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Frequency of thrombotic microangiopathy in COVID-19 patients and its correlation with disease severity at Ndola teaching hospital and levy Mwanawasa university teaching hospital in Zambia

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Abstract

Severe COVID-19 can result in multiorgan dysfunction, with the lungs being the most commonly affected and prominent organ. Recent studies suggest that an exaggerated immune response characterized by a cytokine storm may play a crucial role in the extensive organ damage observed in this disease. Additionally, COVID-19 patients often exhibit hypercoagulability, with a high incidence of thrombosis and a higher-than-expected failure rate of anticoagulation therapy. While macrovascular thrombosis is frequently observed, the presence of extensive microvascular thromboses, as reported in several case series and studies, raises the possibility of Thrombotic Microangiopathy (TMA) contributing to the thrombotic and multiorgan complications associated with COVID-19. Identifying TMA promptly and addressing the underlying pathophysiology may potentially improve outcomes for critically ill patients.

The objective of the study was to determine the incidence of thrombotic microangiopathy (TMA) in COVID-19 patients and its association with COVID-19 disease severity at two tertiary hospitals in Zambia. The study was conducted at Ndola Teaching Hospital (NTH) and Levy Mwanawasa University Teaching Hospitals (LMUTH) using a hospital-based cross-sectional study design. The study recruited 173 COVID-19 positive patients and 167 COVID-19 negative patients using the simple random sampling technique. TMA was diagnosed in any COVID-19 patient presenting with anemia (hemoglobin concentration < 13.0 g/dl for men and < 12.0 g/dL for women), thrombocytopenia (platelet count < 150 x 10^9/L), reduced haptoglobin (<40 mg/dl), and the presence of more than 1% schistocytes on a May-Grunwald-Giemsa stained blood smear. Statistical analysis was performed using SPSS version 21, and the results were summarized in tables and graphs.

According to the study, **22%** of COVID-19 patients had Thrombotic Microangiopathy (TMA), and these patients had significantly elevated levels of cytokines such as Interleukin 1 (IL-1) (77.97±31.71 ng/l), Interleukin 6 (IL-6) (64.92±32.43 ng/l), Interleukin 8 (IL-8) (126.99±64.06 ng/l), and Tumor Necrosis Factor (TNF) (70.91±21.81 ng/l) compared to TMA-negative COVID-19 patients, in which the inflammatory cytokine profiles were Interleukin 1 (IL-1) (32.97±22.53 ng/l), Interleukin 6 (IL-6) (27.01±25.5 ng/l), Interleukin 8 (IL-8) (65.36±32.52 ng/l), and Tumor Necrosis Factor (TNF) (37.09±27.74 ng/l). Additionally, the study reported that all COVID-19 patients in critical condition had TMA.

The authors recommended that a rational stepwise approach be implemented in diagnosing TMA in all COVID-19 patients suspected of thrombosis so as to institute appropriate treatment for TMA other than anticoagulation therapy.

Keywords: COVID-19; Thrombotic Microangiopathy; VwF; ADAMTS13; Endotheliopathy; Hypercoagulability

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1. Introduction

The COVID-19 pandemic has become a global public health emergency, with over 700 million confirmed cases and 6 million fatalities worldwide as of September 2023. COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the Coronaviridae family of positive-sense, single-stranded RNA viruses. Although initially thought to be limited to the respiratory tract, COVID-19 is now recognized as a multisystem disease that can cause coagulopathy, renal failure, hepatic dysfunction, arrhythmias, and heart failure when severe. The pathophysiology of COVID-19 is not yet entirely understood. However, it is known that glycoprotein spikes on the surface of SARS-CoV-2 engage angiotensin-converting enzyme-2 (ACE-2) receptors on human cells, including the respiratory epithelium, macrophages, and cardiac myocytes (Cao & Li, 2020). This engagement leads to virus entry, replication, and cell lysis, initiating an inflammatory process with the release of pro-inflammatory cytokines such as interleukins (IL-6, IL-1, IL-7, IL-8), various glycoproteins (G-CSF, GM-CSF, FGF, VEGF), and acute phase reactants (procalcitonin, C-reactive protein, fibrinogen). In some patients, this inflammatory response can lead to a state of systemic inflammation characterized by an exuberant cytokine storm that triggers the coagulopathy associated with COVID-19 (Cao & Li, 2020). In severe cases of COVID-19, endothelial injury and the presence of antiphospholipid antibodies can further compound the situation and lead to widespread thromboses (Zhang et al.,., 2020; Beyrouti et al., 2020). While venous and arterial thrombotic events have been reported widely in COVID-19, microvascular thromboses have mostly been described in autopsy case series on histopathological exam of affected tissues. Many of these patients do poorly despite therapeutic anticoagulation and a more nuanced approach is therefore necessary- to consider and address possible additional pathophysiological processes that may be contributing to microvascular thromboses, such as complement dysregulation. A study done in Lusaka, Zambia reported that some patients on anticoagulant therapy (enoxaparin) had died of pulmonary thromboembolism with disseminated thrombosis. The reason for disseminated thrombosis despite therapeutic anticoagulation in such patients is still unknown (Himwaze *et al.* 2021). These findings support the hypothesis that there must be an additional pathogenic mechanism of thrombosis, which cannot be sufficiently addressed by anticoagulation therapies. We therefore assume that increased VWF arising from COVID-19 induced endotheliopathy as a result of hyperinflammatory response causes excessive exocytosis of ultra large VWF multimers (ULVWF) from Weibel-Palade bodies present in endothelial cells. This may lead to insufficient ADAMTS13 in relation to VWF concentration and hence increased platelet activation and aggregation resulting in platelet-rich thrombi in the microvasculature of the lungs and other organs leading to a condition known as Thrombotic Microangiopathy (TMA). This may be the reason why there is prophylactic anticoagulation failure in some COVID-19 patients. This assumption was tested in the current study.

