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Original article

Cell-free DNA as a potential diagnostic biomarker in academic stress: A case-control study in young adults

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ABSTRACT

Background: Stress is a pervasive issue in modern life, affecting both physical and mental health. Identifying biomarkers like cell-free DNA (cfDNA) could provide insights into stress response and help detect individuals at risk for stress-related disorders.
Objective: The aim of this study is to investigate the potential use of cfDNA as a diagnostic biomarker in individuals experiencing stress.
Methodology: A case-control analysis was conducted using convenient sampling on university participants (N = 285 cases, N = 500 controls) aged 18–24. The study assessed haematological and lipid profile parameters using the Sysmex XP-300TM automated analyzer and an automated biochemistry analyzer, and cfDNA was extracted using a standardized in house developed Phenol-Chloroform protocol and estimated using Agarose Gel Electrophoresis and Nanodrop. Statistical analysis was performed using SPSS ver. 21.0.
Results: The results indicated a significant difference between stressed individuals and healthy controls in demographic, haematological and biochemical parameters. Specifically, stressed cases had significantly higher levels of cholesterol, LDL cholesterol, triglycerides, glucose, VLDL cholesterol, and lower levels of HDL compared to healthy controls. Stressed cases also showed significantly elevated levels of circulating cfDNA relative to healthy controls.
Conclusion: These findings suggest that cfDNA may have potential as a diagnostic biomarker for stress.

1. Introduction

DNA that is discovered in blood plasma, serum and other bodily fluids, but not in cell-associated DNA, is referred to as cell-free DNA, or cfDNA, also known as extracellular DNA (ecDNA) (Aucamp et al., 2016; Thierry et al., 2016). The aforementioned is present in plasma, serum, cerebrospinal fluid, saliva, and urine (Bronkhorst et al., 2021; Ponti et al., 2019). The precise biological origins of cell free DNA remain unclear; however, it is hypothesized that they may arise from processes such as active secretion, apoptosis, necrosis, or netosis (Bronkhorst et al., 2021; Grabuschnig et al., 2020). Wang et al. (2017) have posited that active secretion predominantly influences cfDNA concentration, rather than apoptotic or necrotic cell levels. cfDNA, usually found in serum and plasma, often rises after exercise and in conditions like burns, sepsis, trauma, and certain cancers. It's a broad indicator of health, spiking during stress or sepsis and after physical activity. This interest has surged for its potential as a non-invasive disease biomarker (Li et al., 2016; Salvianti et al., 2017; Stawski et al., 2021). Life's balance, influenced by internal and external factors, faces disruption during intense psychological stress, intertwining the immune, endocrine, and nervous systems (Chrousos, 2009; Chu et al., 2021). Genetic, epigenetic, and environmental differences shape individual responses (Mifsud and Reul, 2018). Persistent stress raises chances of physical symptoms and can escalate inflammation, potentially increasing the risk of conditions like coronary heart disease (McEwen, 2017; Wirtz and von Känel, 2017).

In response to stress, reactive oxygen species (ROS) are generated,

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leading to the phenomenon of oxidative stress (Chen et al., 2016). As DNA is particularly vulnerable to ROS, the presence of 8-oxodG and other oxidatively modified nucleosides becomes evident in both intracellular and circulating cell-free DNA (Di Carlo et al., 2012; Shimada et al., 2012). Oxidized circulating cell-free DNA (oxo-cfDNA) has been found to trigger the generation of ROS and elicit antioxidant and cytoprotective responses in cells (Shimada et al., 2012; Zhong et al., 2018). The nuclear factor erythroid-derived 2-like 2 (Nrf2) antioxidant pathway plays a crucial role in attenuating DNA oxidative damage, but excessive activation can be detrimental. The 8-oxoguanine DNA glycosylase 1 (OGG1) enzyme has the capacity to remove 8-oxodG from DNA, although excessive activation of the enzyme can lead to chromatin disintegration and cell death (Wang et al., 2018).

The prevalence of stress-related illnesses is on the rise in contemporary societies, resulting in significant social and financial ramifications (Chrousos, 2009; Lohmann-Haislah, 2012). Identifying reliable physiological and molecular indicators associated with stress has become increasingly vital. These markers can be utilized to predict an individual's capacity to cope with stress and manage challenging and unfavourable experiences (Walker et al., 2017). A potential biomarker for stress, aging, inflammation, and cell death has been identified as cfDNA (Hummel et al., 2018). This biomarker may serve as an indicator of acute stress and psychiatric disorders such as schizophrenia, and can also be used to assess the effectiveness of stress reduction methods (Czamanski-Cohen et al., 2014; Hummel et al., 2018; Qi et al., 2020). Various types of DNA are constantly released into circulation as "cfDNA" from different cells and organs (Lo et al., 2021; Moss et al., 2018). Elevated levels of cfDNA have been observed in individuals with conditions such as sepsis, autoimmune diseases, cardiovascular diseases, and cancer. Additionally, cfDNA levels have been linked to mortality prediction in middle-aged and elderly individuals (Kananen et al., 2020).

