/µL)	1.50 – 7.00	4.57±3.13	4.51±3.54	1.36±0.79	1.61±0.75	**.005***
Monocytes (10^3/µL)	0.00 – 0.70	0.49±0.28	0.66±0.50	0.28±0.10	0.42±0.10	.243
Basophiles (10^3/µL)	0.00 – 0.10	0.03±0.02	0.04±0.02	0.03±0.02	0.04±0.02	.106
Eosinophiles (10^3/µL)	0.00-0.4	0.02±0.02	0.01±0.01	0.04±0.02	0.19±0.15	**.004***

Hb=hemoglobin, WBC=white blood cells, RBC=red blood cells, SD=standard deviation. Asterisk (*) indicates statistical significance, confidence interval (CL) = 95%.

4.10 Association between *Plasmodium* parasite density and hematological parameters

Spearman's bivariate correlations analyses showed a negative association between *Plasmodium* parasite density and Hb (*rho* = -.279; p = .063), RBC (*rho* = -.276; p = .066), Platelets (rho = -.719; p =.000), Lymphocytes (rho = -.287; p =.056), Monocytes (rho = -.260; p = .084), and Eosinophils (rho = -.234; p = .113). However, only the association between Platelets and *Plasmodium* parasite density was statistically significant (rho = -.719; p < .001) (**Table 4.8)**, meaning decreasing platelet count corresponded with increasing Plasmodium parasite density. Furthermore, *Plasmodium* parasite density positively correlated with WBC (rho = .004; p = .979), Neutrophils (rho = .174; p = .254), and Basophils (rho = .027; p = .860); however, these correlations are not statistically significant (all p > .05) (**Table 4.8)**

Table 4. 8: Correlation between *Plasmodium* parasite density and hematological parameters

Parameters	Spearman's correlation	
	Spearman's *rho*	P value
Hb	-.279	.063
WBC	.004	.979
RBC	-.276	.066
Platelets	-.719	**.000****
Lymphocytes	-.287	.056
Neutrophils	.174	.254
Basophils	.027	.860
Monocytes	-.260	.084
Eosinophils	-.234	.113

Hb=hemoglobin, WBC=White blood cells, RBC=Red blood cells. (*****) indicates correlation is significant at confidence interval (CL) = 95%, (******) indicates correlation is significant at confidence interval (CL) = 99%.

4.11 Detection of plasma sPD-L1 in participants before treatment, and after treatment.

In general, plasma sPD-L1 was detected and quantified in 45 patients, and five controls, with 44 patients (88%) and 4 (8%) controls falling within the detectable range (25 - 1600 pg/ml), indicating sPD-L1 positivity, and one patient (2%) and one control (2%) falling below the detectable limit (25 pg/ml), indicating sPD-L1 negativity.

At pre-treatment, 40% (n=18/45) of patients had plasma sPD-L1 concentrations less than or equal to 100 pg/ml, 53.3% (n=24/45) of the patients had concentrations between 101 and 500 pg/ml, and only 6.7% (n =3/45) patients had concentrations greater than 500 pg/ml (**Figure 4.7)**, with mean concentrations of 68.05 pg/ml (median: 72.03 pg/ml; range: 17.89-100.11 pg/ml), 186.21 pg/ml (median: 148.54 pg/ml; range: 105.46-486.15 pg/ml), and 815.81 pg/ml (median: 675.98pg/ml; range: 534.74-1236.74 pg/ml), respectively. The overall mean plasma concentration of sPD-L1 was 166.67 pg/ml (median: 112.14 pg/ml; range: 0.00 – 1236.71 pg/ml).

After treatment, plasma sPD-L1 was detected and quantified in the 37 patients and five controls. Of these, 36 patients (85.71%) and 4 (9.52%) controls were within the detectable (25-1600 pg/ml), indicating sPD-L1 positivity, while one patient (2.38%) and one control (2.38%) fell below the detectable limit (25 pg/ml), indicating sPD-L1 negativity. Of these, the plasma sPD-L1 concentration in all 37 (100%) patients was less than 100 pg/ml, with a mean concentration of 38.34 ± 9.44 pg/ml. The control group's concentration was less than 100 pg/ml, with a mean of 38.41 ± 23.13 pg/ml. The study participants' post-treatment mean plasma sPD-L1 concentration was 38.35 ± 11.12 pg/ml (median: 35.11 pg/ml; range: 0.00 – 68.02 pg/ml).

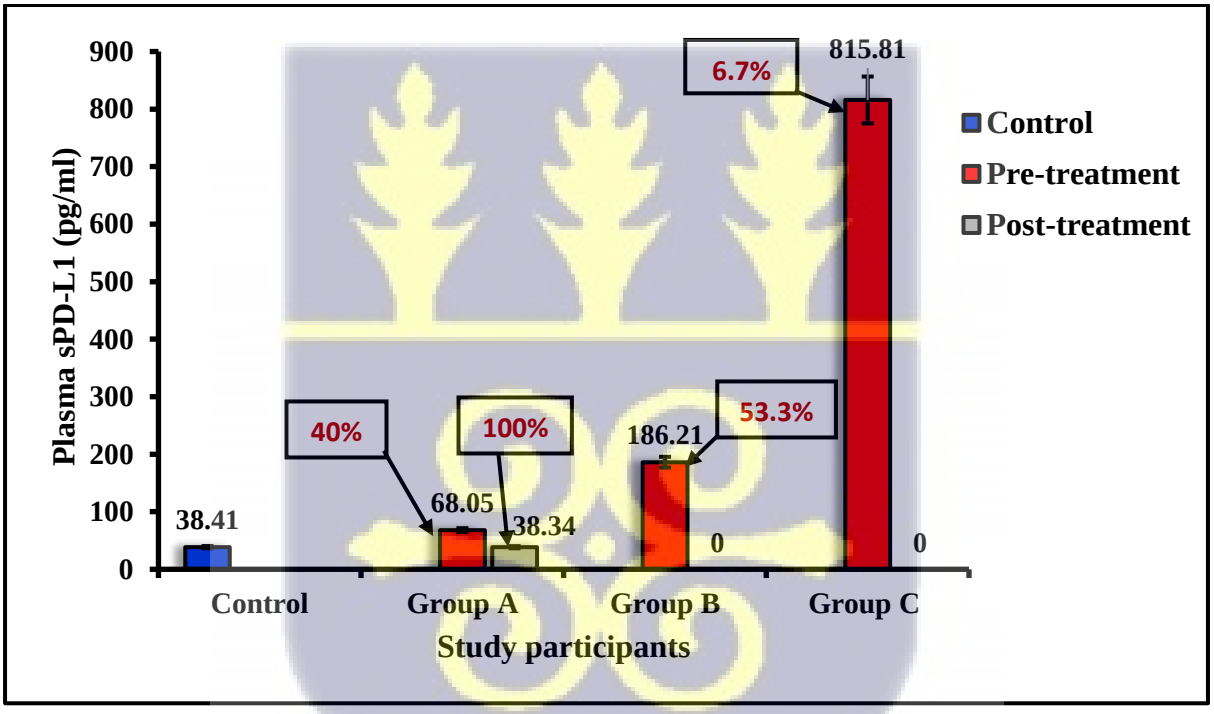


Figure 4. 7: Pre-treatment and post-treatment plasma sPD-L1 concentration.

Group A: sPD-L1 ≤100 pg/ml **Group B:** sPD-L1 from 101 to 500 pg/ml

Group C: sPD-L1 greater than 501 pg/ml

4.12 Detection of urine sPD-L1 in participants before treatment, and after treatment.

Soluble PD-L1 (sPD-L1) was detected, and concentrations were quantified in urine sample of 45 patients and five controls before treatment. Of these, 41 (82%) patients and 2 (4%) controls

had sPD-L1 in their urine. However, only 37 (74%) of the patients and 1(2%) of the controls expressed concentrations that fall within the detectable range (25 -1600 pg/ml), indicating urine sPD-L1 positivity. Contrary, 8 (16%) patients and 4 (8%) controls had sPD-L1 concentrations below the detectable limit (25 pg/ml), indicating sPD-L1 negativity.

Eleven (n=11/45; 24.4%) patients at pre-treatment had urine sPD-L1 concentration below 100 pg/ml, 60% (n=27/45) of the patients had concentrations between 101 and 500 pg/ml, and 15.6% (n=7/45) of the patients had concentrations greater than 500 pg/ml (**Figure 4.8)**, with mean concentrations of 32.55 pg/ml (median: 30.77 pg/ml; range: 0-90.94 pg/ml), 257.12 pg/ml (median: 231.35 pg/ml; range: 114.46-473.97 pg/ml), and 945.03 pg/ml (median: 963.42 pg/ml; range: 619.55-1446.18 pg/ml), respectively. The study participants' mean urine sPD-L1 concentration was 279.37 ± 318.84 pg/ml (median: 190.64 pg/ml; range: 0.00 – 1446.18 pg/ml).

After treatment, urine sPD-L1 was detected and quantified in the 37 patients and five controls. Of these, three patients (7.14%) and 1 (2.38%) control were within the detectable (25 - 1600 pg/ml), indicating sPD-L1 positivity, while 34 patients (80.95%) and four control (9.52%) fell below the detectable limit (25 pg/ml), indicating sPD-L1 negativity. Following treatment, the urine sPD-L1 concentration in all 37 (100%) patients was also less than 100 pg/ml, as observed with plasma sPD-L1, with a mean concentration of 5.4 pg/ml (median: 0.00 pg/ml; range: 0-51.12 pg/ml). Only 27.03% (n=10/37) patients had sPD-L1 in their urine after treatment, while the remaining 72.97% (n=27/37) did not have sPD-L1 expressed in their urine after treatment. The control group's concentration was also less than 100 pg/ml, with a mean of 10.55 ± 14.48 pg/ml.

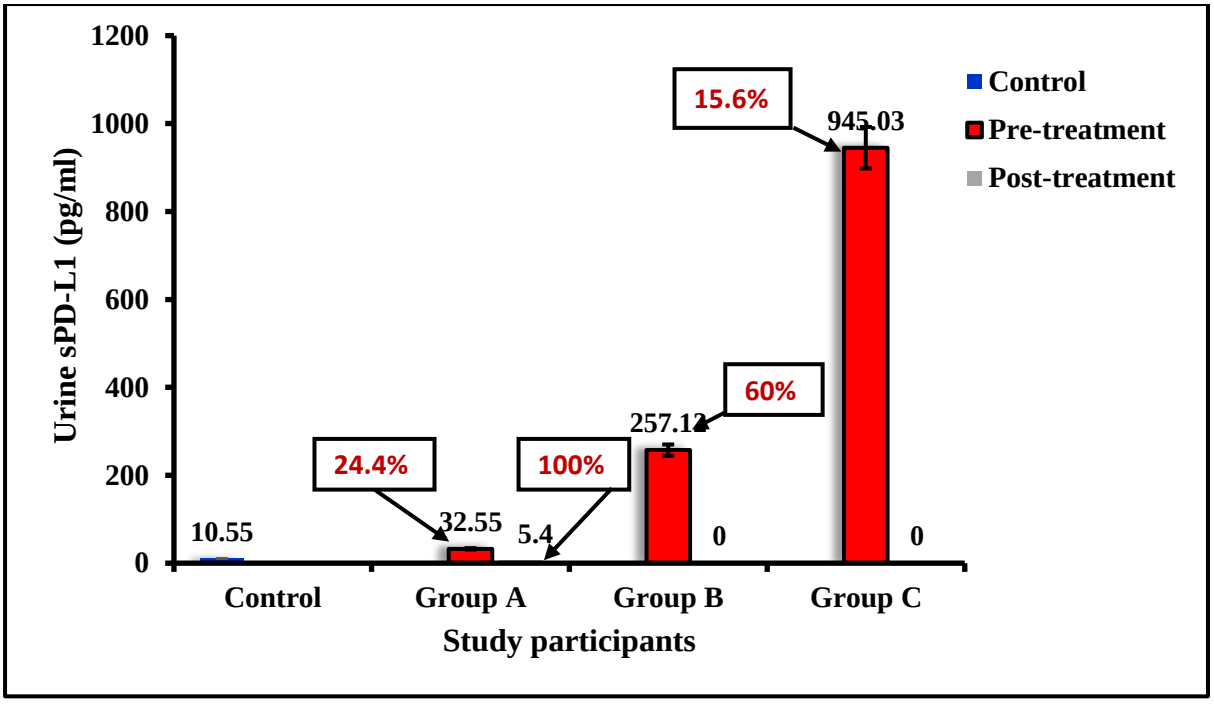


Figure 4. 8: Pre-treatment and post-treatment urine sPD-L1 concentration.

Group A: sPD-L1 ≤100 pg/ml **Group B:** sPD-L1 from 101 to 500 pg/ml

Group C: sPD-L1 greater than 501 pg/ml

4.13 Detection of sPD-L1 in malaria patients and controls before and after treatment

Before treatment, sPD-L1 was detected and quantified in the plasma and urine samples of 40 malaria patients and five controls. Plasma sPD-L1 was detected in all 40 patients at pre-treatment, all falling within the detectable range (25 -1600 pg/ml), with an overall mean of 196.78 pg/ml (median = 122.4, range; 58.59 – 1236.71). Generally, 35% (n=14/40) patients had plasma sPD-L1 concentrations less than 100 pg/ml, and 57.5% (n=23/40) had concentrations between 101 and 500 pg/ml. Only 7.5% (n=3/40) patients had concentrations greater than 500 pg/ml, with mean concentrations of 79.04 pg/ml (median: 78.65 pg/ml; range: 58.59-100.11pg/ml), 187.74 pg/ml (median: 146.14 pg/ml; range: 105.46-486.15 pg/ml), and 815.81 pg/ml (median: 675.98 pg/ml; range: 534.74-1236.71pg/ml), respectively. All the thirty-two (100%) malaria patients that were followed up after treatment had sPD-L1 concentrations less than 100 pg/ml with a mean of 39.57 pg/ml (median: 36.51 pg/ml; range:

27.14-68.02 pg/ml) (**Figure 4.9A**). Among the control group, 4 of the 5 had plasma sPD-L1, while one had no plasma sPD-L1. All four controls had detectable plasma sPD-L1 levels (25-1600 pg/ml), with an overall mean of 38.41 pg/ml (median: 48.71 pg/ml; range: 0.00 - 58.59 pg/ml).

