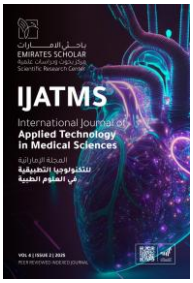

International Journal of Applied Technology in Medical Sciences
Vol 4 Issue 2 (2025)
Pages (26 - 37)

Available at
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Oxidized HDL as a Novel Predictive Biomarker in Conjunction with Selected Inflammatory Variables in Severe Dengue Fever Patients from Lahore, Pakistan

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ARTICLE HISTORY

Received: 16 August 2025.
Accepted: 14 September 2025.
Published: 29 December 2025.

PEER - REVIEW STATEMENT:

This article was reviewed under a double-blind process by three independent reviewers.

HOW TO CITE

Sarwar, M. ., Kamil, N. ., Ashraf, R., Ali, R., Majeed, R. ., Arif, S. ., Sohail, H. B. ., Khan, Z. ., & Rehan, M. . (2025). Oxidized HDL as a Novel Predictive Biomarker in Conjunction with Selected Inflammatory Variables in Severe Dengue Fever Patients from Lahore, Pakistan. *International Journal of Applied Technology in Medical Sciences*, *4*(2), 26-37. https://doi.org/10.54878/1ezq4e90

ABSTRACT

Background: Dengue fever is a deadly disease and represents one of the biggest threats to global health, with persisting uncertainty surrounding its prognosis and treatment standards. The onset of severe dengue fever, characterized by intense inflammation and the production of pro-inflammatory molecules, is currently the only well-established association with disease severity. Therefore, identifying and assessing both new and established biomarkers that can accurately predict the outcome of severe dengue fever is essential. Methods: In this study, 100 age-matched healthy controls and 100 hospitalized dengue patients positive for NS1 and IgM, with a mean age of 45 years (range: 22- 65), were examined. Potential biomarkers were analyzed using a Coulter counter, spectroscopy, and ELISA to determine their prognostic value in assessing dengue fever severityResults: Triglycerides and very-low-density lipoproteins (VLDL) were significantly higher in severe dengue fever patients compared to controls ($p<0.001$). Conversely, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and cholesterol levels were significantly lower in patients compared to controls ($p<0.001$). Albumin levels were 40.9% lower, lactate dehydrogenase (LDH) was 422.1% higher, and C-reactive protein (CRP) levels were 435.6% higher in severe dengue fever patients compared to controls. Unlike HDL, oxidized HDL (oxHDL) levels were 160.4% higher in patients with severe dengue fever compared to controls. Still, the absolute levels of oxHDL did not exceed total HDL levels, as confirmed by corrected data. Conclusion: Oxidized HDL, combined with other lipoproteins, may provide an ideal panel of prognostic indicators that could guide the treatment of severe dengue fever and serve as reliable biomarkers for predicting disease outcomes

Keywords: *Albumin, inflammatory markers, Ox HDL, lipid profile, dengue virus.*

1. INTRODUCTION

Globally, dengue virus (DENV) is currently the most common virus spread by mosquitoes. Similar to the genomes of other flaviviruses, the DENV genome consists of a single strand of positive-sense RNA that codes for three structural and seven nonstructural (NS) proteins [1]. In contrast to other flaviviruses, DENV comprises four serotypes (1-4) that exhibit genetic homology but differ in antigens. The female Aedes aegypti mosquito, prevalent in tropical and subtropical regions, is the primary vector of DENV [2]. Dengue fever affects over 2.5 billion people worldwide. The World Health Organization estimated that 200,000 cases of dengue hemorrhagic fever and 50 million cases of dengue fever occur annually [3]. Southeast Asia and the Western Pacific regions, inhabited by 1.8 billion people at risk of dengue fever, currently account for >75% of dengue fever-related disease burden worldwide [4]. This surge will grow further over the next few decades due to urbanization, travel, and climate change [5]. A small proportion of people develop complications from dengue infection, although most cases resolve without sequelae. Thus, many patients require regular evaluations to recognize complications early [6]. Accurate and timely identification of these individuals during the febrile phase could allow for providing appropriate care and management. Numerous researchers have investigated the possible predictive value of blood biomarkers for severe outcomes of dengue fever and whether biomarkers assessed during the acute phase could be associated with dengue fever severity. Consequently, the focus has shifted to finding ways to predict the course of the disease from the onset [7, 8].