The main aim of this study was to evaluate frequency of Thrombotic Microangiopathy in Covid-19 Patients at Ndola Teaching Hospital and Levy Mwanawasa University Teaching Hospital and Correlation with Disease Severity Levels. Identifying TMA promptly and addressing the underlying pathophysiology may potentially improve outcomes for critically ill patients.

2. Material and method

This was a Hospital based research and utilised cross sectional study design. This study design was chosen because of being relatively cheap and results are obtained quickly especially that we are dealing with a disease outbreak whose research outcomes may be needed in a quickest possible time. The study was conducted at Ndola Teaching Hospital (NTH) and Levy Mwanawasa University Teaching Hospitals (LMUTH). Ndola Teaching Hospital is a third level referral hospital for Copperbelt and Northern part of Zambia. The Hospital is located at the Corner of Broadway and Nkana Roads in Ndola, the Provincial headquarters of the Copperbelt province. It is the second largest Hospital in Zambia. The Hospital has a bed capacity of 851 and acts as a referral Hospital for the Northern part of Zambia. Ndola Teaching Hospital was chosen because of its close proximity to Tropical Diseases Research Centre (TDRC), which was able to undertake confirmatory Molecular Techniques for SARS-CoV2.

LMUTH is situated along the Great East Road around Chainama Hills area in Lusaka, Zambia. LMUTH functions as a Provincial hospital with 3rd level services and was chosen because the Hospital served as a COVID-19 referral centre in Lusaka. The study included Hospitalized or Outpatients at NTH and LMUTH with a confirmed diagnosis of COVID-19 using a reverse transcriptase–polymerase chain reaction (RT-PCR) assay on nasopharyngeal swab samples. For each patient, demographic data, clinical history and some laboratory findings were obtained from the patients' hospital records.

The study recruited a total number of 340 participants comprising of 87 and 86 SARS-Cov-2 positive patients at NTH and LMUTH respectively while 84 and 83 SARS-Cov-2 negative indiviuals were recruited at NTH and LMUTH

respectively. This study adopted a simple random sampling technique to recruit 173 COVID-19 positive patients and 167 COVID-19 negative patients. This type of technique was adopted in this study because it is easy to conduct and when conducted properly, a simple random sample represents an unbiased sample, and therefore is a fair and accurate representation of the population.

2.1. Philosophical Assumption

Positivist research philosophy was adopted in the current research project. As a philosophy, positivism adheres to the view that only "factual" knowledge gained through observation, including measurement, is trustworthy. In positivism studies the role of the researcher is limited to data collection and interpretation in an objective way (Collins, 2010). In other words, the researcher is an objective analyst and he/she distances himself or herself from personal values in conducting the study. In these types of studies research findings are usually observable and quantifiable.

Decision to adopt the positivist research philosophy was based on the four philosophical assumptions of the Positivist paradigm:

At the ontological level, positivists believe that there is a single, defined reality that is fixed, measurable, and observable. They assume that reality is objectively given and can be broken down into measurable variables using properties that are independent of the researcher and instruments (Park, 2020). In the current study, it was hypothesized that SARS-CoV-2 infection leads to a cytokine storm causing hyperinflammation and inducing endothelial activation, cell damage, and increased platelet aggregation. This leads to thrombotic microangiopathy. Cytokine storm and hyperinflammation were measured and quantified by measuring the inflammatory profiles. Thrombotic Microangiopathy was determined by measuring Haemoglobin, platelet count, Haptoglobin, and evaluating schistocytosis on a May-Grunwald Giemsa stained blood smear. Therefore, at the ontological level, our study aligned well with the positivist paradigm.