Of the 40 patients and five controls, 38 patients and two controls had sPD-L1 in their urine prior to treatment. The sPD-L1 concentrations in all 38 patients were within the detectable range (25-1600 pg/ml), and only one control fell within this range. Seven 17.5% (n=7/40) patients had urine sPD-L1 concentrations below 100 pg/ml, 65% (n=26/40) had concentrations between 101 and 500 pg/ml, and 17.5% (n=7/40) had concentrations greater than 500 pg/ml (**Figure 4.9B)**, with mean concentrations of 32.20 pg/ml (median: 31.49 pg/ml; range: 0.00-76.93 pg/ml), 261.50 pg/ml (median: 244.12 pg/ml; range: 114.46-473.97 pg/ml), and 945.03 pg/ml (median: 963.42 pg/ml; range: 619.55-1446.18 pg/ml), respectively. The post-treatment urine sPD-L1 concentrations in the 32 (100%) patients that were successfully followed up were all less than 100 pg/ml, with a mean concentration of 5.85 pg/ml (median: 0.00 pg/ml; range: 0.00-51.12pg/ml) (**Figure 4.9 B**). The overall urine means sPD-L1 concentrations in patients and controls are 340.99 pg/ml (median: 244.12 pg/ml; range: 0.00 – 1446.18 pg/ml) and 10.55 pg/ml (median: 0.00 pg/ml; range: 0.00 – 27.86 pg/ml) respectively.

Plasmodium parasite density increased with increasing plasma and urine sPD-L1 concentration in each group. As the parasite density increased from the control group to Group A to Group D, so did the concentration of sPD-L1 (**Figure 4.10A**) and vice versa (**Figure 4.10B**).

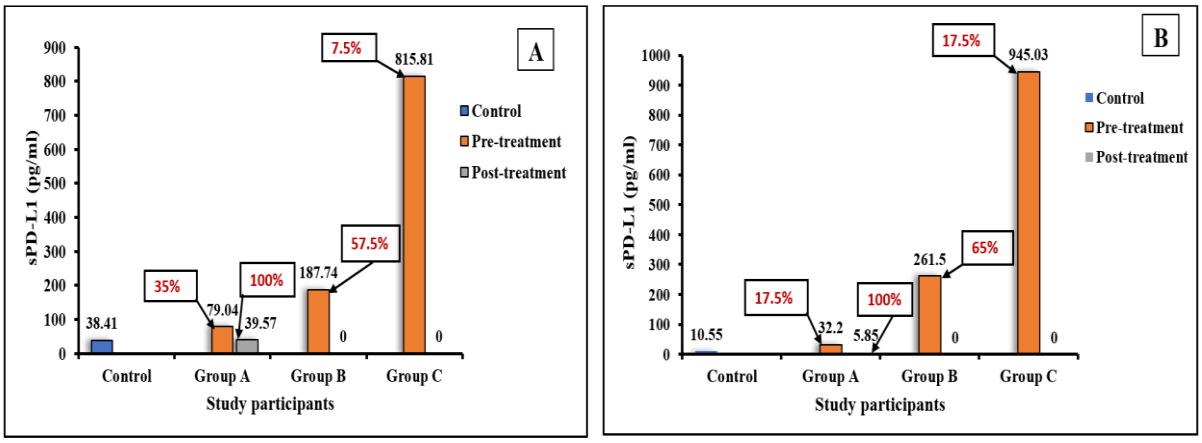


Figure 4. 9: Bar charts showing levels of soluble programmed cell death ligand 1 (sPD-L1) in different groups. (A) Pre-treatment and post-treatment plasma sPD-L1 among malaria patients. **(B)** Pre-treatment and post-treatment urine sPD-L1 among malaria patients**.**

Group A: sPD-L1 ≤100 pg/ml **Group B:** sPD-L1 from 101 to 500 pg/ml

Group C: sPD-L1 greater than 501 pg/ml

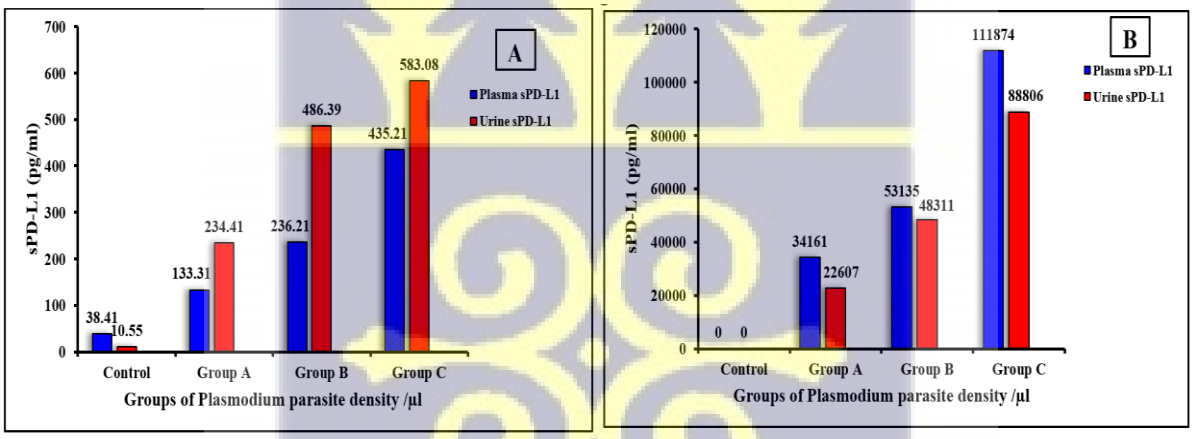


Figure 4. 10: Plasma and urine sPD-L1 concentrations and *Plasmodium* density among malaria patients.

Group A: <40000 *Plasmodium* /µl **Group B:** 40000-119999 *Plasmodium* /µl

Group C: ≥120000 *Plasmodium* /µl (**Figure 4.10A**)

Group A: sPD-L1 ≤100 pg/ml **Group B:** sPD-L1 from 101 to 500 pg/ml

Group C: sPD-L1 greater than 501 pg/ml.

4.14 Levels plasma and urine sPD-L1 and clinical features at pre-treatment.

Mann-Whitney U test analyses showed no statistical difference (p=.129) between plasma levels of sPD-L1 in males (median; 126.95 pg/ml; range: 0.00- 675.98 pg/ml) and females (median; 92.63 pg/ml; range: 17.79 – 1236.71 pg/ml) (**Figure 4.11A, Table 4.9**); additionally, no significant difference (p =.960) was found for urine sPD-L1 levels between males (median; 177.20 pg/ml; range: 0 – 1446.18 pg/ml) and females (median; 220.45 pg/ml: range; 0 – 1033.35 pg/ml) (**Figure 4.11A, Table 4.9**).

Furthermore, Kruskal-Wallis one-way between-groups ANOVA for non-parametric data showed no significant difference between age groups in plasma sPD-L1 levels (p =.516) (**Figure 4.11B, Table 4.8**), as well as urine sPD-L1 levels (p =.682) (**Figure 4.11C, Table 4.9**).

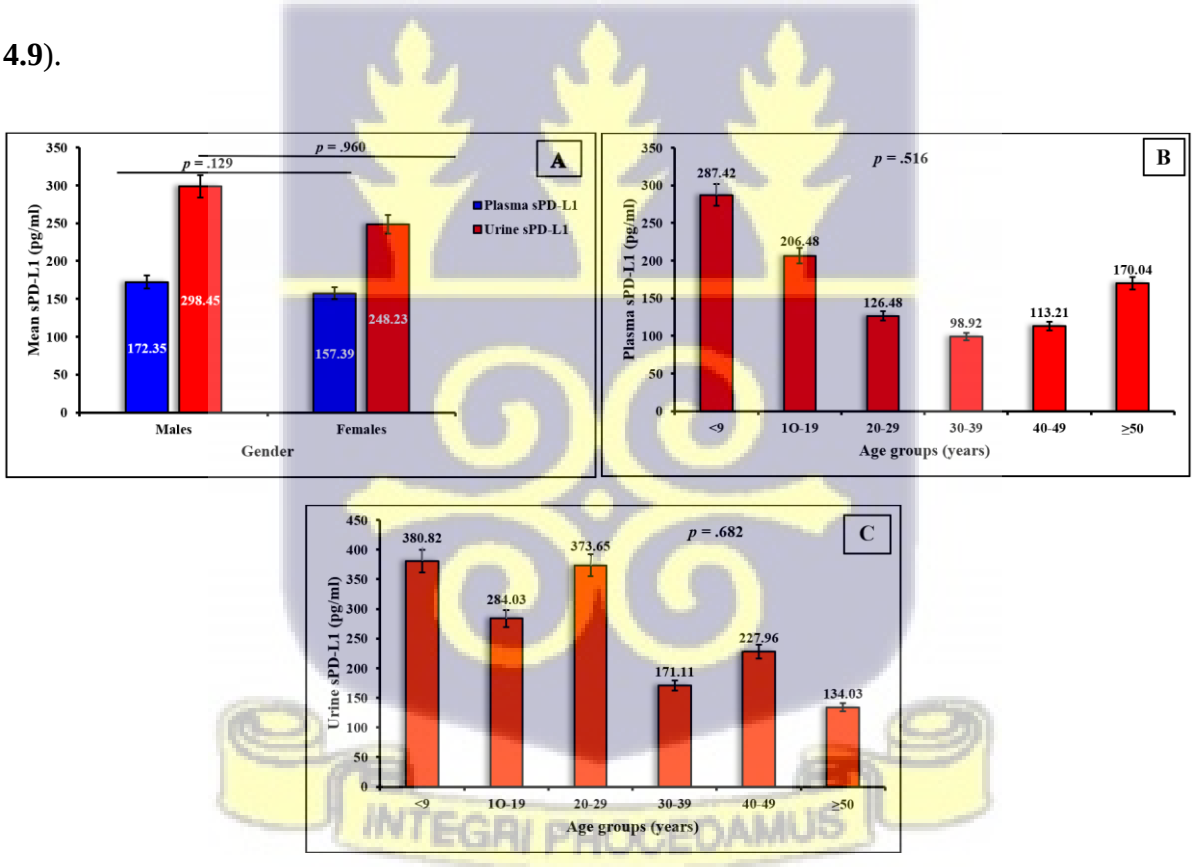


Figure 4. 11. Concentrations of plasma and urine sPD-L1 and clinical features.

(**A**) Plasma and urine sPD-L1 and gender. (**B**) Plasma sPD-L1 and age groups. (**C**) Urine sPD-L1 and age groups.

Table 4. 9: Soluble PD-L1 (sPD-L1) concentrations in different groups

Groups	N	Plasma sPD-L1 (pg/ml)		Urine sPD-L1 (pg/ml)	
		Mean (pg/ml)	Median (Min-Max) pg/ml	Mean (pg/ml)	Median (Min-Max) pg/ml
Gender					
Males	31	172.35	126.95 (0-675.98)	298.45	177.20 (0-1446.18)
Females	19	157.39	92.63 (17.17-1236.71)	248.23	220.45 (0-1033.35)
Age groups					
≤ 9	8	287.42	149.99 (60.95-1236.71)	380.82	324.38 (0-1033.35)
10-19	10	206.48	113.77 (50.12-675.98)	284.03	164.23 (24.87-963.42)
20-29	12	126.42	102.38 (0- 486.15)	373.65	214.41 (0-1446.18)
30-39	7	89.92	96.43 (32.29-146.14)	171.11	220.45 (30.77-285.57)
40-49	7	113.21	105.46 (17.79-282.17)	227.96	149.79 (10.9-619.55)
≥ 50	6	170.04	130.06 (28.55-534.74)	134.03	143.22 (0-292.83)

4.15 Soluble PD-L1 (sPD-L1) levels in patients and controls at pre-treatment.

The concentrations of both plasma and urine sPD-L1 among the patients and the control group are summarized in **Figure 4.12 A and B** and **Table 4.10.** Generally, the plasma and urine sPD-L1 were higher in the patients compared to the control group before treatment (**Figure 4.12A and B**). The plasma sPD-L1 levels were significantly higher in patients (median; 116.43 pg/ml; range: 17.79- 1236.71 pg/ml) (p =.002) compared to the control group (median; 48.71 pg/ml; range: 0.00- 58.59 pg/ml) (**Figure 4.12A, Table 4.10**); also, the urine sPD-L1 levels were significantly higher in the infected patients (median; 244.92 pg/ml; range; 0.00- 1446.18 pg/ml) (p = .002) than the control group (median; 0.00 pg/ml; range 0.00- 27.86 pg/ml) (**Figure 4.12B, Table 4.10**).

Also, the distribution of both plasma and urine sPD-L1 levels in patients' subgroups (Types of exposure: typhoid immunoglobulins only, malaria with typhoid immunoglobulins, and malaria only) and the control group are presented in **Figure 4.12C&D,** respectively and **Table 4.10**. In general, the Kruskal-Wallis test revealed a significant difference between plasma sPD-L1 levels and types of infection

($p < .001$) as well as between urine sPD-L1 levels and infection types ($p = .001$). In addition, Dunn's post hoc analysis showed a significant difference between plasma sPD-L1 levels among the control group and patients with malaria only ($p = .006$), the control group and patients with malaria and typhoid immunoglobulins ($p = .009$), patients with only typhoid immunoglobulins and patients with only malaria infection ($p = .041$), and patients with typhoid immunoglobulins only and malaria with typhoid immunoglobulin patients ($p = .004$) (**Figure 4.12C**). However, no statistical difference was observed between patients with malaria only and patients with malaria and typhoid immunoglobulins, nor between the controls and patients with exposure to typhoid immunoglobulins only (all $p > .05$). Also, a significant difference was observed between urine sPD-L1 levels in the control group and patients with malaria and typhoid infection ($p = .032$), as well as in the control group and patients with malaria only ($p = .004$) (**Figure 4.12D**).

Furthermore, the Kruskal-Wallis test revealed a significant difference between groups of *Plasmodium* parasite densities among the malaria patients and plasma sPD-L1 ($p = .001$) (**Figure 4.12E**), as well as urine sPD-L1($p = .003$) (**Figure 4.12F**). Dunn's post hoc analysis revealed a significant difference in plasma sPD-L1 levels between the control group and patients with *Plasmodium* parasite density less than 40000/µl ($p = .022$), the control group and patients with *Plasmodium* parasite density between 40000 -119999/µl ($p = .001$), and the patients with *Plasmodium* parasite density less than 40000/µl and those with *Plasmodium* parasite density between 40000 -119999/µl (**Figure 4.12E**). A significant difference was also observed in urine sPD-L1 levels between the control group and patients with *Plasmodium* parasite density less than 40000/µl ($p = .035$), the control group and patients with *Plasmodium* parasite density between 40000 -119999/µl ($p = .008$), as well as the control group and patients with *Plasmodium* parasite density greater than 120000/µl ($p = .003$) (**Figure 4.12E**).