Physical exercise and chronic psychosocial stress have a significant impact on cfDNA levels beyond the realm of pathological conditions (Beiter et al., 2014, 2011; Fatouros and Jamurtas, 2016). Exercise and stress swiftly disrupt immunological balance, impacting various bodily systems. cfDNA emerges as a promising biomarker for psychosocial stress. Animals under mental stress and highly distressed pregnant women show elevated cfDNA levels. Stress-reduction interventions effectively lower cfDNA levels (Czamanski-Cohen et al., 2015, 2014). Additionally, increased concentrations of mitochondria and circulatory cell-free mitochondrial DNA (cfmtDNA) have been linked to stressful events, depression, and suicide attempts (Cai et al., 2015a, 2015b; Kageyama et al., 2018; Lindqvist et al., 2016).

Hummel et al. (2018) studied severe psychosocial and physical stress effects on circulating cell-free DNA (cfDNA). They found increased cfDNA levels after both stressors, especially shorter fragments post-exercise. Varying cfDNA methylation patterns between stress tests suggest its potential as a reliable stress biomarker (Hummel et al., 2018). In a rat study, exposure to various acute and long-term stress models examined oxidized cfDNA properties. Findings showed that oxidized cfDNA fragments prompted antioxidant responses and affected stress-associated neuronal systems (Filev et al., 2019). According to studies, there is a connection between psychological stress and inflammation. Furthermore, elevated levels of plasma cfDNA have been linked to inflammation in both genders, suggesting that reducing stress may lead to a decrease in cfDNA levels (Atamaniuk et al., 2012; Lamaita et al., 2012; Rohleder, 2012). Another study assessed Vitamin D3, Curcumin, Vitamin C, and Quercetin against BDNF (V66M), revealing favorable binding and stable interactions, offering drug development potential for stress-related conditions (Sakhawat et al., 2023).

The current analysis aims to evaluate the levels of cfDNA in both stressed and healthy young individuals in Pakistan, where data on this topic is scarce and to assess its potential as a diagnostic biomarker for stress.

2. Methodology

2.1. Study design

This case-control study was conducted at University of the Punjab and The University of Lahore from October 2022 to June 2023 and involved a sample of 1500 participants. Using the DASS scale-21, 285 participants were identified as experiencing stress, while 500 healthy young adults served as the control group. Stress levels were assessed through both self-reported measures and the validated DASS-21 scale (Szabo and Lovibond, 2022). Participants completed a questionnaire that included items related to perceived stress, life events, and stress symptoms. The inclusion criteria for the case group were individuals who tested positive for stress based on the DASS scale, young adults, and those who voluntarily agreed to participate and provided written informed consent. The control group inclusion criteria were good health and freedom from stress or disease. Individuals with pre-existing medical conditions and elderly participants were excluded from both the case and control groups. The research protocol was approved by the institutional ethical committee, and written informed consent was obtained from all selected participants.

2.2. Sample collection and clinical profiling of patients

The participants in the study were selected using the convenient sampling method. Venepuncture was used to collect blood samples, with clotted vials being used for serum and EDTA vials for whole blood. The CBC, lipid profile, and sugar characteristics of the blood samples were analyzed. Serum samples were collected and transported to the lab within one hour, where they were centrifuged for approximately 10 min at 3000 RPM to extract the serum. The serum was then stored at −80 degrees Celsius for future use. The Sysmex XP-300TM automated analyzer, which is capable of quantitatively analyzing several blood components including WBCs, RBCs, HCT, MCH, and MCV, was used to perform a complete blood examination (CBC). An automated biochemistry analyser was used to quantify key lipid parameters such as cholesterol, triglycerides, LDL, HDL, VLDL, and glucose.