At the epistemological level Positivists view knowledge as those statements of belief or fact that can be tested, confirmed and verified or disconfirmed, and are stable and can be generalized (Longino, 2020). According to the positivists, knowledge can be obtained through the use of reliable and valid measurement tools. Positivists believe that researchers only need the right data gathering instrument or tools to produce absolute truth for a given inquiry. The acquisition of knowledge about Thrombotic Microangiopathy in COVID-19 patients is an objective process, one that was measured to give reliable and useful knowledge to the clinicians. Reliable and objective laboratory methods were used to determine Thrombotic Microangiopathy.

At the axiological level, positivists argue that all inquiries should be value-free. This approach aims to exclude a researcher's own values when conducting research, making the observations and interpretations as unbiased as possible. To achieve objectivity and neutrality during the inquiry process, researchers should use scientific methods of gathering data (Collins, 2010). In the current research data was collected by

At the methodological level, positivists assume that the only acceptable method to generate valid knowledge is through the use of quantitative research methods such as experiments, quasi-experiments, exploratory and analytical models, and case studies (Crowther & Lancaster, 2008). Quantitative methods were used in the current proposed study, which required objective measurement and analysis of data that was generated.

2.1.1. Inclusion criteria

This study enrolled individuals who tested positive for COVID-19 by RT-PCR as cases and healthy individuals of both genders aged 18 years or older testing negative for Sars-Cov-2 as control subjects. Only those who provided informed consent were included in the study

2.1.2. Exclusion criteria

Participants who had a history of venous thromboembolism or known inherited coagulation disorders, Cancer and hyperthyroidism were excluded from the study. Others excluded include, those who were Pregnant, had recent surgery, those taking standard anticoagulant treatment, less than 18 years and those not willing to consent.

2.2. Variables and indicators of measurements

For the purpose of this study individuals were considered to have COVID-19 when Reverse Transcription Polymerase Chain Reaction (RT-PCR) for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was positive. In the current study TMA was diagnosed in any COVID-19 patient presenting with Anemia (Haemoglobin concentration < 13.0

g/dl for men and < 12.0 g/dl for women), Thrombocytopenia (Platelet count < 150 x 10^9/l), reduced Haptoglobin (<40 mg/dL) and the presence of more than 1% schistocytes on a blood film (ICSH, 2021).

WHO acceptable classification was used for COVID-19 severity classification (Buonsenso et al., 2021) and is as follows;

2.3. Asymptomatic Infection

Individuals who test positive for SARS-CoV-2 using a nucleic acid amplification test (NAAT) or an antigen test) but who have no symptoms that are consistent with COVID-19.

- **Mild Illness:** Individuals who have any of the various signs and symptoms of COVID-19 (e.g., fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, loss of taste and smell) but who do not have shortness of breath, dyspnea, or abnormal chest imaging.
- **Moderate Illness:** Individuals who show evidence of lower respiratory disease during clinical assessment or imaging and who have oxygen saturation (SpO2) ≥94% on room air at sea level.
- **Severe Illness:** Individuals with clinical signs of pneumonia (fever, cough, dyspnoea, fast breathing) plus one of the following: respiratory rate > 30 breaths/min; severe respiratory distress; or SpO2 < 90% on room air.
- **Critical Illness:** Individuals who have respiratory failure, septic shock, and/or multiple organ dysfunction.

2.4. Data Collection

Good Laboratory Practice (GLP) principles according to the Ministry of Health laboratory quality manual were observed to ensure uniformity, consistency, reliability and reproducibility of all the laboratory test results in the study. Quality control measures were observed in all the laboratory procedures.

2.5. Laboratory Assays

2.5.1. Analysis of Haemoglobin and Platelet count

The Sysmex XT-2000i was used for Full blood count analysis. It uses the electric resistance detecting method (impedance technology) with hydro dynamic focusing to measure Red Blood cells (RBC), platelet (PLT), mean platelet volume (MPV), Mean Red Cell Volume (MCV), and Hemat-ocrit (HCT). Fluorescence flow cytometry is used to measure white blood cells (WBC), Differential WBC, the optical PLT count, and the reticulocyte (RET) count. The system employs a 633 nm semi-conductor laser for flow cytometry analysis. For the measurement by flow cytometry of the proportional count, expressed as percent of the total WBC, of neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), and eosinophils (EOS), white cells are stained with fluorescent dyes that bind to both DNA and RNA. Side Scatter (SSC) is employed to determine the internal complexity of the cell: the size, shape, and density of the nucleus and granules of the cell. Fluorescence and scatter measurements are combined to characterize white cell populations. Basophils (BASO) are measured separately using cell size and SSC properties. Hemoglobin (HGB) is measured photo colorimetrically using SLS-HGB, a cyanide-free method.