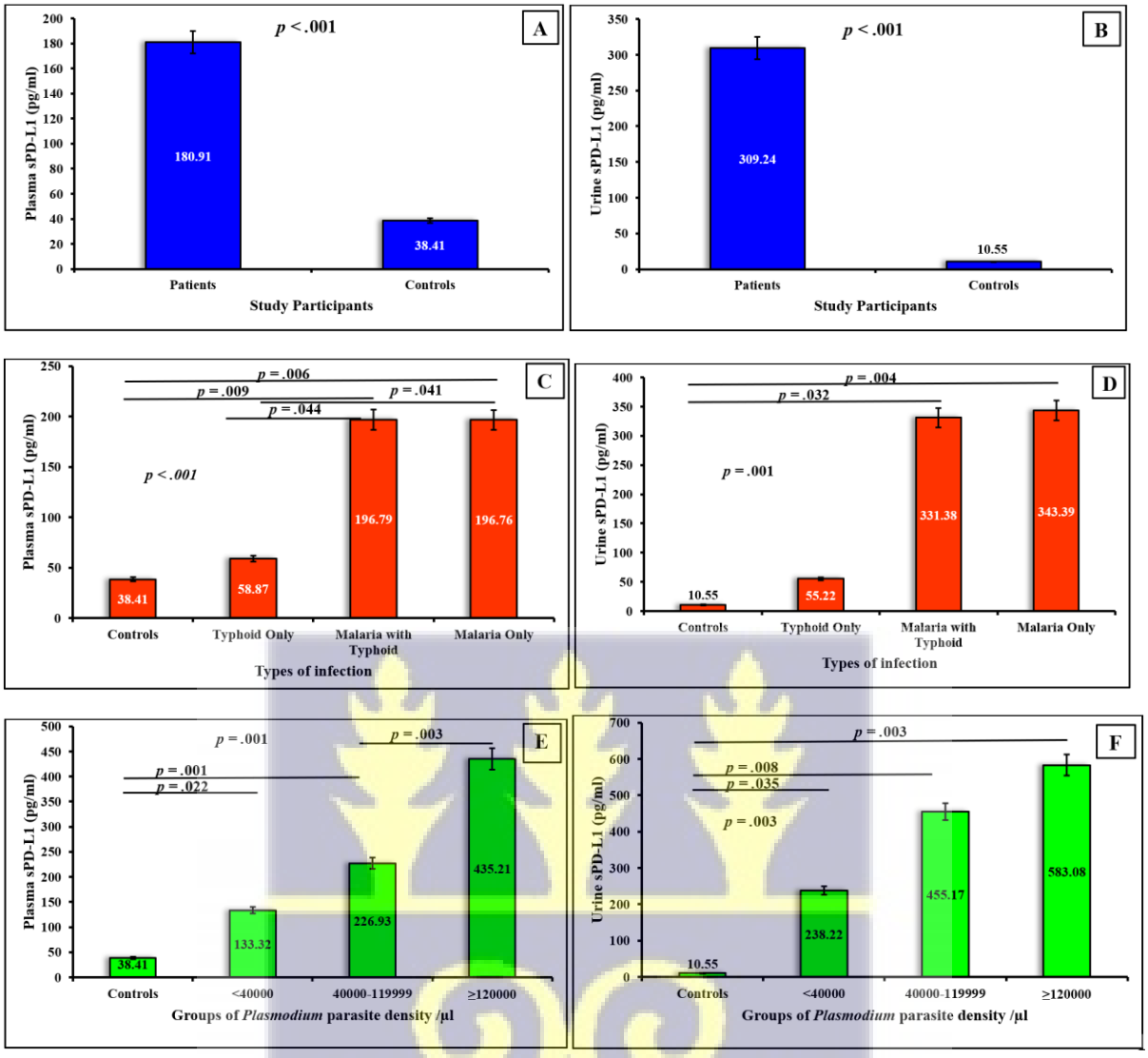


Figure 4. 12. Relationship of plasma and urine sPD-L1 between patients and controls. (**A and B**) Plasma and urine sPD-L1 among patients and controls. (**C and D**) Plasma and urine sPD-L1 between infection types and controls. (**E and F**) Plasma and urine sPD-L1 between the groups of *Plasmodium* parasite density and the controls.

Table 4. 10: Soluble PD-L1 (sPD-L1) concentrations among patients and controls.

Groups	Plasma sPD-L1 (pg/ml)		Urine sPD-L1 (pg/ml)	
	Mean (pg/ml)	Median (Min-Max) pg/ml	Mean (pg/ml)	Median (Min-Max) pg/ml
Participants				
Patients	180.91	116.43 (17.79-1236.71)	10.55	0.00 (0-27.86)
Controls	38.41	48.71 (0-58.59)	309.24	224.92 (0-1446.18)
Infection types				
Controls	38.41	48.71 (0-58.59)	10.55	0.00 (0-27.86)
Typhoid only	53.87	32.29 (17.79-150.95)	55.22	32.29 (0-143.48)
Malaria with typhoid	196.79	130.06 (86.46- 675.98)	331.38	220.26 (37.81-1176.14)
Malaria only	196.76	116.43 (58.59-1236.71)	343.39	244.12 (0-1446.18)
Plasmodium Parasite/µl				
Controls	38.41	48.71 (0-58.59)	10.55	0.00 (0-27.86)
<40000	133.32	110.23 (58.59-534.74)	238.22	229.62 (0-747.14)
40000-199999	226.93	156.73 (92.63-675.98)	455.17	220.45 (31.49-1446.18)
≥ 120000	435.21	278.67 (63.67-1236.71)	583.08	465.34 (139.25-1033.35)

4.16 Association between pre-treatment and post treatment sPD-L1 concentrations.

Out of the 45 patients, 37 were successfully followed up after (post) treatment. There was a significant difference between plasma sPD-L1 levels at pre-treatment (median; 116.43 pg/ml; range: 17.79-534.74 pg/ml) ($p < .001$) compared to post-treatment plasma sPD-L1 levels (median; 35.11 pg/ml; range: 22-68.02 pg/ml) by Wilcoxon Signed-Rank Test analysis (**Figure 4.13A, Table 4.11**). Also, Wilcoxon Signed-Rank Test analysis revealed that pre-treatment urine sPD-L1 levels (median; 227.89 pg/ml; range: 0-1446.18 pg/ml) were significantly higher than post-treatment urine sPD-L1 (median; 0 pg/ml; range: 0-51.11 pg/ml) ($p < .001$) (**Figure 4.13B, Table 4.11**).

Among the 32 malaria patients that were successfully followed up after treatment, a significant difference was observed between levels of plasma sPD-L1 at pre-treatment (median; 122.4 pg/ml; range: 58.59-534.74 pg/ml) ($p < .001$) compared to the post-treatment levels (median;

36.51 pg/ml; range; 27.17-68.02 pg/ml) (**Figure 4.13C, Table 4.11**) by Wilcoxon Signed-Rank test. In addition, urine sPD-L1 levels at pre-treatment were also significantly higher (median; 261.03 pg/ml; range; 0-1446.18 pg/ml) ($p < .001$) compared to the levels of urine sPD-L1 after treatment (median; 0 pg/ml; range; 0-51.12 pg/ml) (**Figure 4.13D, Table 4.11**) by Wilcoxon Signed-Rank test.

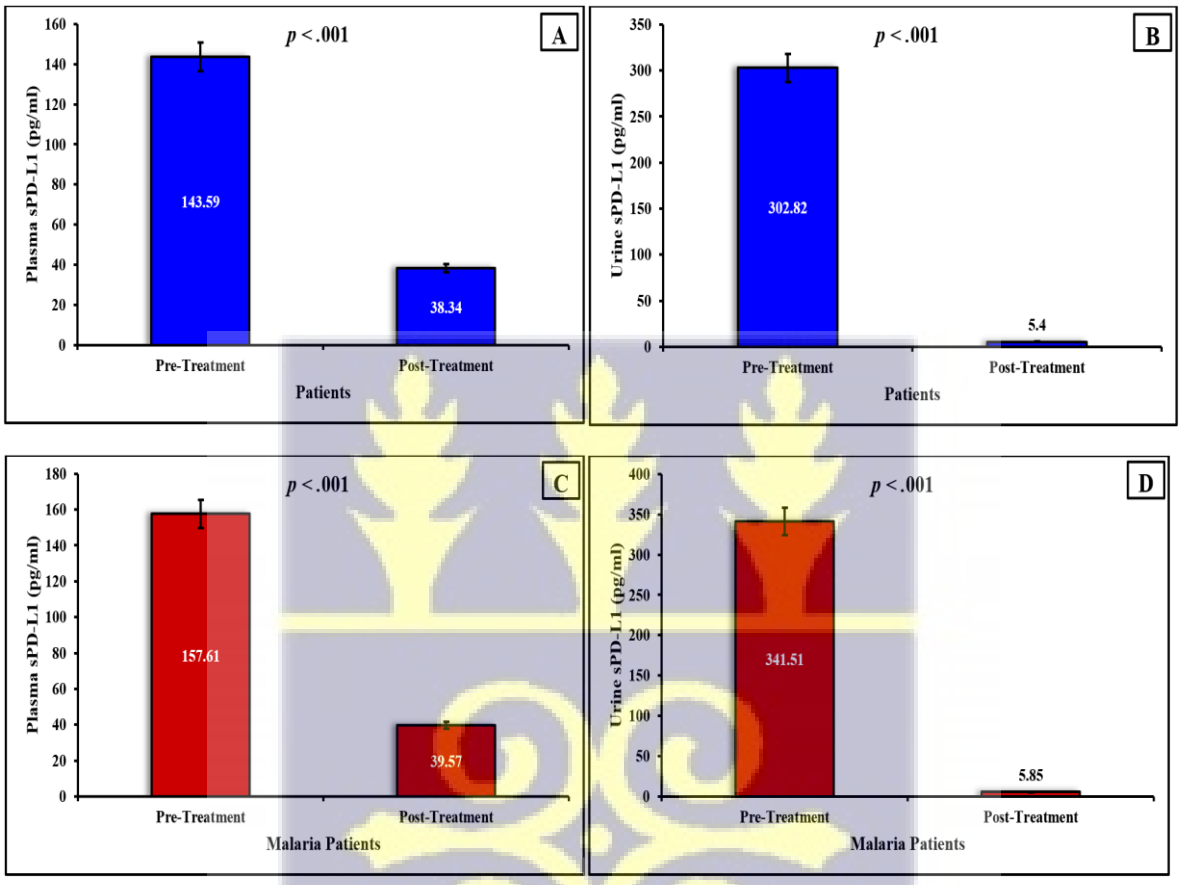


Figure 4. 13. Association between pre-treatment and post treatment sPD-L1 concentrations. **(A and B)** Pre-treatment and post treatment sPD-L1 concentrations in all patients. **(C and D)** Pre-treatment and post treatment sPD-L1 concentrations among malaria patients.

Table 4. 11. Pre-treatment and post-treatment sPD-L1 concentration among patients

Groups	N	Plasma sPD-L1 (pg/ml)		Urine sPD-L1 (pg/ml)	
		Mean (pg/ml)	Median (Min-Max) pg/ml	Mean (pg/ml)	Median (Min-Max) pg/ml
All patients					
Pre-treatment	37	143.59	116.43 (17.79-534.74)	302.82	227.89 (0-1446.18)
Post-treatment	37	38.34	35.11 (22-68.02)	5.4	0 (0-51.11)
Malaria patients					
Pre-treatment	32	157.61	122.40 (58.59-534.74)	341.51	261.03 (0-1446.18)
Post-treatment	32	39.57	36.51 (27.14-68.02)	5.85	0 (0-51.12)

4.17 Association between plasma and urine sPD-L1 among patients.

There was a positive correlation between plasma and urine sPD-L1 at pre-treatment (*rho* = .507, $p < .001$). in all patients, Plasma sPD-L1 positively correlated with urine sPD-L1 post-treatment; however, this was also not statistically significant (*rho* = .221, $p = .160$) (**Table 4.12**).

Also, a positive correlation was established between plasma and urine sPD-L1 at pre-treatment (*rho* = .437, $p = .003$) in malaria patients. Also, post-treatment, a positive correlation was found between plasma and urine sPD-L1 but was also not statistically significant (*rho* = .264, $p = .115$) (**Table 4.12**).

Table 4. 12. Correlation between plasma and urine sPD-L1 among patients.

Groups	Pre-treatment		Post-treatment	
	Spearman's rho	*P* value	Spearman's rho	*P* value
All patients	.507	**.000****	.102	.548
Malaria patients	.437	**.003****	.264	.115

(*****) indicates correlation is significant at confidence interval (CL) = 95%, (******) indicates correlation is significant at confidence interval (CL) = 99%.

4.18 Soluble PD-L1 (sPD-L1) levels among malaria patients with exposure to HSV.

Mann-Whitney U test analysis revealed no statistical difference between the levels of plasma sPD-L1 among patients with malaria only and patients with malaria and HSV immunoglobulins (p=.818) (**Figure 4.14A, Table 4.13**), as well as between patients with malaria only and patients with malaria with exposure to typhoid and HSV (p = .882) (**Figure 4.14B, Table 4.13**), and also between patients with malaria and HSV exposure and patients with malaria with exposure to typhoid and HSV (p = .411). (**Figure 4.14C, Table 4.13**).