Thrombocytopenia, or decreased platelets, is a clinical sign of dengue virus infection. Three distinct processes can cause thrombocytopenia: immune complex lysis, increased platelet consumption, or reduced platelet production [9, 10]. Nevertheless, the precise mechanism causing thrombocytopenia or platelet failure following dengue infection remains unclear. However, thrombocytopenia correlates with different predictable dengue severity biomarkers [8]. The detection of early biomarkers in the blood of dengue fever patients might help direct treatment. Certain biomarkers have been the subject of research, including C-reactive protein (CRP), lactate dehydrogenase (LDH), albumin, oxidized high-density lipoprotein (oxHDL), and lipid profile. The liver generates CRP, an acute-phase protein, six hours after inflammation starts [11]. CRP levels can fluctuate in several conditions, including cancer, inflammatory illnesses, burns, trauma, and infections. Certain studies suggested that measuring CRP levels could help determine the severity of dengue fever [7, 12]. Based on some evidence, individuals with dengue fever and dengue hemorrhagic fever might have elevated serum LDH, which functions as both a diagnostic marker and an independent predictor [13]. In many diseases, serum albumin levels decrease, making it a negative acute-phase reactant. Studies showed that serum albumin is an independent factor linked to both dengue fever mortality and severe dengue fever. Thus, serum albumin was an independent factor associated with severe dengue fever and dengue fever mortality in various studies [14].

DENV modifies lipid metabolism in the host cell. To evade the innate immune system, DENV creates a physical framework to assemble, multiply, and raise the local concentration of necessary cofactors, with virion secreting some complexes in the endoplasmic reticulum called replication complexes, which need phospholipids, cholesterol, and fatty acids [15]. There are a few reports on the relationship between dengue fever severity and lipoprotein changes in affected patients. Previous studies demonstrated that systemic lipid changes might modify the lipid microenvironment and be related to dengue fever symptoms [16]. Clinical research showed a connection between severe dengue infection, thrombocytopenia, and hemorrhagic symptoms, with lipid profiles representing crucial factors for consideration [17]. However, there are contradictory reports regarding lipoproteins, particularly HDL, in severe dengue fever patients [15, 18]. For clinical purposes, many of these indicators are either very short-lived or peak very late in

the disease course. As a result, we decided to add oxHDL, another unique and significant biomarker, to the current biomarker panel. oxHDL is pivotal in the onset and progression of atherosclerotic plaque and cardiovascular disease [19, 20]. A biomarker panel, including oxHDL, is expected to serve as an initial screening procedure for predicting the severity of dengue fever. Therefore, we selected CRP, LDH, albumin, oxHDL, and lipid profile as biomarkers in Pakistan. The proposed panel in this investigation, or oxHDL alone as an independent predictive marker, is expected to serve as a significant factor for determining the risks of complications in dengue fever and, consequently, appropriate management.

2. MATERIALS AND METHODS

The study was performed at the Centre for Research in Molecular Medicine, the University of Lahore, Pakistan

2.1 Sample Size

The sample size of 100 severe dengue fever patients and 100 healthy controls was determined based on previous studies investigating biomarkers in dengue fever and the availability of patients during the study period. A power analysis was conducted using G*Power software (version 3.1) to ensure adequate statistical power. Assuming an effect size of 0.5, a significance level (α) of 0.05, and a power (1-β) of 0.80, the calculated sample size was 64 participants per group. To account for potential dropouts and ensure robust statistical analysis, we increased the sample size to 100 participants per group. This sample size was deemed sufficient to detect significant differences in biomarker levels between severe dengue fever patients and controls.

2.2 Source of Data

The study included dengue fever patients to investigate the relationship between oxHDL and specific inflammatory markers in individuals with severe dengue fever specifically. One hundred dengue fever patients and 100 healthy controls were enrolled. They were chosen based on the exclusion/inclusion criteria from two tertiary care hospitals in Lahore.

2.3 Ethical Approval

The Ethical Committee of the IMBB, the University of Lahore, approved all study protocols, with Ref-IMBB/CRiMM/BBBC/CR/22/1021 as an ethical clearance number.