2.5.2. Analysis of Haptoglobin

Haptoglobins are a group of serum proteins, originally identified by Polonovski and Jayle in 1939 ((Naryzny & Legina, 2021), which have the characteristic property of combining with haemoglobin to form a stable complex. Quantitative estimation of haptoglobins depends upon measuring the amount of this haemoglobin-haptoglobin complex formed when the test serum has been allowed to combine with free haemoglobin either as oxyhaemoglobin or methaemoglobin and is therefore a measure of their haemoglobin-binding capacity. Studies have shown that in patients with excessive haemolysis clearance of haemoglobin from serum leads to absence or reduction of haptoglobins (Warming, 2023). Serum haptoglobin estimation may therefore be of value in the diagnosis of haemolytic disease (Naryzny & Legina, 2021). The Architec Chemistry Analyser by Aboott (USA) was used for analysis of Haptoglobin in the plasma samples of study participants is based on nimmunoturbidimetric procedure that measures increasing sample turbidity caused by the formation of insoluble immune complexes when antibody to haptoglobin is added to the sample. Sample containing haptoglobin is incubated with a buffer (Reagent 1) and a sample blank determination is performed prior to the addition of haptoglobin antibody (Reagent 2). In the presence of an appropriate antibody in excess, the haptoglobin concentration is measured as a function of turbidity.

2.5.3. Determination of Circulatory Cytokines

The plasma samples for determination of cytokines were frozen upon collection and analyzed later after thawing. The types and quantities of cytokines were detected by flow cytometry using a multiplex assay system that included Becton

and Dickinson Cytometric Bead Array (BD CBA) Human Inflammatory Cytokine kit and Becton and Dickinson Fluorescence Activated Cell Sorter (FACS Calibur) flow cytometer (FACS Count System; Plate3.2, BD Biosciences, U.S.A). The BD CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry. Each capture bead in the kit has been conjugated with a specific antibody. The detection reagent provided in the kit is a mixture of phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-8, IL-1ß, IL-6 and TNF proteins. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

2.6. May-Grunwald Giemsa stained Blood Smear for enumeration of Schistocytes

The May-Giemsa stain is a type of Romanowsky stain that iscommonly used for staining air-dried cytological smears, blood, and bone marrow smears. Schistocytes are fragments of red blood cells that are often associated with microangiopathic hemolytic anemia (MAHA). The presence of schistocytes in the peripheral blood smear is an important diagnostic criterion for MAHA.

To enumerate schistocytes using the May-Giemsa stain, a thin smear of the blood sample was prepared and allowed to air dry. The smear was then fixed by immersing it in methanol for 5-10 minutes. The smear was stained in May-Giemsa working solution for 10 minutes and rinsed in pH 6.8 buffer. The smear was then stained with diluted Giemsa stain for 30 minutes, washed with distilled water, and allowed to dry. Schistocytes were detected in the peripheral blood smear stained using standard procedures and observed by microscopy. The number of schistocytes was counted relative to the normal red blood cells and expressed as a percentage.

2.7. Ethical considerations

The study was conducted under a protocol that was reviewed and approved by the Tropical Diseases Research Centre (TDRC) Ethics Review Committee and National Health Research Authority. Written permission was obtained from the Permanent Secretary in the Ministry of Health as well as from the Senior Medical Superintendent of Ndola Teaching Hospital and Levy Mwanawasa University Teaching Hospital. The study participants were informed about the study, its purpose, and their rights to participation. Privacy and confidentiality were maintained by using codes instead of names on the forms, lockable cabinets for storage, and password-protected computers. Only qualified medical professionals such as nurses and laboratory staff working in COVID-19 isolation centers were involved in collecting venous blood samples from study participants. Public health measures of social distancing and masking up to mitigate the transmission of COVID-19 was adhered to. Free masks were distributed to all the study participants. All research assistants were required to be fully vaccinated against COVID-19 and underwent a one week training in laboratory safety with a focus on COVID-19.

2.8. Data Analysis

Statistical analysis was performed using SPSS version 21, and the results were summarized in tables and graphs. All statistical tests were performed at a 5% significance level or 95% confidence interval with a p-value of less than 0.05 to determine statistical significance. The distribution of the data was analyzed using the Kolmogoroff-Smirnoff test. To analyze the frequency of Thrombotic Microangiopathy among SARS-Cov-2 patients and its correlation with disease severity, we used descriptive statistics such as frequency distribution to summarize our data. Chi-square test was used to determine if there was significant association between Thrombotic Microangiopathy and disease severity.

3. Results

Figure 2 report results of the frequency of Thrombotic Microangiopathy (TMA) in Sars-CoV-2 patients. The results show that out of a total of 173 Sars-Cov-2 positive patients 38 patients representing 22% had Thrombotic Microangiopathy while 138 patients representing 78% had no TMA.