Also, Mann-Whitney U test analysis found no statistically significant difference between urine sPD-L1 levels in patients with malaria only and patients with malaria and HSV immunoglobulins (p =.426) (**Figure 4.15A, Table 4.13**), as well as between patients with malaria only and patients with malaria with exposure to typhoid and HSV (p = .349) (**Figure 4.15B, Table 4.13**), and also between patients with malaria and HSV exposure and patients with malaria with exposure to typhoid and HSV (p = .290) (**Figure 4.15C, Table 4.13).**

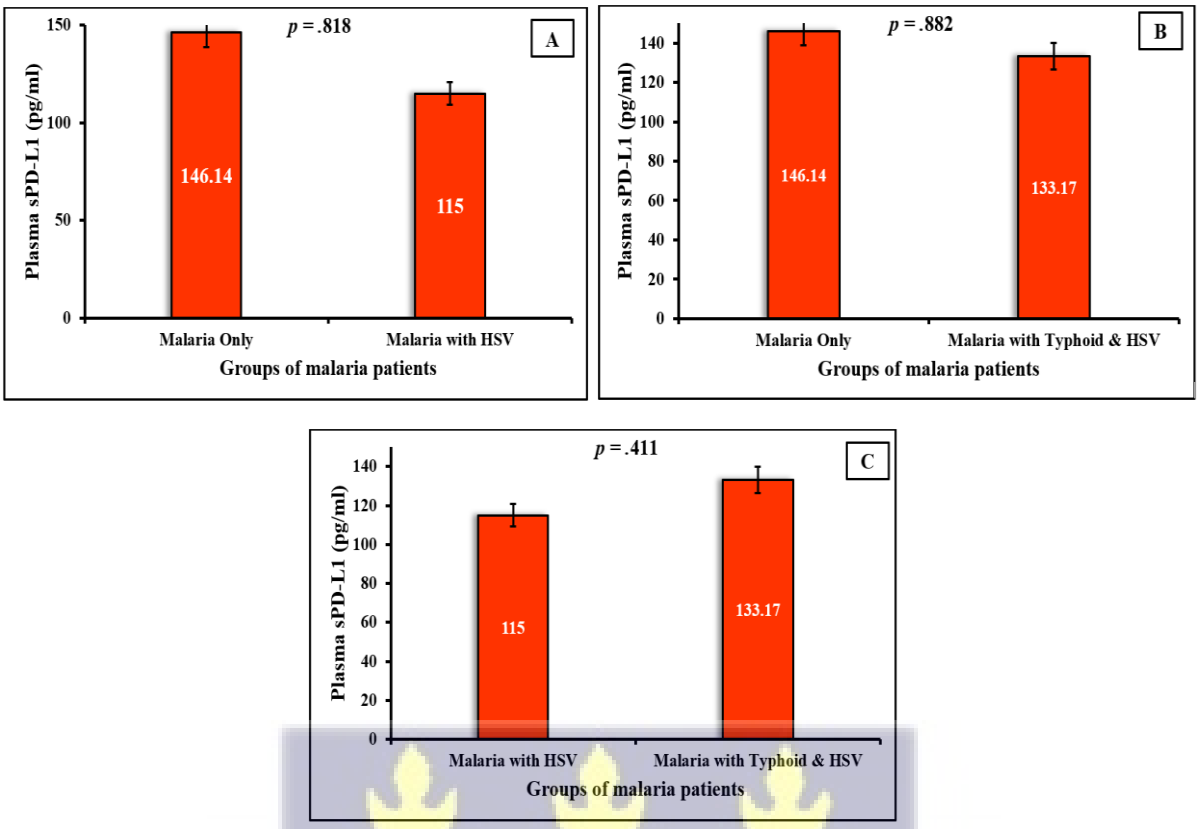


Figure 4. 14. Soluble PD-L1 (sPD-L1) levels among malaria patients with exposure to HSV. (A) Plasma sPD-L1 levels among patients with malaria only and patients with malaria and HSV immunoglobulins. **(B)** Plasma sPD-L1 levels between patients with malaria only and patients with malaria with exposure to typhoid and HSV. **(C)** Plasma sPD-L1 levels between patients with malaria and HSV exposure and patients with malaria with exposure to typhoid and HSV.

Table 4. 13: Levels of sPD-L1 among malaria patients with exposure to HSV and typhoid

Groups	Plasma sPD-L1 (pg/ml)		Urine sPD-L1 (pg/ml)	
	Mean (pg/ml)	Median (Min-Max) pg/ml	Mean (pg/ml)	Median (Min-Max) pg/ml
Malaria only	231.73	146.14 (58.59-1236.71)	421.24	288.96 (0.0-1446.18)
Malaria with HSV	182.45	115 (63.78-534.74)	300.6	220.45 (0-963.41)
Malaria with typhoid & HSV	233.76	133.17 (81.46-675.98)	316.31	142.95 (37.81-1176.14)

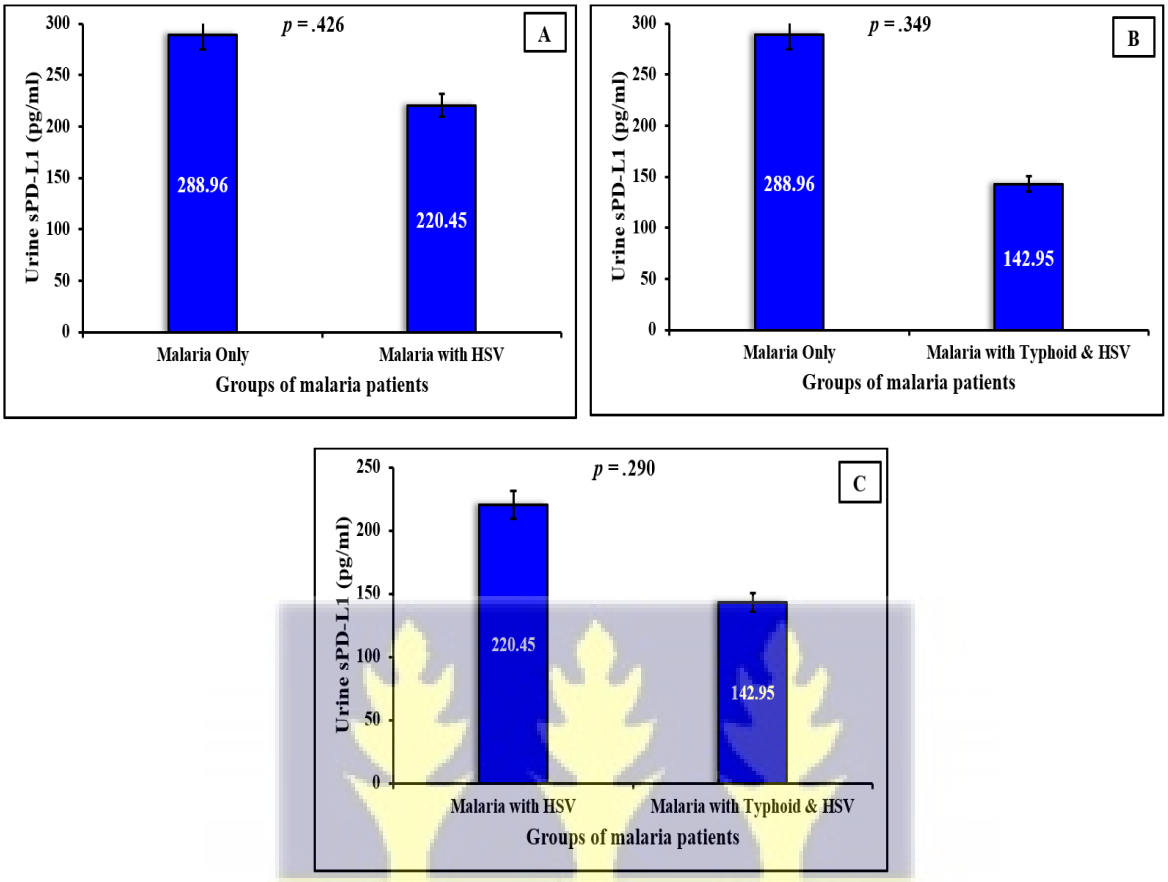


Figure 4. 15. Soluble PD-L1 (sPD-L1) levels among malaria patients with exposure to HSV. (A) Urine soluble PD-L1 concentration among malaria patients only and malaria with HSV immunoglobulins patients**. (B)** Concentrations of urine soluble PD-L1 between only malaria patients and patients with malaria with exposure to typhoid and HSV**. (C)** Urine sPD-L1 levels between patients with malaria and HSV exposure and patients with malaria with exposure to typhoid and HSV.

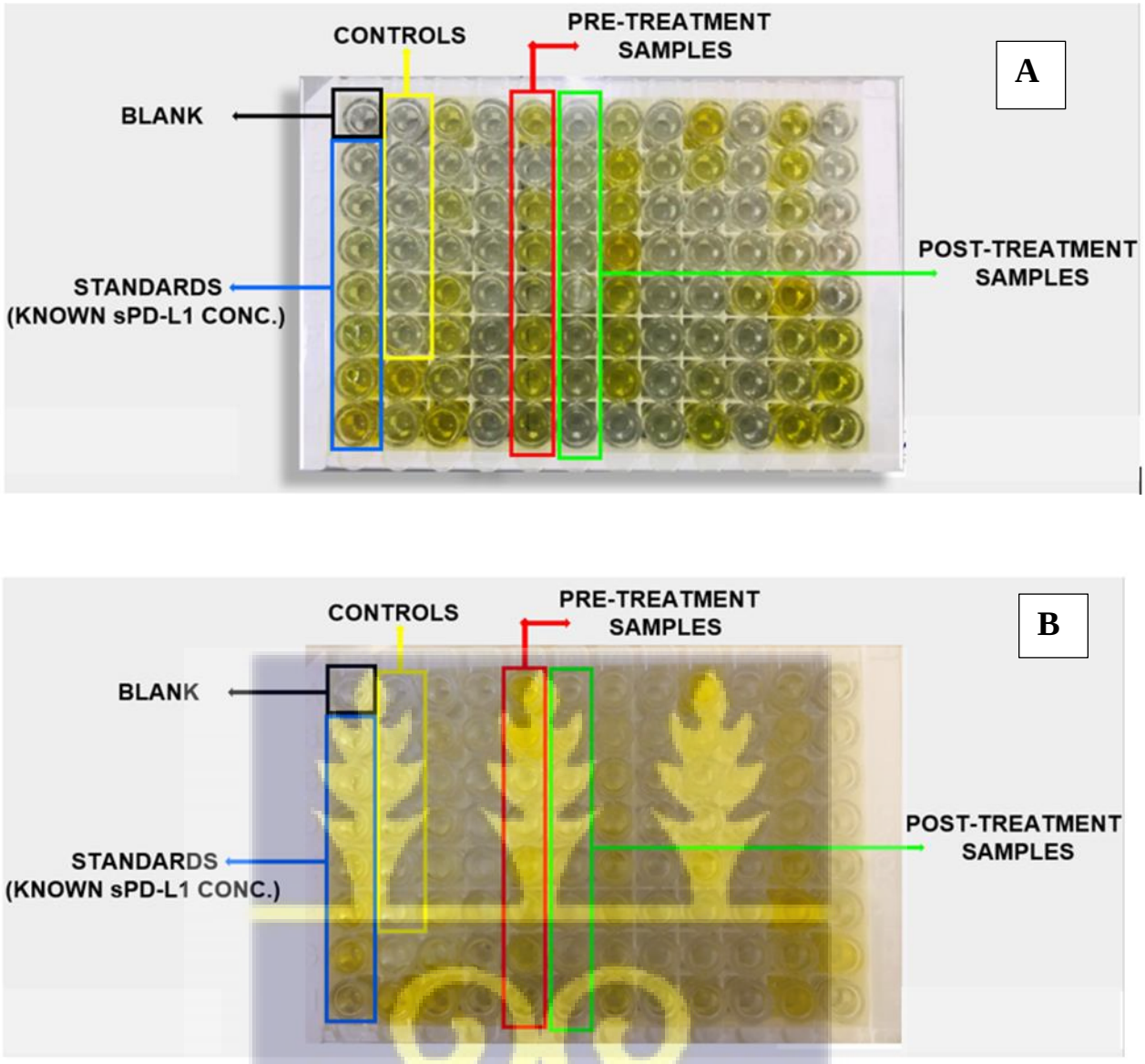


Figure 4. 16: **Pictures of soluble PD-L1 (sPD-L1) ELISA plates**. (A) urine sPD-L1 ELISA plate. (B) Plasma sPD-L1 ELISA plate

Black colour: Blank **Blue colour:** Standard dilutions with known concentrations

Yellow colour: Controls among study participants

Red colour: Pre-treatment samples

Green colour: Matched post-treatment samples

4.19 Associations between pre-treatment and post-treatment plasma and urine sPD-L1 and hematological parameters

Before treatment, the mean HB, WBC, RBC, platelets, lymphocytes, neutrophils, basophiles, monocytes, and eosinophiles were 12.05 × g/dL (median: 12.15 × g/dL; range: 6.1-19.0 × g/dL), 6.08 $\times 10^9$/L (median: 5.07 $\times 10^9$/L; range: 1.45-21.88 $\times 10^9$/L), 4.49 $\times 10^6$/hpf (median: 4.64 $\times 10^6$/hpf; range: 2.24-7.25 $\times 10^6$/hpf), 115.24 $\times 10^9$/L (median: 79.5 $\times 10^9$/L; range: 6-361 $\times 10^9$/L), 1.58 $\times 10^3$/µL (median: 1.32 $\times 10^3$/µL; range: 0.46-5.36 $\times 10^3$/µL), 3.94 $\times 10^3$/µL (median: 3.14 $\times 10^3$/µL; range: 0.31-14.84 $\times 10^3$/µL), 0.03 $\times 10^3$/µL (median: 0.03 $\times 10^3$/µL; range: 0.00-0.08 $\times 10^3$/µL), 0.49 $\times 10^3$/µL (median: 0.42 $\times 10^3$/µL; range: 0.04-1.6 $\times 10^3$/µL), and 0.03 $\times 10^3$/µL (median: 0.1 $\times 10^3$/µL; range: 0.00-0.36 $\times 10^3$/µL) respectively. Plasma sPD-L1 did not correlate with RBC, lymphocytes, monocytes, or eosinophils (all $P > 0.05$). (**Table 4.14).** However, plasma sPD-L1 concentration was negatively correlated with HB (*rho* = -.286, p =.044), platelet count (*rho* = -.667, $p < .001$), and positively correlated with WBCs (*rho* = .439, p = .001), neutrophiles (*rho* = .494, $p < .001$), and basophiles (*rho* =.355, p =.011). (**Table 4.14).**

After treatment, the mean HB, WBC, RBC, platelets, lymphocytes, neutrophils, basophiles, monocytes, and eosinophiles were 12.37 × g/dL (median: 12.15 × g/dL; range: 6.8-15.6 × g/dL), 5.41 $\times 10^9$/L (median: 4.88 $\times 10^9$/L; range: 3.26-10.99 $\times 10^9$/L), 4.57 $\times 10^6$/hpf (median: 4.67 $\times 10^6$/hpf; range: 2.06-5.60 $\times 10^6$/hpf), 246.48 $\times 10^9$/L (median: 234.0 $\times 10^9$/L; range: 140-374 $\times 10^9$/L), 2.58 $\times 10^3$/µL (median: 2.33 $\times 10^3$/µL; range: 0.83-6.23 $\times 10^3$/µL), 2.06 $\times 10^3$/µL (median: 1.90 $\times 10^3$/µL; range: 0.93-4.95$\times 10^3$/µL), 0.03 $\times 10^3$/µL (median: 0.04 $\times 10^3$/µL; range: 0.00-0.06 $\times 10^3$/µL), 0.5 $\times 10^3$/µL (median: 0.39 $\times 10^3$/µL; range: 0.22-1.13 $\times 10^3$/µL), and 0.25 $\times 10^3$/µL (median: 0.13 $\times 10^3$/µL; range: 0.02-1.6 $\times 10^3$/µL) respectively. There was a positive association between plasma sPD-L1 and Hb, WBC, RBC, platelets, lymphocytes, basophils, monocytes, and eosinophils after treatment, as well as a negative association

between plasma sPD-L1 and neutrophils. However, these associations were not statistically significant (all p > .05) **(Table 4.14)**. In addition, there was a negative correlation between plasma sPD-L1 and basophil count after treatment, which was statistically significant (*p* = .037).