2.4. Selection of Patients and Sample Size

One hundred samples were taken from men and women of all ages diagnosed with dengue fever (NS1- and IgM-positive). Severe dengue fever was defined as thrombocytopenia with a platelet count <1,000,000/mm³ and a hematocrit increase <20% above baseline. An equal number of age- and weight- matched healthy controls were also included in the study. All patients were residents of Lahore, Pakistan. Participants voluntarily provided informed consent to participate in the study.

2.5. Inclusion Criteria

We recruited participants aged 20-70 years (males and females) who tested positive for both dengue NS1 and IgM.

2.6. Exclusion Criteria

The exclusion criteria comprised individuals with unconfirmed dengue fever, diabetes, heart disease, thyroid disease, cancer, and other infectious disorders, such as COVID-19 or HIV.

2.7. Blood Collection

A disposable syringe was used to extract 5 mL of venous blood from each patient's antecubital vein. Half of the extracted blood was transferred to a serum separator vial, whereas the other half was transferred to an ethylenediaminetetraacetic acid (EDTA) vial. Both vials were pre-labeled with patient identification numbers. The vial sample

was immediately centrifuged to separate the serum. The serum samples were stored at -70°C until use. The levels of oxHDL and other biochemical parameters investigated in this study were measured using serum specimens.

2.8. Biochemical Assays

OMEGA IV, Labomed, Inc., NY, USA, was used to enzymatically measure total cholesterol and triglycerides, expressing results in mg/dL [21]. The level of HDL, which was expressed in mg/dL, was measured following the precipitation of lipoproteins containing apo B (Human GmbH German). Friedewald et al.'s method [22] was used to measure low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL). The platelet count was calculated using an auto-analyzer (Beckman Coulter Counter, Coulters Corporation, FL, USA). The Alinity ci-series (Abbott, IL, USA) was used for bromocresol green colorimetry to measure albumin levels in g/dL. Alinity C (1200T) from Abcam was used to calculate LDH (mg/dL). CRP levels were measured using a human CRP ELISA kit (ab260058), Abcam. oxHDL was measured in mg/dL using an ELISA kit from Zokeyo, China.

2.9. Statistical Analysis

Data analysis was performed using SPSS 25.0. The normal distribution was employed to test the relevance of the parameters. An independent sample t-test was used to determine the significance of the determinants. The Pearson correlation was used to establish a relationship between the obtained results. The mean values of the patients were compared to the control group, and the following formula was used to get the total percentage increase or decrease: % Increase or decrease = (Mean of patients - mean of control) / mean of control × 100 The percentage of values of the dengue patients that were greater than the upper limit of the reference range was determined as below: % increase = [(mean value above higher reference range - upper limit of reference range) / upper limit of reference range] × 100 Similarly, the percentage of values data. Then (%) format is used to display some results, where n is the number of samples and % is the corresponding percentage of variables. A pertaining to the dengue patients that fell below the lower limit of the reference range was determined as follows: % decrease =[(mean value less than lower limit of reference range-lower limit of reference range)/ lowerlimitofreferencerange]×100[(meanvalueless thanlo werlimitofreferencerange-lower limit of reference range)/lowerlimitofreferencerange]×100[(meanv alueles sthanlowerlimitofreferencerange-lower limit of reference range)/lower limit of reference range]×100[(mean value less than lower limit of reference range-lower limit of reference range)/lower limit of reference range]×100

3. RESULTS

Severe dengue fever patients in this study were chosen from hospitalized subjects on the seventh day of their illness based on clinical and laboratory data shown in Table 1. Severe dengue fever was associated with plasma leakage, percentage of petechiae, and an increase in haemoglobin >20% over baseline. Remarkably, the majority of the severe dengue fever patients had thrombocytopenia, which is defined as having a platelet count of fewer than 150,000 platelets/ µL, the standard value. In our study population, there was a non-significant difference in the mean age between patients (Mean 44.76 ± 15.26) and controls (Mean 44.02 ± 16.62). Fig. (1) shows the lipid profiles for both groups. Severe dengue fever patients had considerably higher VLDL (74.9%) and triglyceride (66.58%) levels than controls ($p<0.001$). Conversely, there was a significant drop in HDL (33.09%), LDL (30.06%), and cholesterol (24.4%) levels in severe dengue fever patients compared to controls ($p < 0.001$). There has never been any research done on oxHDL as a dengue disease biomarker. Consequently, we examined oxHDL as a novel biomarker of severe dengue fever. As shown in Fig. (2), oxHDL was higher (160.43%) in patients compared to controls. However, the absolute levels of oxHDL did not exceed total HDL levels, as confirmed by corrected