Table 1 present results of the relationship between frequency of Thrombotic Microangiopathy (TMA) and Covid-19 disease severity. The table shows that none of the TMA positive patients were asymptomatic, while only 1 (3.1%) had a mild case of Covid-19. On the other hand, 6 (9.8%) TMA positive Covid-19 patients had moderate disease severity, while 20 (51.3%) and 11 (73.3%) TMA positive Covid-19 patients were in severe and critical condition, respectively. Chi-

square test showed that there was significant relationship between TMA and COVID-19 disease levels $X^2 = 61.8$; $p = 0.000$.

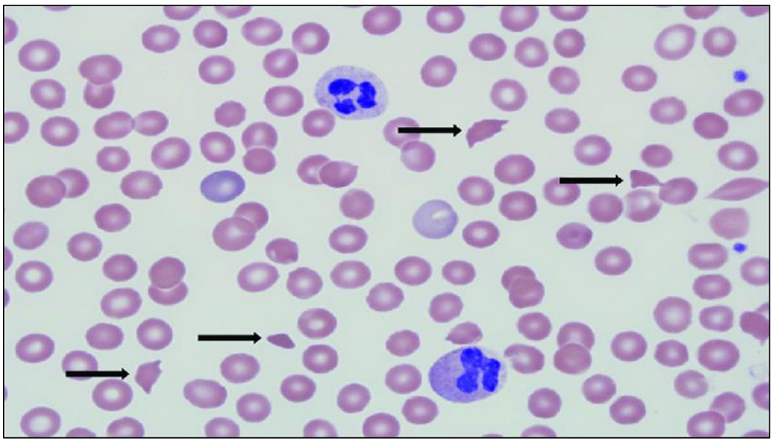

Figure 1 May-Grunwald stained Peripheral blood film showing classic findings of thrombotic microangiopathy. The image shows features of thrombocytopenia and microangiopathic hemolysis, original magnification × 50). Arrows show red blood cell fragments (schistocytes)

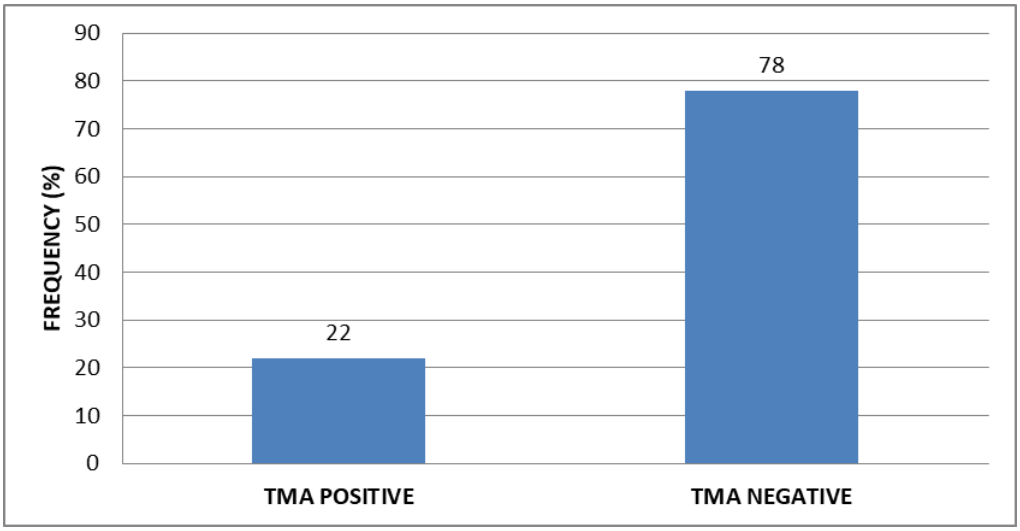

Figure 2 Frequency of Thrombotic Microangiopathy among SARS-Cov-2 patients

3.1. Relationship between TMA and COVID-19 Severity

Table 1 Frequency of TMA according to Covid-19 Disease Severity

	Asymptomatic	**Mild**	**Moderate**	**Severe**	**Critical**
	N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**
TMA Negative	26(100)	31(96.9)	55(90.7)	19(48.7)	4(26.7)
TMA Positive	0(0)	1(3.1)	6(9.8)	20(51.3)	11(73.3)
Total	26(100)	32(100)	61(100)	39(100)	15(100)

An independent sample t-test was conducted to compare VWF plasma levels in TMA Positive patients and TMA negative patients. The results indicate that mean VwF factor concentration for Sars-CoV-2 TMA Positive patients (39.25 IU/ml) was significantly higher than Sars-Cov-2 patients without TMA (10.14 IU/ml), t-value= 8.2; $P<0.05$.