Furthermore, no association was established between urine sPD-L1 and Hb, RBC, basophiles, or monocytes, prior to treatment (all *P* > 0.05). (Table 4.). However, there was a negative correlation between urine sPD-L1 concentration and platelet count (*rho* = -.536, *p* < .001), as well as between urine sPD-L1 and eosinophiles (*rho* = -.380, *p* = .009), and between urine sPD-L1 and lymphocytes (*rho* = -.326, *p* = .021) (**Table 4.15**). There was positive association was established between urine sPD-L1 and WBCs (*rho* = .281, *p* = .048), urine sPD-L1 and neutrophiles (*rho* = .457, *p* = .001)

A positive correlation was established after treatment association between urine sPD-L1 and Hb, WBC, RBC, platelets, lymphocytes, neutrophils, basophiles, monocytes, and eosinophiles after, however, these associations were not statistically significant (all p > .05) **(Table 4.15)**

Table 4. 14: Association between plasma sPD-L1 and hematological parameters

Parameters	**Pre-treatment Plasma sPD-L1 pg/ml**		**Post-treatment Plasma sPD-L1 pg/ml**	
	Spearman's *rho*	P value	Spearman's *rho*	P value
Hb	-.286	**.044***	.111	.450
WBC	.439	**.001***	.028	.697
RBC	-.158	.273	.147	.953
Platelets	-.667	**.000****	.100	.423
Lymphocytes	-.052	.721	.079	.340
Neutrophils	.494	**.000****	-.122	.577
Basophils	.335	**.011***	.232	**.037***
Monocytes	-.114	.432	.053	.443
Eosinophils	-.228	.128	.275	.085

Hb=hemoglobin, WBC=White blood cells, RBC=Red blood cells. (*****) indicates correlation is significant at confidence interval (CL) = 95%, (******) indicates correlation is significant at confidence interval (CL) = 99%.

Table 4. 15: Association between urine sPD-L1 and hematological parameters

Parameters	Pre-treatment Urine sPD-L1 pg/ml		Post-treatment Urine sPD-L1 pg/ml	
	Spearman's *rho*	P value	Spearman's *rho*	P value
Hb	-.090	.537	-.120	.450
WBC	.281	**.048***	-.062	.697
RBC	-.096	.508	-.009	.953
Platelets	-.536	**.000****	.127	.423
Lymphocytes	-.326	**.021***	-.151	.340
Neutrophils	.457	**.001****	.089	.577
Basophils	-.143	.323	-.033	.834
Monocytes	-.092	.526	-.130	.443
Eosinophils	-.380	**.009***	.153	.347

Hb=hemoglobin, WBC=White blood cells, RBC=Red blood cells. (*****) indicates correlation is significant at confidence interval (CL) = 95%, (******) indicates correlation is significant at confidence interval (CL) = 99%.

4.20 Case studies demonstrating screening for pathogen exposure and sPD-L1 levels in three patients before and after treatment, as well as a control.

CASE ONE

A 15-year-old schoolboy presented to the hospital's Out Patients Department (OPD) with fever, chills, and headache, as well as an axillary body temperature of 37.8° C. Rapid diagnostic kits confirmed exposure to *Plasmodium* spp., *Salmonella Typhi*, Herpes Simplex virus serotypes 1 and 2 (HSV-1&2), and *Toxoplasma gondii* (**Figure 4.17**). Malaria microscopic examination revealed the presence of *Plasmodium* parasites with a parasite density of 24,488/µL. Prior to treatment, a full blood count analysis revealed a low platelet count (61×10^9/L), but all other blood cell parameters were within normal range. A urine routine examination also revealed no anomalies.

Simultaneously, a healthy 23-year-old woman was screened for exposure to the selected pathogens. Laboratory test results for pathogen exposure were negative (**Figure 4.17**).

Furthermore, the results of the full blood count analysis and the routine urine examination were normal.

Levels of plasma and urine were detected and quantified in the clinical samples of the patient prior to treatment and that of the healthy donor (control) using an sPD-L1 ELISA kit. Generally, the patient had higher levels of both plasma and urine sPD-L1. The patient had 100.11 pg/ml of plasma sPD-L1 and 412 pg/ml urine sPD-L1, while the control (healthy donor) had 34.64 pg/ml plasma sPD-L1 and 0.00 pg/ml urine sPD-L1

Twenty-one days after treatment, the patient (a 15-year-old boy) was again screened for the presence of either antigens or antibodies of the selected pathogens by rapid diagnostic kits. Laboratory test results were negative for all the selected pathogens (indicated by the presence of only the control band on the RDT (**Figure 4.17**). Again, the levels of both plasma and urine sPD-L1 were detected and quantified, revealing 31.36 pg/ml plasma sPD-L1 and 0.00 pg/ml urine sPD-L1. Post-treatment sPD-L1 levels in the patient were almost the same when compared to the control (healthy donor)

CASE TWO

The second case involves a 21-year-old male employee of a private company who presented to the hospital's Adult Emergency Unit with fever, chills, and general malaise, as well as an axillary body temperature of 39.3° C. Rapid diagnostic kits confirmed exposure to *Plasmodium* spp., and Herpes Simplex virus serotypes 1 and 2 (HSV-1&2). Malaria microscopic examination revealed the presence of *Plasmodium* parasites with a parasite density of 114,731/µL. Prior to treatment, a full blood count analysis revealed a low platelet count (20×10^9/L), but all other blood cell parameters were normal. A routine urine examination also revealed no anomalies.

At the same time, a healthy 23-year-old healthy donor (donor) was screened for exposure to the selected pathogens. Laboratory test results for pathogen exposure came out negative. Furthermore, the results of the full blood count analysis and the routine urine examination were normal.

Levels of plasma and urine were detected and quantified in the clinical samples of the patient prior to treatment and that of the healthy donor (control) using an sPD-L1 ELISA kit. Generally, the patient had higher levels of both plasma and urine sPD-L1. The patient had 486.15 pg/ml of plasma sPD-L1 and 629.40 pg/ml urine sPD-L1, while the control (healthy donor) had 48.71 pg/ml plasma sPD-L1 and 0.00 pg/ml urine sPD-L1

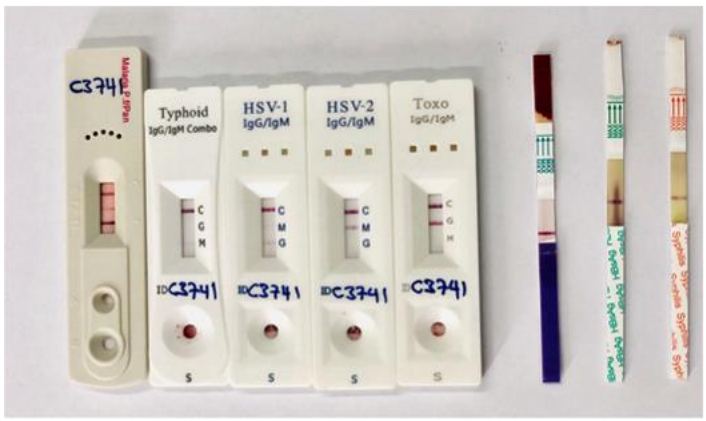
Twenty-one days after treatment, the patient was again screened for the presence of either antigens or antibodies of the selected pathogens by rapid diagnostic kits. Laboratory test results were negative for all the selected pathogens (indicated by the presence of only the control band on the RDT). Again, the levels of both plasma and urine sPD-L1 were detected and quantified, revealing 51.07 pg/ml plasma sPD-L1 and 8.11 pg/ml urine sPD-L1. Post-treatment sPD-L1 levels in the patient were almost the same when compared to the control (healthy donor).

CASE THREE

This was a case of a 7-year-old girl who was rushed to the hospital's Pediatric Emergency Unit. The patient presented with a 38.2° C axillary body temperature, fever, chills, and vomiting on arrival. Laboratory investigation revealed exposure to *Plasmodium* spp only among the selected pathogens. Malaria microscopy showed the presence of *Plasmodium* parasite, with a parasite density of 211,283 /µL. The patient's hematological profile after a full blood count analysis revealed low Hb and platelets count, among other parameters, which were all normal.

Detection of sPD-L1 levels was done using a commercial sPD-L1 ELISA kit. The sPD-L1 ELISA result showed 1236.71 pg/ml plasma sPD-L1 and 1033.35 pg/ml urine sPD-L1.

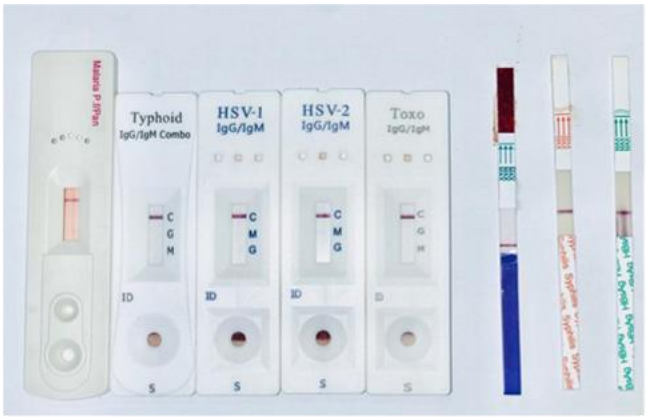
Despite the fact that the patient in case one was exposed to a greater number of the selected pathogens than the case-two patient, the sPD-L1 levels expressed by the patient in case two are generally higher than the levels expressed by the patient in case one. As a result of this, we can say that exposure to *Plasmodium* species is the primary cause of upregulated sPD-L1 levels. This deduction is also supported by the fact that, patient two had a higher *Plasmodium* parasite density and higher sPD-L1 levels than patient one. These findings are supported by the results of patient three, who was the only patient among the three cases to be exposed to the only *Plasmodium* parasite. Furthermore, patient three had the highest *Plasmodium* parasite density as well as the highest plasma and urine sPD-L1, indicating that the *Plasmodium* parasite is indeed responsible for the elevated levels of sPD-L1. Sadly, patient three (7-year-old girl) died 30 minutes after clinical sampling.



Pre-treatment Screening

Results At Pre-treatment

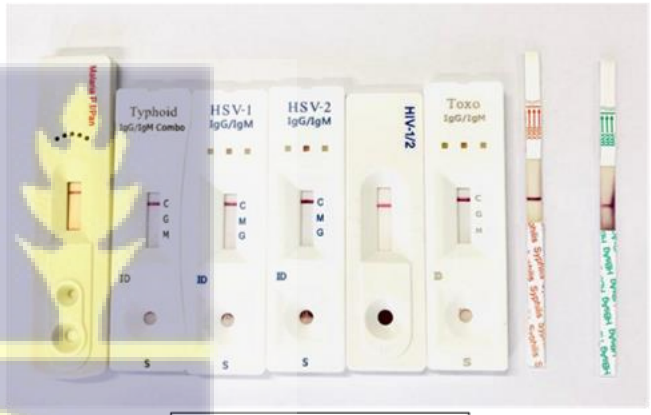
- Malaria: Positive (Pf/PAN)
- Typhoid: Positive (IgM)
- HSV-1: Positive (IgG & IgM)
- HSV-2: Positive (IgM)
- Toxoplasma: Positive (IgG)
- HIV: Negative
- Syphilis: Negative
- Hepatitis B virus: Negative



Post-treatment Screening

Results At Post-treatment

All negative (**presence of only the control band**)



Control Screening

Screening Results of Control

All negative (**presence of only the control band**)

Figure 4. 17: A representative picture demonstrating screening for pathogen exposure in a patient before and after treatment, as well as a control.

CHAPTER FIVE

5.0 DISCUSSION

There is increasing evidence of the involvement of soluble programmed cell death ligand 1 (sPD-L1) in various forms of cancers, including hepatocellular cancer, renal cell cancer, and lung cancer, among others (Okuma *et al*., 2017; Finkelmeier *et al*., 2016; Frigola *et al*.,2017), and some infectious diseases such human immunodeficiency virus (HIV), hepatitis B and C viral infections, and yet it is under-investigated in relation to common infectious diseases such as malaria, and typhoid which are endemic to Ghana and many sub-Saharan Africa countries.

Despite implementing international and national malaria control and elimination programs, the disease remains a significant global burden (Al-Awadhi *et al*., 2021). Over 200 million clinical cases and approximately 655,000 deaths are reported annually (Nureye and Assefa., 2020; Moxon *et al*., 2020; WHO., 2011), with an estimated 20,000 of these mortalities been recorded in children. Children less than five years old bear a tremendous burden of the disease. In this study, 85 of 1200 febrile patients reporting to LEKMA hospital in the Greater Accra region of Ghana had *Plasmodium falciparum* malaria, yielding a prevalence of 7.08% (85/1200) by rapid diagnostic test and microscopy. This finding suggests that human malaria infection continue to be a major health challenge in many Sub-Sahara African countries including Ghana.