correlation analysis between oxHDL and components of the lipid profile was performed. A strong relationship was found between LDL and

oxHDL (r = 0.484, p=0.007), whereas oxHDL was not related to the other components of the lipid profile. Pearson correlation analysis showed that triglycerides, HDL, and LDL exhibited a substantial relationship with each other (Table 2). In most of the patients, the percentage increase of triglyceride and VLDL relative to their normal upper limit of the reference range was 84 (58%) and 86 (48.7%), respectively. In the majority of the patients, the

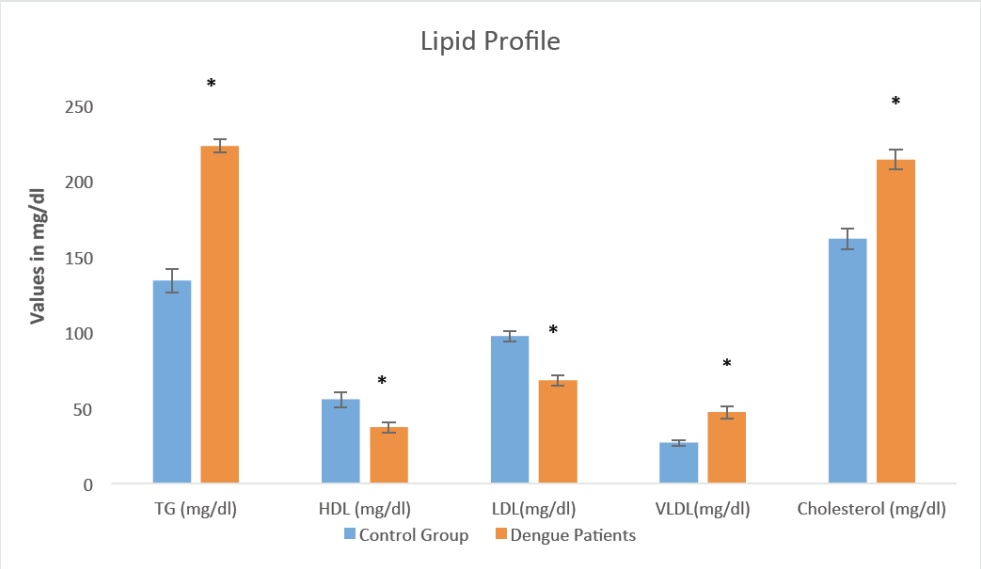

Fig. (1). Alterations in lipid profile in study participants *p<0.001. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

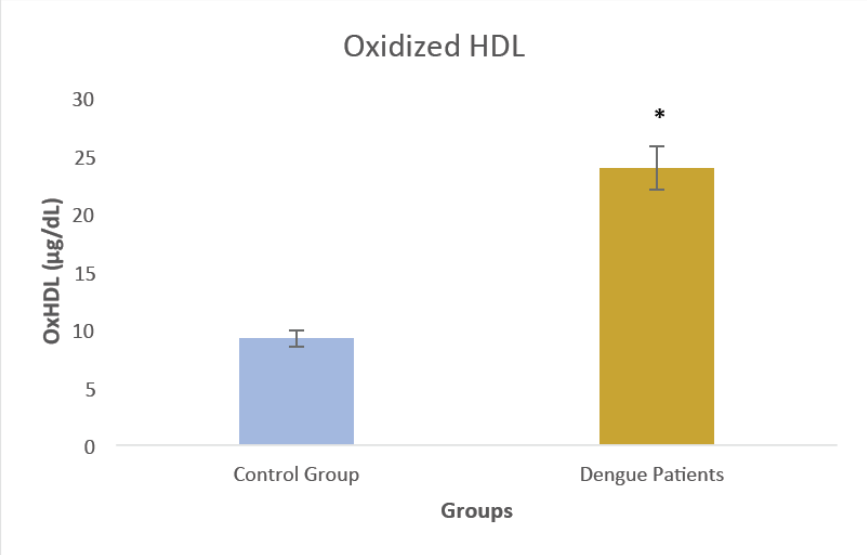

Fig. (2). Alterations in oxidized HDL levels in study participants *p<0.001. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Diagnostic criteria for severe dengue fever.