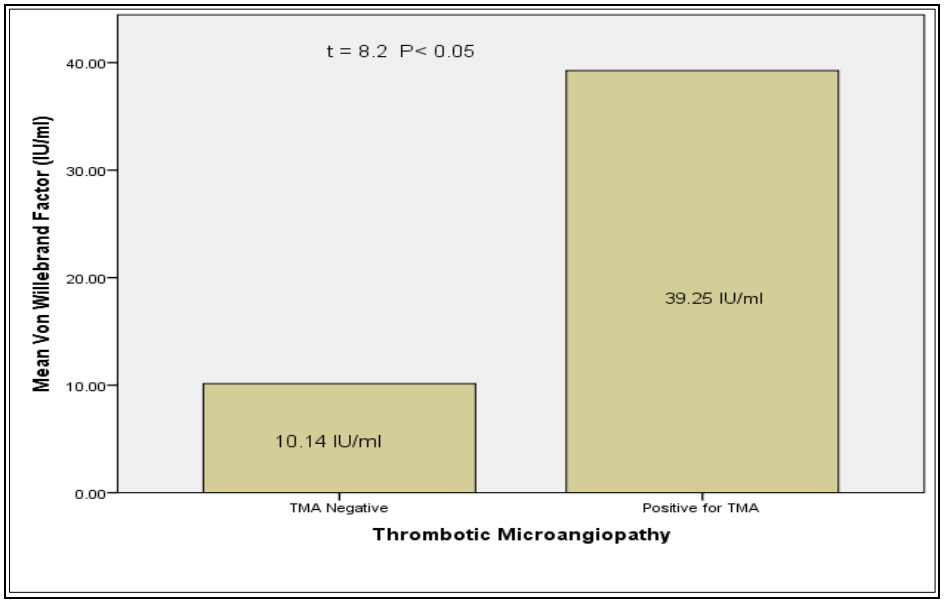

Figure 3 Mean VwF in COVID-19 Patients TMA Negative and Positive

Table 2 Independent T-test for analysis of Cytokine profiles in TMA Positive COVID-19 patients and TMA Negative COVID-19 Patients

Biomarker	TMA POSITIVE COVID-19 PATIENTS (ng/l)	TMA NEGATIVE COVID-19 PATIENTS (ng/l)	t-value	P-Value
IL-1	77.97±31.71	32.97±22.53	6.80	0.004
IL-6	64.92±32.43	27.01±25.5 2	5.56	0.000
IL-8	126.99±64.06	65.36±32.52	5.91	0.000
TNF	70.91±21.81	37.09±27.7 4	5.35	0.000

Table 2 show results of an independent sample t-test conducted to compare the Cytokine profiles in Sars-CoV-2 TMA Positive patients and TMA negative patients. The results indicate that mean concentration of Interleukin 1 (IL-1) in TMA Positive COVID-19 patients was higher (77.97±31.71 ng/l) than in the TMA negative COVID-19 patients (32.97±22.53 ng/l) and this differences in the concentration was significant, t = 6.80, P<0.05.

The table additionally shows that the mean circulatory levels of Interleukin 6 (IL-6) was increased in TMA positive COVID-19 patients (64.92±32.43ng/l) than the TMA negative patients (27.01±25.5 2ng) and the differences in the mean concentration of IL-6 between the TMA Positive COVID-19 patients and TMA negative patients was significant, t = 5.56, P<0.05.

The mean Interleukin-8 (IL-8) circulatory levels in TMA positive COVID-19 was significantly higher (126.99±64.06ng/l) than the TMA negative COVID-19 patient (65.36±32.52ng/l), t =5.91, P<0.05.

Similary, table 3 shows that mean circulatory Tumour Necrosis Factor (TNF) in TMA positive COVID-19 was significantly increased (70.91±21.81 ng/l) than in the control subjects (37.09±27.7 4 ng/l), t =5.35, P<0.05.

4. Discussion

TMA is a rare and potentially fatal disorder of the microvascular system, with an incidence of 1 to 3 per million (McFarlane et al., 2021). It is characterized by microvascular thrombosis, endothelial injury, organ dysfunction, and hematologic abnormalities such as thrombocytopenia and hemolytic anemia (Noutsos, et al., 2020). COVID-19 can induce TMA, which can worsen the prognosis and outcome of the infection. The main hypothesis of this cross-Sectional

study is that endothelial activation is associated with coagulation and microvascular thrombosis in COVID-19. Endothelial activation was determined by plasma values of VwF. The mean VwF in TMA Positive COVID-19 patients was significantly higher in TMA Positive COVID-19 patients in TMA negative COVID-19 Patients. This confirms our assumption that microthrombosis is normally triggered by the damage to endothelial cells which leads to an increased release of VwF into the blood circulation and this promotes adhesion and activation of the platelets.