The current study also investigated the proof of exposure to *Salmonella Typhi* infection, with a recorded seroprevalence of 1.5% (18/1200) for typhoid immunoglobulins (IgM or IgG) by rapid diagnostic kit, also suggesting that *S. Typhi* exposure is quite common among febrile patients in Ghana. The seropositivity of typhoid IgM was 1%, 0.25% for IgG and 0.25% for IgG and IgM. The fact that out of the 18 febrile patients who had exposure to typhoid, 66.67% (12/18) also had exposure to *Plasmodium falciparum* showed that the cause of fever could be

multifactorial, and in our part of the world, it can be caused by common endemic infections such as malaria and typhoid fever.

Proof of HSV-1 and HSV-2 exposure was also investigated in 91 febrile patients with a rapid diagnostic test confirmed diagnosis of malaria or typhoid. Herpes simplex virus (HSV) is a globally recognized virus that causes lifelong infection, with approximately 60% to 90% of adults in the general population being carriers or affected by associated infections, typically laying in the host in a dormant state. People worldwide are infected with either serotype 1 (HSV-1) or serotype 2 (HSV-2), with 19.2 million new infections occurring yearly. In addition, serotype one has been linked to orofacial infections, while serotype two has been linked to genital herpes, which in most cases leads to genital ulcer disease.

The high prevalence of HSV-1 and HSV-2 (52.8% and 46.17%, respectively) among the 91 febrile patients with malaria or typhoid demonstrated that herpes simplex virus remains dormant in approximately 60% to 90% of the global population, causing lifelong infections by reactivation caused by agitation of the host's immune system. In the present study, HSV-1 recorded 14.3 % (13/91) seropositivity for IgM, while IgG was 23.1% (21/91) and 15.4% for both IgG and IgM. In addition, HSV-2 detected 28.6% (26/91) IgM seropositivity, 14.3% (13/91) IgG seropositivity, and 3.3% (3/91) IgG and IgM seropositivity. Furthermore, there was a 33% cross-seropositivity for HSV-1 and HSV-2. The prevalence of HSV among patients with malaria infection (n = 85) is 51.8% for HSV-1, 47% for HSV-2, and 34.1% cross-seropositive for HSV-1&2. Herpes simplex virus types 1 and 2 are known to be latent in immunocompetent individuals and to be reactivated as recurrent infections during moderate illness (Chêne *et al.*, 2011). Chêne *et al.* (2011) demonstrated that *Plasmodium falciparum* malaria could reactivate latent state HSV-1 because of the parasite's ability to profoundly compromise the host's immune system.

The seroprevalence of *Toxoplasma gondii* from our study among the 91 positive patients was 11% (10/91). The seropositivity for immunoglobulins was 11% for IgG.

The fact that only one positive case of HIV-1 and hepatitis B was recorded, respectively, and two syphilis positive cases were recorded among the 91 febrile patients suggests that HIV, hepatitis B virus, and syphilis may not be the most common causes of febrile illnesses reported in our hospitals. Another plausible reason for the low prevalence of hepatitis B virus among our study participants may be partly due to infant vaccination which began in 2003 (almost two decades ago).

The hematological disparities seen in febrile patients with confirmed malaria and typhoid exposure in this study suggest that hematological abnormalities are common complications encountered in febrile illnesses such as malaria, as *Plasmodium* was the most common pathogen exposed to by study participants. Furthermore, because most of these observations were made in patients infected with *Plasmodium* sp., the frequently observed hematological abnormalities such as thrombocytopenia, anemia, leucopenia, lymphopenia, and increased monocyte count (monocytosis) may suggest the probable involvement of these abnormalities in the pathogenesis of malaria.

The findings of this study confirmed decreased hemoglobin concentration in 54.95% of the patients, indicating anemia. The observation of decreased hemoglobin levels from our findings are similar to those of Sakzabre *et al*. 2020, Osaro *et al*. 2014., and Maina *et al*. 2010. In malaria patients, anemia has been linked to malaria parasitemia (Osaro *et al*., 2014; Maina *et al*., 2010). In addition, studies have shown that anaemia resulting from malaria infection can result from reduced red blood cell count and the rapid removal of both parasitized and non-parasitized red blood cells resulting from lysis of infected RBC (Sakzabre *et al*., 2020; Bawah *et al*., 2018).

The frequently observed thrombocytopenia (which was defined as a platelet count less than 150×10^9/L) in most patients suggests the significant influence of platelets in febrile illnesses such as malaria. This fact is also supported by the recorded 70.33% thrombocytopenia among the 91 positive patients of which 68.13% occurred in malaria patients and the remaining 2.2% in patients who were seropositive to *S. Typhi* antibodies. Our findings agree with those of Sakzabre et al (2020)., who, in their work on the haematological profiles of adults with malaria parasitaemia attending the Volta Regional Hospital in Ghana, also found a higher proportion of their participants with low platelet count. Again, our findings are consistent with those of Kotepui *et al*. (2014), who also reported low platelets count in greater than 50 percent of study population in their research into the effects of malaria infection on haematological parameters. Similar findings were also found in the work of Srivastava *et al*. (2011) among malaria patients in Kenya. According to Srivastava *et al*. (2011), thrombocytopenia may occur as a result of platelet sequestration, splenic platelet pooling, antibody-mediated platelet destruction, and adenosine diphosphate (ADP) release after parasitized RBC haemolysis, dysmegakaryopoiesis, platelet aggregation and activation, platelet invasion by parasites, platelet phagocytosis, platelet adhesion to erythrocytes, and oxidative stress.

Furthermore, the current study's higher percentage of leukopenia (19.78%) than leukocytosis (16.48%) is consistent with those of Srivastava *et al*. (2011), who found a higher percentage of leukopenia than leukocytosis among their participants (Srivastava *et al*.,2011). However, our findings contradict those of Sakzabre *et al*. 2020, who found a higher percentage of leukocytosis (17.37%) than leukopenia (8.47%). According to Ali *et al*. (2017), these differences could be due to additional factors such as disease severity, parasite density, co-infection, and host immunity. In addition, the current study found a high percentage of monocytosis (26.37%) and lymphopenia (38.46%) compared to monocytopenia and

lymphocytosis, respectively. The high percentage of monocytosis among the patients may be attributed to the fact that monocytes play an essential role in blood infection by controlling parasite burden and contributing to host-defence through phagocytosis, secretion of pro-inflammatory cytokines (such as tumour necrosis factor, interleukin-1 and interleukin-6), and antigen presentation, but they also promote inflammation and the sequestration of infected red blood cells (iRBCs) in organs (such as the brain, placenta, or lungs) (Ortega-Pajares & Rogerson, 2018; Wickramasinghe and Abdalla., 2000). The study also found a higher percentage of neutrophilia (16.48%) than neutropenia (12.09%). Similar results were reported in a study by Sakzabre *et al*., 2020. Lymphocytes and neutrophils, according to research, play critical roles in host defence during various diseases (Wang *et al*., 2018). As a result, neutrophil levels have been proposed to reflect the inflammatory state as the disease progresses, whereas lymphocyte levels represent the result of controlled immune responses. (Kwon *et al*., 2015).

Urine routine examination of 91 patients revealed the following urinalysis alterations: increased specific gravity (41.8%), proteinuria (24.2%), blood presence (20.7%), urobilinuria (8.8%), bilirubinuria (11%), ketones (19.8%), and leukocytes (13.2%). Similar findings were reported by Tobón-Castao *et al*. (2017), who found that 41.2% of the participants had increased specific gravity, 39.2% had proteinuria, 29.8% had blood in their urine, 22.7% had ketones, and 14.86% had leukocytes. However, Tobón-Castao *et al*. (2017) reported higher urobilinuria and bilirubinuria (30.6% and 24.3%, respectively), whereas the current study recorded low urobilinuria (8.8%) and bilirubinuria (11%). Bilirubinuria and urobilinogenuria may indicate hepatic involvement or haemolysis in malaria patients, as demonstrated by Ugwuja and Ugwu (2011), who reported that urobilinogenuria and bilirubinuria were found to be positively associated with malaria parasite density, with bilirubinuria increasing as parasite density increased. According to Hendrickse & Adeniyi (1979), the presence of haematuria and proteinuria in malaria patients may be associated with complex immune nephritis.

Our findings demonstrated that plasma sPD-L1 was detectable in all the patients and the majority (80%) of the controls before treatment, whereas urine sPD-L1 was detectable in 82% of patients and 40% of controls. At post-treatment, plasma sPD-L1 was detectable in all patients successfully followed up, whereas urine sPD-L1was only detectable in 27.03% of patients. Studies by Okuma *et al.* (2017) in lung cancer patients reported detectable plasma sPD-L1 samples of all participants, and the mean concentration was 6950 pg/ml (median 7930 pg/ml; range: 2300–20000 pg/ml). A study by Vikerfors *et al*. (2021) also detected sPD-L1 in the urine of 56 of 163 (34.4%) bladder cancer patients, and the median concentration was 74.2 pg/ml (57.5–669.2 pg/ml). These studies showed that sPD-L1 is detectable in plasma and urine under different clinical pathologies. A plausible explanation for the differences in sPD-L1 concentrations is that different clinical pathologies have different baseline sPD-L1 levels and may be affected by different expressions of different infiltrating immune cells or other immunosuppressive molecules.

Generally, the overall mean sPD-L1 concentration in patients before treatment was 180.92 pg/ml (median 116.43 pg/ml; range: 17.79-1236.71pg/ml), while in controls, it was 38.41 pg/ml (median 48.71pg/ml; range: 0-58.59pg/ml). Before treatment, the overall mean urine sPD-L1 concentration in patients was 309.24 pg/ml (median 224.92 pg/ml; range: 0-1446.18pg/ml), while in controls, it was 10.55 pg/ml (median 0.00 pg/ml; range: 0-27.86 pg/ml). The significantly higher levels of plasma ($p < .001$) and urine ($p <. 001$) sPD-L1 in patients than controls (all $p < .05$) suggests that sPD-L1 levels are upregulated during infection and may partake in immune suppression of the patients. These findings are also buttressed by the fact that significantly higher levels of plasma (median; 116.43 pg/ml, range: 17.79-534.74) ($p < .001$) and urine (median; 227.89 pg/ml, range; 0-1446.18) (median; 0 pg/ml, range; 0-51.11) ($p <.001$) sPD-L1 was recorded prior to treatment in all patients than post-treatment plasma (median; 35.11 pg/ml, range: 22-68.02) and urine sPD-L1 levels. In addition, after

treatment, there was no significant difference between plasma sPD-L1 concentrations in patients compared to the controls (p =.362) and between urine sPD-L1 concentrations in patients and control (p = .546) also corroborated the fact that sPD-L1 levels were elevated during infection. These findings agree with a study by Okuma *et al*. (2017), Finkelmeier *et al*. (2016), and Frigola *et al*. (2011) also reported significantly higher levels of sPD-L1 in patients than controls, which is consistent with our findings, even though their work was done on cancer patients.

The fact that no significant difference was observed in the level of plasma (**Figure 4.12C**) and urine (**Figure 4.12D**) sPD-L1 between malaria patients and malaria patients with typhoid immunoglobulins nor between controls and typhoid only (all p >.05), suggests that typhoid immunoglobins may not necessarily contribute to elevated levels of sPD-L1 in the presence of *Plasmodium* infection. Another interesting fact that supports this finding is the significant difference between plasma sPD-L1 of patients with malaria patients and controls (p = .006), which suggests that sPD-L1 is upregulated during human malaria infection. Similarly, a statistically significant difference between malaria patients with typhoid immunoglobulins and controls (p = .009), between patients with malaria only and those with typhoid only (p = .041), as well as between patients who had malaria with exposure to typhoid and those who had exposure to typhoid only (p = .044) (**Figure 4.12C**), also buttress the fact as mentioned above that typhoid immunoglobins may not necessarily contribute to elevated levels of sPD-L1 in the presence of *Plasmodium* infection as it was assumed that malaria patients, the presence of typhoid immunoglobulins might influence either upward or downward regulation of sPD-L1 levels than patients with only malaria.

Having demonstrated that typhoid immunoglobulins in the presence of *Plasmodium* infection may not necessarily contribute to further upregulated expression of sPD-L1, it was essential also to evaluate the possible effect of exposure to herpes simplex virus on sPD-L1 levels during

Plasmodium infection since the majority of the patients were exposed to either herpes simplex serotype 1 (HSV-1) or herpes simplex serotype 2 (HSV-2), or both. However, no statistical difference was seen in plasma (p = .818) and urine (p = .426) sPD-L1 levels between malaria patients and malaria patients exposed to the herpes simplex virus. This also showed that HSV-1&2 antibodies might not contribute to further upregulated expression of sPD-L1 during human malaria infection.

Having shown and ruled out the possible contribution of typhoid and HSV-1&2 immunoglobulins to further upregulation of sPD-L1 during human malaria infection, the effect of *Plasmodium* infection on the upregulation of sPD-L1 was determined. From our findings, the statistically significant higher levels of plasma sPD-L1 and urine sPD-L1 in malaria patients compared to the controls showed that *Plasmodium* parasites may partake in inducing upregulated levels of sPD-L1 during human malaria infection. Also, there was a statistically significant difference between pre-treatment plasma sPD-L1 (median 122.40 pg/ml, range: 58.59-1236.71 pg/ml) and post-treatment (median; 36.51 pg/ml, range; 27.17-68.02) ($p < .001$) sPD-L1 levels, when there was complete *Plasmodium* parasite clearance. Similarly, urine sPD-L1 among patients at pre-treatment was significantly higher (median; 261.03 pg/ml, range; 0-1446.18) ($p < .001$) than controls (median 0.00 pg/ml, range: 0.00-28.89 pg/ml), as well between post-treatment urine sPD-L1 (median; 0 pg/ml, range; 0-51.12) ($p < .001$) after antimalaria therapy. These findings also buttress that infection with human malaria parasites contributes to the upregulation of sPD-L1. Also, elevated levels of sPD-L1 during *Plasmodium* spp infection may be a probable indicator of the involvement of sPD-L1 in immune evasion of human malaria parasites, as these molecules are shown to regulate T cell function to suppress immunity to malaria infection (Illingworth *et al.*, 2013; Butler *et al.*, 2012). Furthermore, studies by Gonçalves-Lopes *et al.* (2016) have also demonstrated the involvement of checkpoint proteins such as CTLA-4 and OX40 in human malaria infection.