Parameter	Control (n=100)	Severe Dengue (n=100)	Normal Reference Range
Sampling Day	7th	7th	-
Hemoglobin (g/dL)	12.9 ± 1.2	15.6 ± 1.4	Male: 13.8-17.2, Female: 12.1-15.1
Hematocrit (%)	39.8 ± 3.1	45.0 ± 3.8	Male: 41-50, Female: 36-48
Platelets (10^3/µL)	160 ± 25	57 ± 18	150-450
ALT (IU/L)	38 ± 9	97 ± 15	<40
Petechiae (%)	0	74%	>20/square inch

Table 2. Correlation between oxHDL and lipid profile parameters.

Parameter	oxHDL	Triglyceride	HDL	LDL	VLDL	Cholesterol
oxHDL	1	0.226	0.053	0.484	0.185	0.113
p-value	-	0.230	0.781	0.007	0.328	0.554
Triglyceride	0.226	1	0.553	0.776	0.328	0.213
p-value	0.230	-	0.002	<0.001	0.077	0.259
HDL	0.053	0.553	1	0.401	-0.108	0.020
p-value	0.781	0.002	-	0.028	0.571	0.918
LDL	0.484	0.776	0.401	1	0.391	0.131
p-value	0.007	<0.001	0.028	-	0.033	0.491
VLDL	0.185	0.328	-0.108	0.391	1	0.177
p-value	0.328	0.077	0.571	0.033	-	0.350
Cholesterol	0.113	0.213	0.020	0.131	0.177	1
p-value	0.554	0.259	0.918	0.491	0.350	-

Note: Bold values indicate statistical significance at $p < 0.05$. Pearson correlation was applied after verifying normality distribution using the Shapiro-Wilk test. The correlation coefficient (r = 0.484) between oxHDL and LDL is reported, with a *p*-value of 0.007.

Table 3. Comparison of inflammatory markers between patients and controls.

Marker	Group	Mean ± SD	t-test	p-value	Reference Range
Albumin (g/dL)	Control	3.91 ± 0.39	18.93	<0.001	3.5-5.5 g/dL
	Patient	2.31 ± 0.24			
LDH (U/L)	Control	159.52 ± 10.65	-120.69	<0.001	140-280 U/L
	Patient	832.87 ± 28.64			
CRP (mg/dL)	Control	1.46 ± 0.26	-41.4	<0.001	0.9-4.8 mg/dL
	Patient	7.82 ± 0.80			
oxHDL (µg/dL)	Control	9.19 ± 0.73	-70.28	<0.001	-
	Patient	23.95 ± 1.85			

Note: $p < 0.05$ is considered statistically significant. Data are presented as mean ± standard deviation (SD).

Table 4. Pearson's correlation between oxhdl and other inflammatory markers.

Variable	oxHDL *vs* Albumin	oxHDL *vs* LDH	oxHDL *vs* CRP
Correlation (r)	0.037	-0.406	0.291
95% CI	-0.327 – 0.392	-0.668 – -0.053	-0.077 – 0.589
R^2 (Coefficient of Determination)	0.0014	0.165	0.085
p-value (two-tailed)	0.844	0.026	0.119
Significance (α = 0.05)	Not Significant	Significant	Not Significant
Number of Observations (n)	100	100	100

Note: Bold values indicate statistical significance at $p < 0.05$. oxHDL has a significant negative correlation with LDH, suggesting a potential mechanistic link in the inflammatory response.