We observed a 22% prevalence of TMA among COVID-19 patients in this study, with higher rates in severely and critically ill patients than in asymptomatic, mild, and moderately ill patients. Our findings are in agreement with previous studies (Jhaveri et al. 2020; Sweeney et al., 2020; Tiwari et al., 2021). However, they contrast with Falter et al. (2021), who reported no evidence of classic TMA in COVID-19 patients and suggested that TMA in severe COVID-19 is mainly confined to the alveolar microvasculature with a low blood pressure gradient. Nevertheless, Falter et al. had a smaller sample size of 22 COVID-19 patients, compared to our 173 COVID-19 patients, and a lower proportion of severely and critically ill patients. The current study further reported mean elevated VwF plasma concentration in TMA positive COVID-19 patients than TMA negative patients. Our results accords that of previous studies that reported an imbalance between VWF and ADAMTS13 showing markedly increased VWF antigen levels and VWF collagen-binding capacity but normal or slightly decreased ADAMTS13 activity, (Escher et al.,2020., Martinelli et al.,2020 & Morici et al.,2020) resulting in the formation of large VWF multimers, (Doevelaar et al., 2021., Turecek et al.,2021 & Sweeney et al., 2021)., with the latter biomarkers sometimes correlating with mortality (Morici et al.,2020). This VWF/ADAMTS13 imbalance may support the concept of a secondary TMA-like syndrome potentially present in some critically ill COVID-19 patients and contributing to the microthrombi formation in pulmonary alveolar capillaries (Doevelaar et al., 2021). Also, the link between a TMA process and the COVID-19-associated microthrombosis may be supported by the clinical improvement and the reduction of circulating thromboinflammatory markers (i.e., the VWF/ADAMTS13 ratio) induced by plasma exchange undertaken in some critically ill COVID-19 patients. Arulkumaran et al., 2021). One ex vivo study suggested that purified recombinant ADAMTS13 should be considered as a potential therapy for COVID-19 patients (Turecek et al., 2021).

Our search of the current literature revealed no study done to analyse inflammatory cytokines in TMA positive COVID-19 patients and TMA negative patients. However the current study revealed that the levels of inflammatory cytokines was higher in TMA Positive COVID-19 than TMA negative patients. Besides directly infected by SARS-CoV-2, the endothelial cells also undergo injury by systemic inflammation caused by over-activation of innate immune response, referring to “cytokine storm” (Hu et al., 2021;, 92 Huang et al., 2020). Severe COVID-19-induced cytokine storm (such as IL-6, IL-1β, TNF-α, MCP-1, etc) is a good predictor of the severity of COVID-19, which also aggravates multi-organ injury by propagating the vicious cycle of endothelial cells damage, inflammation and thrombosis (Huang et al., 2022).

The current study reported increased cytokines in COVID-19 patients with TMA than in TMA negative COVID-19 patients. Varga et al., (2020) reported evidence of direct SARS-CoV-2 infection of endothelial cells in several organs and diffuse endothelial inflammation associated with apoptosis in COVID-19 patients in Switzerland. Damage to the endothelium further exposes the sub-endothelial layer which is rich in collagen and initiates the intrinsic pathway of coagulation and the end result is formation of fibrin which leads to thrombosis. Endothelial cell damage within the pulmonary blood vessels increases the release of Von Willebrand factor (VWF) and this overwhelms the ADAMTS13, thus the ratio of ADAMTS13 to VWF keeps on reducing and this culminates in relative deficiency of ADAMTS13 and consequently increased Ultra Large Von Willebrand Factor (ULVWF) (Chen et al.,2018). ULVWF multimers secreted from endothelial cells are anchored to the cell surface as extraordinarily long string-like structures capable of inducing platelet adhesion and aggregation. This initial platelet adhesion to the subendothelial matrix at the site of vascular injury promotes a series of downstream signaling responses, which switch platelets from an inactivated to activated state. The change in platelet shape and platelet dense granule secretion promotes platelets to form aggregates within the microvasculature leading to Thrombotic Microangiopathies (TMA) (Tiwari et al., 2021). Activated platelets further release Plasminogen Activator Inhibitor (PAI-1) leading to hypo fibrinolysis. Destruction of the endothelium also results in loss of heparan sulfates at the surface of injured blood vessels, lack of generation of nitric oxide (NO), prostaglandin E2, and prostacyclin. These mediators are natural anti-aggregating agents and therefore loss of these mediators lead to increased platelet aggregations which may lead to microthrombosis (Friedman et al., 2014).