Generally, T-cells are an integral part of the immune system's response to infection during adaptive immune response. However, these T-cells must be turned off once the disease is under control. T cells are known to be turned off by inhibitory pathways such as CTLA-4 and PD-1 (Hafalla *et al*., 2012). Studies by Illingworth *et al*. (2013) and Butler *et al*. (2012) in malaria-endemic regions of Kenya and Mali found that Plasmodium falciparum-infected persons have PD-1 expressed on their CD4+ and CD8+ T lymphocytes. These findings and the elevated levels of sPD-L1 reported in this study suggest that the elevated levels of sPD-L1 play a role in *Plasmodium* spp infection's immune evasion.

Furthermore, a positive correlation between *Plasmodium* parasite density and plasma sPD-L1 (rho =.554, p =.001) and urine sPD-L1 (rho =.412, p =.005), where increasing *Plasmodium* parasite density corresponded with increasing levels of both plasma and urine sPD-L1, with patients having high *Plasmodium* parasite density recording higher sPD-L1 concentrations, and the statistically significant differences observed between pre-treatment and control sPD-L1 and between pre-treatment and post-treatment sPD-L1 implies that both plasma and urine sPD-L1 increases with increasing *Plasmodium* parasite density. More data must be collected on elevated plasma and urine sPD-L1 among human malaria patients. However, a study by Krafft *et al*. (2021) on their work on urothelial cancer patients demonstrated that patients with a higher stage of the disease had higher levels of sPD-L1 compared to patients with a low stage of the disease. In this current study, the origin and function of circulating sPD-L1 still need to be fully elucidated. Interestingly, studies by Castello *et al*. (2020), Costantini *et al*. (2018) and Krafft *et al*. (2021) found no association between soluble and tissue PD-L1 levels. This finding may imply that increased tissue expression is not the primary source of sPD-L1. However, members of the matrix metalloproteinase family, such as MMP-7, -9, -10, and -13, have been shown to proteolytically cleave membrane-bound PD-L1, releasing the extracellular domain of the molecule into the bloodstream (Aguirre *et al*., 2020; Hira-Miyazawa *et al*., 2018). However,

the contribution of this cleaved membrane-bound PD-L1 to the circulating PD-L1 concentrations in human malaria infection is unknown.

In addition to these findings, the present study has shown for the first time a positive correlation between plasma and urine sPD-L1 in all patients (rho =.470, p =.002), as well as in malaria patients (rho =.393, p =.016) where increasing plasma sPD-L1 concentrations corresponded to increasing urine sPD-L1 concentrations, suggesting that urine samples alone are enough to assess sPD-L1 expression in malaria patients.

Inflammatory factors such as WBC, neutrophils, and lymphocytes were thought to be linked to changes in the immune microenvironment. The inflammatory immune cell response to infection includes neutrophilia and lymphopenia. The present study found a positive correlation between sPD-L1 and leukocytes, neutrophils and basophils. Results showed that patients with high WBC had higher plasma and urine sPD-L1 concentrations than those with low WBC. Furthermore, our results also showed that patients with high neutrophil counts had higher sPD-L1 concentrations in plasma and urine than those with low neutrophil counts. A positive correlation between plasma and urine sPD-L1 and WBC, neutrophils, and basophiles suggests that the sPD-L1 may be linked to the inflammatory response to Plasmodium falciparum infection.

Results from the present study also show that patients with high basophil counts had higher plasma sPD-L1 concentrations than those with low basophil counts, however, the mechanisms underlying this is not clearly elucidated in this study. A study by Takahashi *et al*. (2016) also reported a positive correlation between leukocytes and neutrophils in their patients. Their results showed that patients with high WBC had higher levels of sPD-L1 than those with low WBC. In addition, patients with high neutrophil counts had higher sPDL1 levels than those with low ones. In contrast to these results, Yang *et al*. (2019) reported no correlation between

sPD-L1 and He WBC, neutrophils, and lymphocytes. In this study, there was no correlation between sPD-L1 and lymphocytes, consistent with the findings of Yang *et al*. (2019).

Our findings revealed also revealed a negative relationship between haemoglobin and plasma sPD-L1 concentrations and eosinophils and urine sPD-L1 concentrations. Patients with low haemoglobin levels had higher plasma sPD-L1 concentrations than patients with normal haemoglobin levels. In addition, patients with a low eosinophil count had higher urine sPD-L1 concentrations than patients with a high eosinophil count. The data on the relationship between sPD-L1 concentrations and inflammatory factors in malaria patients is limited and needs further exploration.

We investigated the relationship between urinalysis parameters and urine sPD-L1 concentrations further because of the changes in urinalysis in malaria patients. Our findings revealed a link between urine sPD-L1 and proteinuria and urobilinuria. The fact that urine sPD-L1 is negatively associated with proteinuria and urobilinuria suggests that the presence of protein and urobilinogen in urine contributed in some effect to the levels of sPD-L1 in the urine of the participants.

It is important to note that the present study has some limitations that need to be addressed in future studies. First, other sensitive testing methods, polymerase chain reaction (PCR), are required for some of the selected pathogens, such *as S. Typhi, T. gondii*, HSV-1, and HSV-2, in order to rule out their presence or absence as possible confounders to upregulated elevated expression sPD-L1in malaria infection. Second, more inflammatory cytokines should be studied to support the idea that plasma and urine sPD-L1 are linked to inflammatory responses in malaria. However, the specific mechanisms underlying this link are unknown and require further investigation. Third, although sPD-L1 appears to be elevated in malaria infection, indicating that the PD-1/PD-L1 pathway may be necessary for malaria infection, other immune

inhibitory pathways in malaria pathogenesis must be investigated. Furthermore, the contribution of sPD-L1 in malaria pathogenesis still needs to be fully elucidated in the present study. Hence more studies are required to investigate the possible role of sPD-L1 in malaria pathogenesis, when it is expressed, and how it is expressed during human malaria infection.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, exposure to the selected pathogens in the blood of febrile patients is distributed as follows; *Plasmodium* species 7.08% (85/1200), *Salmonella Typhi* 1.5% (18/1200), HSV-1 and HSV-2 (52.8% and 46.17 %, respectively), *Toxoplasma gondii* 11% (10/91), HIV-1 1.1% (1/91), hepatitis B virus 1.1% (1/91), and syphilis 2.2% (2/91).

The present study has also demonstrated that plasma and urine sPD-L1 are detectable during infections associated with febrile illness such as human *Plasmodium* species infection, with patients having higher concentration at pre-treatment than post-treatment, and controls.

Our findings have revealed that upregulated expression of sPD-L1 concentrations in malaria patients is associated with increased in *Plasmodium* parasite density. This molecule may play an important role in immune dysregulation in malaria infection, although its exact biochemical function is yet to be determined. Urine sPD-L1 may serve as a useful biomarker for evaluating therapeutic efficacy and prognosis in human *Plasmodium* infection. These findings may also provide the basis and rationale for therapeutics-driven research in the fight against malaria infection, and other clinical pathologies under which sPD-L1 is upregulated. Since sPD-L1 was detectable in urine and blood samples of all the patients. The present study has shown for the first time that urine samples alone are enough to assess sPD-L1 expression in malaria patients

6.2 Limitations

It is important to note that the present study has some limitations. Exposure to selected pathogens in the current study was assessed by the presence of the antigen or antibodies. However, the presence of an immunoglobulin (such as IgM) by RDT does not necessarily mean current infection. Therefore, it would have been better having an antigen testing kit in addition to the immunoglobulin testing kits for some of the selected pathogens, such *as S. Typhi*, *T. gondii*, HSV-1, and HSV-2, to see if the presence of the antigen rather than antibodies may be a possible confounder to upregulated elevated expression sPD-L1 during human malaria infection. Furthermore, although sPD-L1 appears to be elevated during malaria infection, indicating that sPD-L1 may play an important role in malaria infection, the design of the current study will not be able to explain the specific mechanisms of action of sPD-L1 or its role in the pathogenesis in human malaria infection.

6.3 Recommendations

The following recommendation are given based on the results of this current research study,

1. Further studies are needed to compare the concentrations of membrane-bound PD-L1 and soluble PD-L1 in human malaria infection in order to determine the origin of sPD-L1 in malaria infection.
2. In future, studies on sPD-L1 in malaria patients, the stage at which the concentration of sPD-L1 is upregulated and its biochemical function must be explored for therapeutic purposes.
3. Additional studies are necessary, including studies exploring the link between sPD-L1 upregulation and inflammatory response to malaria infection.

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APPENDICES

APPENDIX 1: Kruskal-Wallis one-way between-groups ANOVA

A. Differences in hematological parameters among study participants with exposure to different

Ranks

	Malaria and Typhoid Test	N	Mean Rank
Hb	Malaria Only	32	22.42
	Malaria with Tyhoid	8	29.19
	Typhoid only	5	29.10
	Control	5	35.70
	Total	50	
White blood cells	Malaria Only	32	28.53
	Malaria with Tyhoid	8	27.25
	Typhoid only	5	11.20
	Control	5	17.60
	Total	50	
Red blood cells	Malaria Only	32	23.42
	Malaria with Tyhoid	8	28.56
	Typhoid only	5	28.20
	Control	5	31.20
	Total	50	
Platelet count	Malaria Only	32	23.08
	Malaria with Tyhoid	8	16.19
	Typhoid only	5	36.60
	Control	5	44.80
	Total	50	
Lymphocyte	Malaria Only	32	23.03

	Malaria with Tyhoid	8	23.81
	Typhoid only	5	30.10
	Control	5	39.40
	Total	50	
Neutrophils	Malaria Only	32	29.39
	Malaria with Tyhoid	8	28.19
	Typhoid only	5	9.10
	Control	5	12.70
	Total	50	
Basophils	Malaria Only	32	22.38
	Malaria with Tyhoid	8	34.25
	Typhoid only	5	23.70
	Control	5	33.30
	Total	50	
Monocytes	Malaria Only	32	26.38
	Malaria with Tyhoid	8	30.69
	Typhoid only	5	14.50
	Control	5	22.60
	Total	50	
Eosinophils	Malaria Only	32	20.59
	Malaria with Tyhoid	8	22.94
	Typhoid only	4	37.50
	Control	3	45.17
	Total	47	

Test Statistics[a,b]

	Hb	White blood cells	Red blood cells	Platelet count	Lymphocyte	Neutrophile	Basophile	Monocytes	Eosinophiles
Kruskal-Wallis H	4.698	7.784	1.941	15.815	6.073	12.740	6.120	4.179	13.919
df	3	3	3	3	3	3	3	3	3
Asymp. Sig.	.195	.051	.585	.001	.108	.005	.106	.243	.003

a. Kruskal Wallis Test

b. Grouping Variable: Malaria and Typhoid Test

B. Difference between sPD-L1 among age groups

Ranks

	Age Group	N	Mean Rank
Baseline plasma sPd-L1 concentration	≤9	8	32.88
	10-19	10	27.85
	20-29	12	23.21
	30-39	7	19.43
	40-49	7	22.29
	≥50	6	27.17
	Total	50	
Baseline urine sPD-L1 concentration	≤9	8	31.31
	10-19	10	26.80
	20-29	12	26.21
	30-39	7	23.29
	40-49	7	24.29
	≥50	6	18.17
	Total	50	

Test Statistics[a,b]

	Baseline plasma sPd-L1 concentration	Baseline urine sPD-L1 concentration
Kruskal-Wallis H	4.238	3.114
df	5	5
Asymp. Sig.	.516	.682

a. Kruskal Wallis Test

b. Grouping Variable: Age Group

C. Levels of sPD-L1 among participants with exposure to different pathogens

Ranks

	Malaria and Typhoid Test	N	Mean Rank
Baseline plasma sPd-L1 concentration	Malaria Only	32	29.17
	Malaria with Tyhoid	8	32.50
	Typhoid only	5	10.20
	Control	5	6.10
	Total	50	
Baseline urine sPD-L1 concentration	Malaria Only	32	29.88
	Malaria with Tyhoid	8	29.00
	Typhoid only	5	11.50
	Control	5	5.90
	Total	50	

Test Statistics[a,b]

	Baseline plasma sPd-L1 concentration	Baseline urine sPD-L1 concentration
Kruskal-Wallis H	18.241	17.023
df	3	3
Asymp. Sig.	.000	.001

a. Kruskal Wallis Test

b. Grouping Variable: Malaria and Typhoid Test

D. Levels of plasma and urine sPD-L1 between the groups of *Plasmodium* parasite density and the controls.

Ranks

	Plasmodium density	N	Mean Rank
Baseline plasma sPd-L1 concentration	Controls	5	3.10
	<40000	26	22.62
	40000-119999	9	29.94
	≥120000	5	32.40
	Total	45	
Baseline urine sPD-L1 concentration	Controls	5	4.80
	<40000	26	22.73
	40000-119999	9	28.11
	≥120000	5	33.40
	Total	45	

Test Statistics[a,b]

	Baseline plasma sPd-L1 concentration	Baseline urine sPD-L1 concentration
Kruskal-Wallis H	16.581	14.129
df	3	3
Asymp. Sig.	.001	.003

a. Kruskal Wallis Test

b. Grouping Variable: Plasmodium density

APPENDIX 2: Spearman's bivariate correlations between *Plasmodium* parasite density and haematological parameters.