percentage decrease of total cholesterol, LDL, and HDL compared to their lower limit of the reference range was 82 (25%), 79 (32%), and 86 (7.5%), respectively. In addition to the lipid profile, we also analyzed inflammatory markers. LDH, CRP, and albumin are the inflammatory indicators of severe dengue fever that are investigated in this study. Within the population under investigation, LDH and CRP were markedly elevated (p = 0.001), whereas albumin levels dropped in severe dengue fever patients compared to controls (p = 0.001). A substantial increase in oxHDL occurred in patients with severe dengue fever, whereas albumin was 40.9% lower, LDH was 422.1% higher, and CRP was significantly higher (435.6%) in severe dengue fever patients compared to controls (p<0.001). As mentioned earlier in this section, oxHDL was 160.4% higher in patients compared to controls (Table 3). The majority of patients had CRP and LDH levels that were higher than the upper limit of the reference range, which was 84 (62.7%) and 81 (197.45%). Conversely, most of the studied patients had an albumin percentage below the lower limit of the reference range, which was 87 (31.4%). Correlation of albumin, LDH, and CRP with oxHDL was assessed in severe dengue fever patients. Statistical significance was set at p < 0.05. Data showed an extremely weak correlation between albumin and serum oxHDL. However, a weak but insignificant correlation between oxHDL and CRP was identified. Furthermore, a statistically significant correlation between oxHDL and LDH was found in patients with severe dengue fever (Table 4). Previous studies demonstrated a relationship between circulating lipids and DENV infection. However, the association of altered lipid profiles and oxHDL with severe dengue fever remains unclear.

4. DISCUSSION

Dengue fever is a potentially fatal disease affecting a substantial part of the global population. Pakistan is endemic for dengue fever, with sporadic outbreaks. In this study, we investigated oxHDL, a novel prognostic marker, in conjunction with other inflammatory markers to assess the risk of severe dengue fever. We also examined lipid profiles and albumin levels in severe dengue fever cases. Prognostic predictors used previously are helpful but not robust tools for effectively managing dengue fever. In the current study, the average age of patients was 44.76 ± 15.26 years (range, 22-70 years), while that of controls was 44.02 ± 16.62 years (range, 20-70 years). The age difference between these participants was insignificant. Blood was collected and processed on the 7th day of hospital admission to analyze the biomarkers. In cases of severe dengue fever, serum HDL and LDL levels were lower, while triglycerides, VLDL, and total cholesterol levels were higher. These results validate the reasoning presented in a previous study that viruses target lipid signaling, synthesis, and metabolism and establish an ideal cellular environment for replication [23]. Despite some prior research suggesting an association between elevated HDL levels and severe dengue fever [24],

the current study found that HDL levels were significantly decreased in patients with severe dengue fever. Additionally, some studies demonstrated contradictory results regarding LDL in dengue fever. We found considerably decreased LDL levels compared to previous studies [25]. These variations in the association between HDL and LDL cholesterol and dengue severity might stem from differences in study populations. However, our study indicated that serum lipoprotein changes could be used as predictive markers to determine the severity of dengue fever. In our study, patients' serum LDH levels were significantly higher than those of controls. In severe disease, oxHDL was 161% higher than in controls. Additionally, patients' albumin levels were 40.9% lower than those of the controls. Furthermore, CRP and LDH levels dramatically increased by 435.6% and 422.1%, respectively, as previously reported [26]. Increased LDH levels represent a sign of a high viral load. Any infection or inflammation causes the liver to produce CRP, an acute-phase reactant. Infections caused by bacteria or viruses have been linked to elevated CRP levels [27]. When compared to controls, oxHDL was significantly higher in severe dengue fever. Although our knowledge of the roles that host lipids play in the life cycle of viruses has recently expanded, there is still much to learn about the function of oxHDL. However, it might be a reliable prognostic indicator of dengue fever severity. Pearson analysis revealed a significant correlation between oxHDL, LDL, and LDH, all of which were identified as biomarkers of dengue severity. Heart issues, such as non-specific electrocardio- graphic abnormalities, arrhythmias, myocarditis, peri- carditis, functional abnormalities, and atrioventricular conduction disorders, have been reported in people with severe dengue fever. After HDL oxidizes (oxHDL), it loses some capacity to trigger intracellular cholesterol release from foam cells. Accordingly, cardiac issues in severe dengue fever patients might also be related to increased oxHDL. Moreover, oxidative stress caused by DENV might be responsible for increased oxHDL. Therefore, we suggest that oxHDL is a better predictor of severe dengue fever than LDH and CRP. Non- steroidal anti-inflammatory drugs, statins, and magnesium supplements are among the medications that decrease CRP levels in severe dengue fever. On the other hand, severe dengue fever might be linked to elevated CRP levels due to other recent illnesses or injuries [28]. Similarly, LDH levels in patients with severe dengue might be mistakenly elevated if they are receiving anesthetics, aspirin, opioids, or certain other medications. Furthermore, medications containing ascorbic acid can lower LDH levels [29]. In this case, when combined with other panels of predictive bio- markers, oxHDL can be used as a reliable biomarker to assess the severity of dengue and its outcomes. As reactive oxygen species initiate peroxidation of HDL, the propagation of chain reactions occurs, thereby increasing oxHDL. Furthermore, oxHDL has a reduced capacity for cholesterol efflux and some other anti- atherogenic properties of HDL [30]. To our knowledge, this is the first study reporting oxHDL as a good predictive marker in severe dengue fever. Nevertheless, research on the mecha-nism of oxHDL action in dengue fever is still lacking. Our next research will focus on the function of oxHDL in the pathophysiology of dengue fever. The study also highlights the potential clinical implications of oxHDL as a predictive biomarker. Incorporating oxHDL into a panel of established biomarkers, such as NS1 and IgM, could enhance the accuracy of dengue severity prediction and improve patient outcomes. Future studies should explore the integration of oxHDL into clinical practice, particularly in resource-limited settings where rapid and reliable biomarkers are essential for timely intervention.