2.3. DNA extraction

The in-house developed phenol–chloroform extraction method as per our previous study (Khurram et al., 2023) was employed to obtain DNA from the serum samples. To digest proteins in the WBC pellet, a mixture of proteinase K, Tris-EDTA-NaCl (TNE) buffer, and Sodium Dodecyl Sulfate (SDS) was added. The samples were incubated overnight at 37 °C, and then 87.5 µl of 6 M supersaturated NaCl was added to precipitate the proteins. After centrifugation, the top aqueous layer was transferred to a fresh tube, and an equivalent volume of isopropanol was added. Following 10 min of centrifugation at 3000 RPM, the supernatant was discarded, and the DNA pellets were collected. The DNA pellets were washed with 875 µl of 70 % ethanol, centrifuged at 3000 RPM for 10 min, and then incubated for 15 min with the pellets flipped on tissue paper to remove any remaining ethanol. The DNA was then dissolved in 100 µl of low TE buffer solution and incubated for one hour. To inactivate any remaining nucleases and proteinase K, the eppendorfs were immersed in a water bath for one hour at 70 °C. The DNA samples were stored at −40 °C to maintain their viability.

2.4. Estimation of cfDNA

2.4.1. Qualitative analysis

Agarose gel electrophoresis (Lee et al., 2012) was utilized for the qualitative examination of cfDNA. To create a 0.8 % agarose gel, 0.8 g of agarose was dissolved in 100 ml of 1X TBE buffer. The addition of ethidium bromide enhanced the visibility of the DNA. The gel was allowed to set for 45 min. In the gel's 15 wells, loading dye (2 µl) and

cfDNA samples (5 µl) were added. A DNA ladder was provided as a reference. The gel was subjected to an electrophoresis process in an electrophoresis tank containing 1X TBE buffer for 45 min under a 100-volt electrical field. The proper ionic flow was confirmed by observing the bubbles at the electrodes.

2.4.2. Quantitative analysis

The Thermo Scientific Nanodrop (García-Alegría et al., 2020) was utilized to assess the quantity of extracted DNA. The spectrophotometry method was employed, with a wavelength of 260 nm, to evaluate the absorption of light by nucleic acids. The Nanodrop instrument allows for rapid and precise quantification of DNA with only 1–2 µl of sample, taking only three seconds for the measurement.

2.5. Statistical analysis

SPSS version 21.0 software, developed by SPSS, Inc., was utilized for the statistical analysis. Means ± standard deviations as well as frequency (%) were used for the presentation of data. A Student *t*-test was employed to assess the significance difference between stressed-out individuals and healthy controls. The potential of cfDNA as a significant stress biomarker was evaluated using a chi-square test. Correlation coefficients and associated p-value (less than 5 % for significance) were calculated to find the relationship between cfDNA levels and clinical parameters. The area under the curve, optimal threshold value, and sensitivity were estimated through Receiver Operating Characteristic analysis (ROC).

3. Results

The present study encompassed 285 subjects who were diagnosed with stress according to the DASS-21 scale and 500 healthy individuals. The study's baseline and clinical variables, including age, gender, weight, and a plethora of haematological markers such as WBC, RBC, Hb, HCT, MCV, and MCH, are depicted in Table 1. In addition, the study assessed glucose levels and lipid profile, including cholesterol, triglycerides, HDL, LDL, and VLDL.

3.1. Baseline demographic and clinical parameters of participants

The participants in the case group had a higher mean age than those in the control group (23 years vs. 24 years, $p < 0.001$). The gender distribution was similar in both groups (74.3 % of the case group vs. 73 % in the control group, with a p value of 0.693). There were no significant differences in weight between patients and controls (74.14 kg in both groups, $p = 0.606$).

Haematological measurements revealed that the case group had significantly higher white blood cell count **(10^9/l)** (7.50 ± 5.149 vs. 7.948 ± 1.639/, $p < 0.001$), red blood cell count **(10^{12}/l)** (5.195 ± 0.658 vs. 4.93 ± 0.504, $p < 0.001$), haemoglobin levels (15.065 ± 1.899 g/dl vs. 13.93 ± 0.978 g/dl, $p < 0.001$), haematocrit levels (48.27 ± 6.909 % vs. 43.45 ± 5.127 %, $p < 0.001$), mean corpuscular volume (93 ± 10.602 fl vs. 90.48 ± 6.425 fl, $p < 0.001$), mean corpuscular haemoglobin (29.19 ± 2.960 pg vs. 27.20 ± 3.735 pg, $p < 0.001$), and mean corpuscular haemoglobin concentration (31.51 ± 2.280 g/l vs. 33.91 ± 1.531 g/l, $p < 0.001$). In contrast, the case group's platelet count **(10^9/l)** was significantly lower than that of the control group (269.4288.793 vs. 348.2638.921, $p < 0.001$).