4.1. Treatment options for TMAs in COVID-19

According to the literature, the treatment of TMAs is based on four modalities: plasma exchange, immunosuppression, monoclonal antibodies, and management of the underlying cause. The lack of specific treatment for COVID-19 infection, higher mortality in TMAs associated with COVID-19, and availability of effective therapies for TMAs make a strong case for exploring potential treatment options in this specific group of patients. Although anticoagulants like Low Molecular Weight Heparin (LMWH) and rivaroxaban are cornerstones for treating and preventing venous thromboembolism, their

efficacy in managing or preventing TMAs is debatable. At this point, evidence supports the use of anticoagulants in critically ill COVID-19 patients to prevent large vessel thrombosis. However, their role in preventing TMAs is unclear and unelucidated. To date, limited evidence in the form of case reports/series is available for treating TMAs associated with COVID-19. The following are different treatment options available for such patients:

4.1.1. Immunosuppression

Traditionally, corticosteroids were used in combination with plasma exchange for the treatment of a spectrum of disorders, then called – Thrombotic Thrombocytopenic Purpura- Hemolytic Uremic Syndrome TTPHUS (Bell et al., 1991). Currently, they are the agents of choice to achieve rapid immunosuppression in patients with TTP and some cases of acquired Atypical Hemolytic Uremic Syndrome (aHUS) (Noris, & Remuzz, 2009). Mechanisms of their action in patients with TMA are as follows, 1. Suppression of acquired inhibitors to the ADAMTS13 (acquired TTP) Limitation of the endothelial inflammation, by attenuating the production of cytokines and decreasing the expression of adhesion molecules (Zielinska et al., 2016).

While corticosteroids are an important part of treating severe COVID-19 infection, patients with certain TMAs like TTP may need more aggressive immunosuppression with additional agents. Concurrent use of targeted immunosuppressants and corticosteroids puts patients at a significantly higher risk for infection and related complications. Rituximab is one of the commonly used immunosuppressants in the treatment of acquired-TTP. Lack of robust clinical data on the effect of immunosuppression by targeted molecules on COVID-19 patients further complicates the situation. Anecdotal cases of severe disease in patients on rituximab have been published (Guilpain et al., 2020).

4.1.2. Plasma exchange

Despite gaps in the understanding of the pathophysiology, TPE has shown benefit in the management of almost all types of TMA by replacing the defective/deficient proteins (ADAMTS13, complement, etc.) with a functional one (Winters et al., 2017). Therapeutic Plasma Exchange (TPE) has established efficacy in the treatment of TMA in multiple RCTs, with the most robust improvements being demonstrated in patients with TTP (Winters et al., 2017). All three patients reported in the literature having acquired TTP associated with COVID-19 responded well to treatment with TPE [38–40]. As such, the use of TPE in patients with COVID-19 who have also been clinically diagnosed with a TMA should be considered standard, just as it would be in patients without COVID-19. Some unique concerns that come up regarding TPE in patients with COVID-19 however include the question of whether it may have deleterious effects by removing any anti-SARS-CoV-2 antibodies that may be in circulation. This has not been proven or reported in any studies of TPE in COVID-19 so far, but the theoretical concern could be addressed to some extent by administering COVID convalescent plasma (CCP) in between TPE sessions although the limited availability of this product may preclude prolonged use. Ongoing randomized trials of TPE in COVID-19 patients may provide clearer answers to these questions. Besides its use in COVID-19 patients with TMA, the empiric deployment of TPE in severe COVID-19 in general, without documented TMA, has also suggested clinical benefit, presumably by removing inflammatory cytokines like IL-1, IL-6, GCSF, TNF and other deleterious elements (Khamis et al.,2020; Gucyetmez et al., 2020(.

4.1.3. Monoclonal antibodies

Caplacizumab is a bivalent, humanized immunoglobulin fragment used in the treatment of TTP. It binds to the A1 domain of the VWF, intercepting its interaction with the platelet glycoprotein Ib-IX-V receptor. By doing so, it blunts a pathway to microvascular thrombosis (Bae et al., 2022). The efficacy of Caplacizumab in the management of acute and refractory TTP is well established, and it is now FDA approved for the treatment of TTP (Scully et al., 2019) and was also included in the ISTH guidelines in 2020 for the management of this disease. The drug gains particular importance in the management of COVID-19 associated TTP as it is not an immunosuppressant (Bae et al., 2022). The American society of hematology (ASH) recommends its use in conjugation with TPE and corticosteroids if the patient has TTP in the setting of an active COVID-19 infection

5. Conclusion

Our study revealed an increased prevalence of TMA in COVID-19 patients at the two tertiary Hospitals and TMA was associated with disease severity in these patients. Currently treatment and prevention of thrombosis in COVID-19 patients involve the near-universal use of prophylactic or therapeutic doses of anticoagulation in such cases. However, the ineffectiveness of conventional anticoagulation in preventing thrombotic complications in a sizeable portion of patients suggests additional mechanisms such as TMA. These seem to be present in nearly a third of the patients and

would not be expected to respond to plain anticoagulation. Therefore, a rational stepwise approach to these patients is proposed, which involves definitive assessment for the presence of TMA and treatment effected accordingly.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The study was conducted under a protocol that was reviewed and approved by the Tropical Diseases Research Centre (TDRC) Ethics Review Committee and National Health Research Authority (NHRA). *Informed consent was obtained from all individual participants included in the study.*

Statement of informed consent

Informed consent was obtained from all the study participants.

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