CONCLUSION

oxHDL might play a role in identifying early warning indicators of dengue fever outcomes. Lipid profile components related to oxHDL might also function as predictive markers for the prognosis of dengue virus infection. Similarly, CRP, LDH, and albumin might be helpful in Pakistani or other populations as early prognostic markers for severe dengue fever. The role of oxHDL as a predictive marker has never been studied before. An increase in oxHDL in dengue fever patients might represent a predictive biomarker and pathophysiological factor influencing the severity of dengue fever. oxHDL, rather than HDL, had a

statistically significant correlation with LDL and LDH. Moreover, oxHDL might be added to the list of already known biomarkers to help predict the severity of dengue fever, possibly improving patient care. However, the inadequate design and limited sample of the current study prevented it from clarifying the full extent of oxHDL function, requiring further research that will overcome these issues.

STUDY LIMITATIONS

The current study has several limitations that should be acknowledged. First, the sample size of 100 severe dengue fever patients and 100 controls, while sufficient for initial exploratory analysis, may limit the generalizability of the findings. A larger sample size, including asymptomatic and mild dengue cases, would provide a more comprehensive understanding of the role of oxHDL across the spectrum of dengue severity. Second, the study was conducted in a single geographic region (Lahore, Pakistan), which may introduce regional biases. Including patients from diverse geographic locations would enhance the external validity of the results. Third, blood samples were collected only on the 7th day of hospitalization, which limits our ability to observe dynamic changes in biomarkers throughout the disease course. Future studies should include multiple time points, such as the initial febrile phase and the critical phase, to better understand the temporal relationship between oxHDL and disease progression. Fourth, while the correlation between oxHDL and other biomarkers was analyzed, the underlying mechanisms remain unclear. Further mechanistic studies are needed to elucidate how oxHDL interacts with the inflammatory and lipid metabolism pathways in dengue fever. Finally, the study did not compare oxHDL with other established biomarkers, such as NS1 and IgM, which are commonly used in dengue diagnosis. Incorporating these comparisons would strengthen the clinical relevance of oxHDL as a predictive biomarker.

AUTHORS' CONTRIBUTIONS

It is hereby acknowledged that all authors have accepted responsibility for the manuscript's content and consented to its submission. They have meticu- lously reviewed all results and unanimously approved the final version of the manuscript.

LIST OF ABBREVIATIONS

NS	=	Nonstructural
DENV	=	Dengue Virus
EDTA	=	Ethylenediaminetetraacetic Acid
LDL	=	Low-density Lipoprotein
VLDL	=	Very Low-density Lipoprotein

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethical Committee of the IMBB, the University of Lahore, Lahore, Pakistan approved all study protocols, with Ref-IMBB/CRiMM/BBBC/CR/22/1021 as an ethical clearance number.

HUMAN AND ANIMAL RIGHTS

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or research committee and with the 1975 Declaration of Helsinki, as revised in 2013.

CONSENT FOR PUBLICATION

Informed consent was obtained from all participants in this study.

STANDARDS OF REPORTING

STROBE guidelines were followed.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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