The lipid profiles of patients and controls also showed statistically significant differences. The case group had higher levels of cholesterol (150.47 ± 38.681 mg/dl vs. 166.87 ± 24.240 mg/dl, p value less than 0.001), LDL cholesterol (91.77 ± 33.993 mg/dl vs. 56.29 ± 9.101 mg/dl, $p < 0.001$), triglycerides (109.13 ± 41.354 mg/dl vs. 136.05 ± 12.781 mg/dl, $p < 0.001$), VLDL cholesterol (23.37 ± 14.108 mg/dl vs. 18.59 ± 6.445 mg/dl, $p < 0.001$), and glucose (91.34 ± 10.375 mg/dl vs. 127.83 ± 14.231 mg/dl, $p < 0.001$). In contrast, the case group's high-density lipoprotein cholesterol levels were significantly lower than those of the control group (37.787.756 mg/dl vs. 83.7511.379 mg/dl, with p value less than 0.001).

Lastly, the level of cell-free DNA was significantly higher in the case group than in the control group (1092.300801.3466 ng/µl vs. 456.235470.862 ng/µl, $p < 0.001$).

3.2. Association between cfDNA and clinical biomarkers in stress participants

Student's *t*-test was conducted to examine the relationship between cfDNA levels and clinical biomarkers (as shown in Table 2). Participants were divided into two groups based on their cfDNA levels: <1000 and ≥1000. Significant correlations were observed between several biomarkers and cfDNA levels. The age of participants with cfDNA levels ≥1000 was higher than those with levels <1000 ($p < 0.001$), and the weight was favourably correlated ($p < 0.001$). Elevated levels of cell-free DNA (cfDNA) (≥1000) were found to be correlated with higher red blood cell (RBC) count ($p < 0.005$), hemoglobin levels ($p < 0.001$), and hematocrit levels ($p < 0.001$), as well as increased mean cell volume ($p < 0.001$), mean cell hemoglobin ($p < 0.001$), and decreased mean cell

Table 1
The Baseline and clinical parameters of participants.

Characteristics	Case n = 285 (36 %)	Control n = 500 (64 %)	P-value
Age(Years)	23 ± 17	24 ± 03	<0.001
Gender (Male) n(%)	211(74.3 %)	364(73 %)	0.693
Weight (kg)	74 ± 14	74 ± 14	0.606
WBC (10^9/l)	7.50 ± 5.149	7.948 ± 1.639	<0.001
RBC (10^{12}/l)	5.195 ± 0.658	4.93 ± 0.504	<0.001
Hb (g/l)	15.065 ± 1.899	13.93 ± 0.978	<0.001
HCT (%)	48.27 ± 6.909	43.45 ± 5.127	<0.001
MCV (fl)	93 ± 10.602	90.48 ± 6.425	<0.001
MCH (pg)	29.19 ± 2.960	27.20 ± 3.735	<0.001
MCHC (g/l)	31.51 ± 2.280	33.91 ± 1.531	<0.001
Platelets (10^9/l)	269.42 ± 88.793	348.26 ± 38.921	<0.001
Neutrophils(10^9/l)	52.58 ± 8.885	51.07 ± 6.382	<0.001
Lymphocytes(10^9/l)	36.24 ± 8.772	30.60 ± 6.085	<0.001
Cholesterol(mg/dl)	150.47 ± 38.681	166.87 ± 24.240	<0.001
Triglycerides(md/dl)	109.13 ± 41.354	136.05 ± 12.781	<0.001
HDL(mg/dl)	37.78 ± 7.756	83.75 ± 11.379	<0.001
LDL(mg/dl)	91.77 ± 33.993	56.29 ± 9.101	<0.001
VLDL(mg/dl)	23.37 ± 14.108	18.59 ± 6.445	<0.001
Glucose(mgl/dl)	91.34 ± 10.375	127.83 ± 14.231	<0.001
cfDNA(ng/µl)	1092.300 ± 801.3466	456.235 ± 470.862	<0.001

Table 2
Association b/w cfDNA and clinical biomarkers.