A. *Plasmodium* parasite and Hb

Correlations

			Plasmodium parasite /µl	Hb
Spearman's rho	Plasmodium parasite /µl	Correlation Coefficient	1.000	-.279
		Sig. (2-tailed)	.	.063
		N	45	45
	Hb	Correlation Coefficient	-.279	1.000
		Sig. (2-tailed)	.063	.
		N	45	45

B. *Plasmodium* parasite and WBC

Correlations

			Plasmodium parasite /µl	White blood cells
Spearman's rho	Plasmodium parasite /µl	Correlation Coefficient	1.000	.004
		Sig. (2-tailed)	.	.979
		N	45	45
	White blood cells	Correlation Coefficient	.004	1.000
		Sig. (2-tailed)	.979	.
		N	45	45

C. *Plasmodium* parasite and RBC

Correlations

			Plasmodium parasite /µl	Red blood cells
Spearman's rho	Plasmodium parasite /µl	Correlation Coefficient	1.000	-.276
		Sig. (2-tailed)	.	.066
		N	45	45
	Red blood cells	Correlation Coefficient	-.276	1.000
		Sig. (2-tailed)	.066	.
		N	45	45

D. *Plasmodium* parasite and Platelet

Correlations

			Plasmodium parasite /µl	Platelet count
Spearman's rho	Plasmodium parasite /µl	Correlation Coefficient	1.000	-.719**
		Sig. (2-tailed)	.	.000
		N	45	45
	Platelet count	Correlation Coefficient	-.719**	1.000
		Sig. (2-tailed)	.000	.
		N	45	45

**. Correlation is significant at the 0.01 level (2-tailed).

E. *Plasmodium* parasite and Lymphocytes

Correlations

			Plasmodium parasite /µl	Lymphocyte
Spearman's rho	Plasmodium parasite /µl	Correlation Coefficient	1.000	-.287
		Sig. (2-tailed)	.	.056
		N	45	45
	Lymphocyte	Correlation Coefficient	-.287	1.000
		Sig. (2-tailed)	.056	.
		N	45	45

F. *Plasmodium* parasite and Neutrophils

Correlations

			Plasmodium parasite /µl	Neutrophile
Spearman's rho	Plasmodium parasite /µl	Correlation Coefficient	1.000	.174
		Sig. (2-tailed)	.	.254
		N	45	45
	Neutrophils	Correlation Coefficient	.174	1.000
		Sig. (2-tailed)	.254	.
		N	45	45

G. *Plasmodium* parasite and Basophils

Correlations

			Plasmodium parasite /µl	Basophile
Spearman's rho	Plasmodium parasite /µl	Correlation Coefficient	1.000	.027
		Sig. (2-tailed)	.	.860
		N	45	45
	Basophils	Correlation Coefficient	.027	1.000
		Sig. (2-tailed)	.860	.
		N	45	45

H. *Plasmodium* parasite and Monocytes

Correlations

			Plasmodium parasite /µl	Monocytes
Spearman's rho	Plasmodium parasite /µl	Correlation Coefficient	1.000	-.260
		Sig. (2-tailed)	.	.084
		N	45	45
	Monocytes	Correlation Coefficient	-.260	1.000
		Sig. (2-tailed)	.084	.
		N	45	45

I. *Plasmodium* parasite and Eosinophils

Correlations

			Plasmodium parasite /µl	Eosinophiles
Spearman's rho	Plasmodium parasite /µl	Correlation Coefficient	1.000	-.234
		Sig. (2-tailed)	.	.113
		N	50	47
	Eosinophils	Correlation Coefficient	-.234	1.000
		Sig. (2-tailed)	.113	.
		N	47	47

J. Plasma and urine sPD-L1 among all participants before treatment

Correlations

			Baseline plasma sPd-L1 concentration	Baseline urine sPD-L1 concentration
Spearman's rho	Baseline plasma sPd-L1 concentration	Correlation Coefficient	1.000	.507**
		Sig. (2-tailed)	.	.000
		N	50	50
	Baseline urine sPD-L1 concentration	Correlation Coefficient	.507**	1.000
		Sig. (2-tailed)	.000	.
		N	50	50

**. Correlation is significant at the 0.01 level (2-tailed).

K. Plasma and urine sPD-L1 among all participants after treatment

Correlations

			Post-treatment plasma sPD-L1 concentration	Post-treatment urine sPD-L1 concentration
Spearman's rho	Post-treatment plasma sPD-L1 concentration	Correlation Coefficient	1.000	.221
		Sig. (2-tailed)	.	.160
		N	42	42
	Post-treatment urine sPD-L1 concentration	Correlation Coefficient	.221	1.000
		Sig. (2-tailed)	.160	.
		N	42	42

L. Plasma and urine sPD-L1 among malaria patients prior to treatment

Correlations

			Baseline plasma sPd-L1 concentration	Baseline urine sPD-L1 concentration
Spearman's rho	Baseline plasma sPd-L1 concentration	Correlation Coefficient	1.000	.437**
		Sig. (2-tailed)	.	.003
		N	45	45
	Baseline urine sPD-L1 concentration	Correlation Coefficient	.437**	1.000
		Sig. (2-tailed)	.003	.
		N	45	45

**. Correlation is significant at the 0.01 level (2-tailed).

M. Plasma and urine sPD-L1 among malaria patients after treatment

Correlations

			Post-treatment plasma sPD-L1 concentration	Post-treatment urine sPD-L1 concentration
Spearman's rho	Post-treatment plasma sPD-L1 concentration	Correlation Coefficient	1.000	.264
		Sig. (2-tailed)	.	.115
		N	37	37
	Post-treatment urine sPD-L1 concentration	Correlation Coefficient	.264	1.000
		Sig. (2-tailed)	.115	.
		N	37	37

APPENDIX 3: Mann-Whitney U Test Analyses

A. Levels plasma and urine sPD-L1 between males and females before treatment

Ranks

	Gender	N	Mean Rank	Sum of Ranks
Baseline plasma sPd-L1 concentration	Male	31	27.95	866.50
	Female	19	21.50	408.50
	Total	50		
Baseline urine sPD-L1 concentration	Male	31	25.58	793.00
	Female	19	25.37	482.00
	Total	50		

Test Statistics[a]

	Baseline plasma sPd-L1 concentration	Baseline urine sPD-L1 concentration
Mann-Whitney U	218.500	292.000
Wilcoxon W	408.500	482.000
Z	-1.519	-.050
Asymp. Sig. (2-tailed)	.129	.960

a. Grouping Variable: Gender

B. Levels plasma and urine sPD-L1 between males and females before treatment

Ranks

	Study participants	N	Mean Rank	Sum of Ranks
Baseline plasma sPd-L1 concentration	Patients	45	27.66	1244.50
	Controls	5	6.10	30.50
	Total	50		
Baseline urine sPD-L1 concentration	Patients	45	27.68	1245.50
	Controls	5	5.90	29.50
	Total	50		

Test Statistics[a]

	Baseline plasma sPd-L1 concentration	Baseline urine sPD-L1 concentration
Mann-Whitney U	15.500	14.500
Wilcoxon W	30.500	29.500
Z	-3.137	-3.172
Asymp. Sig. (2-tailed)	.002	.002
Exact Sig. [2*(1-tailed Sig.)]	.000[b]	.000[b]

a. Grouping Variable: Study participants

b. Not corrected for ties.

C. Plasma sPD-L1 levels between malaria patients and patients with malaria and HSV

Ranks

	Types of infection	N	Mean Rank	Sum of Ranks
Baseline plasma sPd-L1 concentration	Malaria only	13	15.92	207.00
	Malaria with HSV	17	15.18	258.00
	Total	30		

Test Statistics[a]

	Baseline plasma sPd-L1 concentration
Mann-Whitney U	105.000
Wilcoxon W	258.000
Z	-.230
Asymp. Sig. (2-tailed)	.818
Exact Sig. [2*(1-tailed Sig.)]	.837[b]

a. Grouping Variable: Types of infection, b. Not corrected for ties.

D. Malaria only and patients with malaria, typhoid and HSV

Ranks

	Types of infection	N	Mean Rank	Sum of Ranks
Baseline plasma sPd-L1 concentration	Malaria only	13	9.38	122.00
	Malaria with Typhoid and HSV	5	9.80	49.00
	Total	18		

Test Statistics[a]

	Baseline plasma sPd-L1 concentration
Mann-Whitney U	31.000
Wilcoxon W	122.000
Z	-.148
Asymp. Sig. (2-tailed)	.882
Exact Sig. [2*(1-tailed Sig.)]	.924[b]

a. Grouping Variable: Types of infection

b. Not corrected for ties.

E. Patients with malaria and HSV v patients with malaria, typhoid and HSV

Ranks

	Types of infection	N	Mean Rank	Sum of Ranks
Baseline plasma sPd-L1 concentration	Malaria with HSV	17	10.88	185.00
	Malaria with Typhoid and HSV	5	13.60	68.00
	Total	22		

Test Statistics[a]

	Baseline plasma sPd-L1 concentration
Mann-Whitney U	32.000
Wilcoxon W	185.000
Z	-.823
Asymp. Sig. (2-tailed)	.411
Exact Sig. [2*(1-tailed Sig.)]	.446[b]

a. Grouping Variable: Types of infection

b. Not corrected for ties.

F. Urine sPD-L1 in patients with malaria only and patients with malaria and HSV

Ranks

	Types of infection	N	Mean Rank	Sum of Ranks
Baseline urine sPD-L1 concentration	Malaria only	13	16.96	220.50
	Malaria with HSV	17	14.38	244.50
	Total	30		

Test Statistics[a]

	Baseline urine sPD-L1 concentration
Mann-Whitney U	91.500
Wilcoxon W	244.500
Z	-.795
Asymp. Sig. (2-tailed)	.426
Exact Sig. [2*(1-tailed Sig.)]	.432[b]

a. Grouping Variable: Types of infection

b. Not corrected for ties.

G. Malaria patients and patients with malaria, typhoid and HSV

Ranks

	Types of infection	N	Mean Rank	Sum of Ranks
Baseline urine sPD-L1 concentration	Malaria only	13	10.23	133.00
	Malaria with Typhoid and HSV	5	7.60	38.00
	Total	18		

Test Statistics[a]

	Baseline urine sPD-L1 concentration
Mann-Whitney U	23.000
Wilcoxon W	38.000
Z	-.936
Asymp. Sig. (2-tailed)	.349
Exact Sig. [2*(1-tailed Sig.)]	.387[b]

a. Grouping Variable: Types of infection

b. Not corrected for ties.

H. Patients with malaria with HSV and patients with malaria, typhoid and HSV

Ranks

	Types of infection	N	Mean Rank	Sum of Ranks
Baseline urine sPD-L1 concentration	Malaria with HSV	17	12.29	209.00
	Malaria with Typhoid and HSV	5	8.80	44.00
	Total	22		

Test Statistics[a]

	Baseline urine sPD-L1 concentration
Mann-Whitney U	29.000
Wilcoxon W	44.000
Z	-1.058
Asymp. Sig. (2-tailed)	.290
Exact Sig. [2*(1-tailed Sig.)]	.319[b]

a. Grouping Variable: Types of infection

b. Not corrected for ties.

APPENDIX 4: Wilcoxon Signed-Rank Test

A. Pre-treatment and post-treatment plasma sPD-L1 levels among all patient

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The median of differences between Baseline plasma sPd-L1 concentration and Post-treatment plasma sPD-L1 concentration equals 0.	Related-Samples Wilcoxon Signed Rank Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

B. Pre-treatment and post-treatment urine sPD-L1 levels among all patients

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The median of differences between Baseline urine sPD-L1 concentration and Post-treatment urine sPD-L1 concentration equals 0.	Related-Samples Wilcoxon Signed Rank Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

C. Pre-treatment and post-treatment plasma sPD-L1 levels among malaria patients

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The median of differences between Baseline plasma sPd-L1 concentration and Post-treatment plasma sPD-L1 concentration equals 0.	Related-Samples Wilcoxon Signed Rank Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

D. Pre-treatment and post-treatment urine sPD-L1 among malaria patients

Hypothesis Test Summary

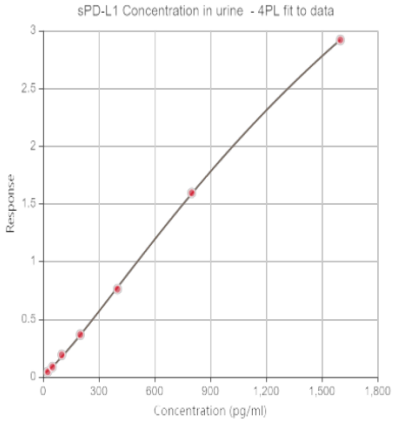
	Null Hypothesis	Test	Sig.	Decision
1	The median of differences between Baseline urine sPD-L1 concentration and Post-treatment urine sPD-L1 concentration equals 0.	Related-Samples Wilcoxon Signed Rank Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

APPENDIX 5: Standard curves for determining sPD-L1 concentrations

A. Urine sPD-L1

$$y = d + \frac{a - d}{1 + (\frac{x}{c})^b}$$



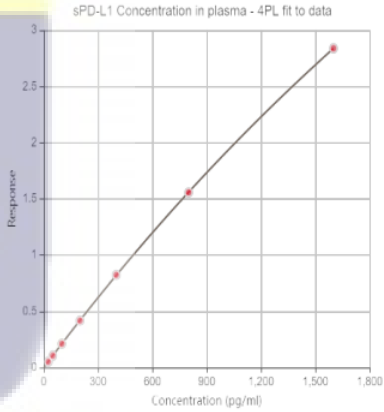
a	b	c	d
0.026288	1.235097	2470.456601	7.874281

$R^2 = 1.0$

a: Theoretical response at zero concentration
b: Slope factor
c: Inflection point (EC50/IC50)
d: Theoretical response at infinite concentration

B. Plasma sPD-L1

$$y = d + \frac{a - d}{1 + (\frac{x}{c})^b}$$



a	b	c	d
0.0	1.003544	7036.3368	15.392923

$R^2 = 1.0$

a: Theoretical response at zero concentration
b: Slope factor
c: Inflection point (EC50/IC50)
d: Theoretical response at infinite concentration

APPENDIX 6: College Ethical Clearance Form

UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

ETHICAL AND PROTOCOL REVIEW COMMITTEE

EPRC/ APR/2022

April 29, 2022

Mr. Jonathan Klutse
Department of Medical Microbiology
University of Ghana Medical School
Korle-Bu.

ETHICAL CLEARANCE

Protocol Identification Number: ***CHS-ET/M.9-P 5.6/2021-2022***

FWA: 000185779 **IORG: 0005170** **IRB: 00006220**

The College of Health Sciences Ethical and Protocol Review Committee (EPRC) at its April 28, 2022 full board meeting reviewed and approved your re-submitted research protocol.

Title of Protocol: **"Expression of Soluble Programmed Cell Death Ligand 1 (sPD-L1) and Complement Activity in Selected Systemic Infections"**

Principal Investigator: Mr. Jonathan Klutse

This approval requires that you submit six-monthly review report(s) of the study to the Committee and a final full review report to the EPRC at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study before, during and after implementation.

Please note that any significant modification(s) to this project/study must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the EPRC within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till April 29, 2023.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: .....

Professor **Andrew** Anthony Adjei
Chair, Ethical and Protocol Review Committee

cc: Provost, CHS
Dean, UGMS
Head, Medical Microbiology

INTEGRI PROCEDAMUS

P. O. Box LG 52, Legon, Accra, Ghana | Tel: +233 (0) 303 665 103/4 | Fax: +233 (0) 302 660 762
Email: admin.chs@ug.edu.gh / provost.chs@ug.edu.gh | Website: www.chs.ug.edu.gh

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