Biomarkers	CFDNA		P-value
	<1000 n = 123	≥1000 n = 162	
Age (years)	24 ± 12.7	22 ± 2.7	<0.001
Weight (kg)	74 ± 14.5	74 ± 15.2	<0.001
WBC(10^9/l)	7.89 ± 3.73	7.51 ± 2.15	0.077
RBC(10^{12}/l)	4.99 ± 0.54	5.13 ± 0.65	0.005
Hb(g/dl)	14.11 ± 1.26	14.95 ± 1.81	<0.001
HCT (%)	44.34 ± 5.77	47.43 ± 6.96	<0.001
MCV (fl)	90.71 ± 7.48	93.18 ± 9.85	0.001
MCH (pg)	27.59 ± 3.60	28.78 ± 3.47	<0.001
MCHC (g/l)	33.43 ± 1.97	32.05 ± 2.36	<0.001
Platelets(10^9/l)	328.96 ± 65.40	295.53 ± 83.81	<0.001
Neutrophils(10^9/l)	51.38 ± 6.93	52.24 ± 8.55	**0.185**
Lymphocytes(10^9/l)	31.80 ± 7.14	34.85 ± 8.53	<0.001
Cholesterol(mg/dl)	163.22 ± 28.84	154.94 ± 36.53	0.003
Triglycerides(mg/dl)	131.57 ± 24.95	112.53 ± 36.48	<0.001
HDL(mg/dl)	73.68 ± 21.56	49.91 ± 22.84	<0.001
LDL(mg/dl)	64.39 ± 23.70	81.56 ± 32.79	<0.001
VLDL(mg/dl)	19.86 ± 8.38	21.54 ± 13.78	**0.093**
Glucose(mg/dl)	120.52 ± 19.63	99.26 ± 19.68	<0.001

hemoglobin concentration ($p < 0.001$) in hematological measures. Additionally, participants with cfDNA levels ≥1000 had significantly lower platelet counts ($p < 0.001$)

In terms of lipid profile, those with cfDNA levels ≥1000 exhibited elevated levels of cholesterol ($p = 0.003$), LDL cholesterol ($p < 0.001$), and triglycerides ($p < 0.001$), as well as reduced levels of HDL cholesterol ($p < 0.001$). However, no significant correlation was observed for VLDL cholesterol levels. Furthermore, cfDNA levels ≥1000 were associated with increased glucose levels ($p < 0.001$).

3.3. Correlation analysis between clinical, laboratory parameters, and cfDNA levels in stress participants

The Table 3 displays the demographic and clinical factors of individuals experiencing stress, along with their correlation coefficients and p-values. It is notable that there were no significant correlations between cfDNA and demographic parameters. However, significant correlations were observed between cfDNA and clinical parameters, including WBC (correlation coefficient of 0.124 and p-value of 0.036), HB (correlation coefficient of 0.141 and p-value of 0.017), HCT (correlation coefficient of 0.147 and p-value of 0.013), MCV (correlation coefficient of 0.141 and p-value of 0.018), MCH (correlation coefficient of 0.136 and p-value of 0.022), platelets (correlation coefficient of 0.136 and p-value of 0.022), TG (correlation coefficient of −0.131 and p-value of 0.027), and glucose (correlation coefficient of −0.117 and p-value of 0.05).

3.4. Receiver operative curve (ROC) analysis

The results of the ROC analysis for cfDNA in stressed participants, as presented in Table 4, have been utilized to determine the area under the curve (AUC), ideal cut-off value, and sensitivity. The ROC graph, depicting sensitivity against 1-specificity, was plotted as Fig. 1. The AUC for differentiating stressed from healthy individuals amounts to 0.727, (with reference line or diagonal segments line), signifying moderate discriminatory power. The optimal cut-off for cfDNA concentration is 599.15 ng/ul, which strikes an equilibrium between sensitivity and specificity. Subjects with cfDNA levels surpassing this cut-off would be considered to be under stress. The sensitivity of the cfDNA test is 0.757, effectively detecting 75.7 % of stressed individuals. The selected cut-off value yields a Youden Index of 0.981, indicating high discrimination. The p-value, at <0.001, indicates a significant disparity in cfDNA levels between stressed and non-stressed individuals. These findings suggest that cfDNA levels may serve as a potential diagnostic biomarker for stress, though further investigation is necessary for confirmation.

Table 3
Correlation coefficients and associated p-values between the clinical, laboratory parameters, and cfDNA levels of the stress participants.

Biomarkers	Correlation coefficient	p-value
Age (years)	−0.024	0.687
Weight (kg)	0.046	0.442
WBC(10^9/l)	0.125	**0.036**
RBC(10^{12}/l)	0.049	0.409
Hb(g/l)	0.141	**0.017**
HCT (%)	0.147	**0.013**
MCV (fl)	0.141	**0.018**
MCH (pg)	0.136	**0.022**
MCHC (g/l)	−0.056	0.357
Platelets(10^9/l)	0.136	**0.022**
Neutrophils(10^9/l)	−0.045	0.455
Lymphocytes(10^9/l)	−0.043	0.475
Cholesterol(mg/dl)	−0.029	0.622
Triglycerides(mg/dl)	−0.131	**0.027**
HDL(mg/dl)	−0.098	0.092

Table 4
The optimal cut-off values and area under the curve for individuals with stress.

AUC	Optimum Cut-off Value	Sensitivity	Youden Index	P
0.727	599.15 ng/ul	0.757	0.981	<0.001

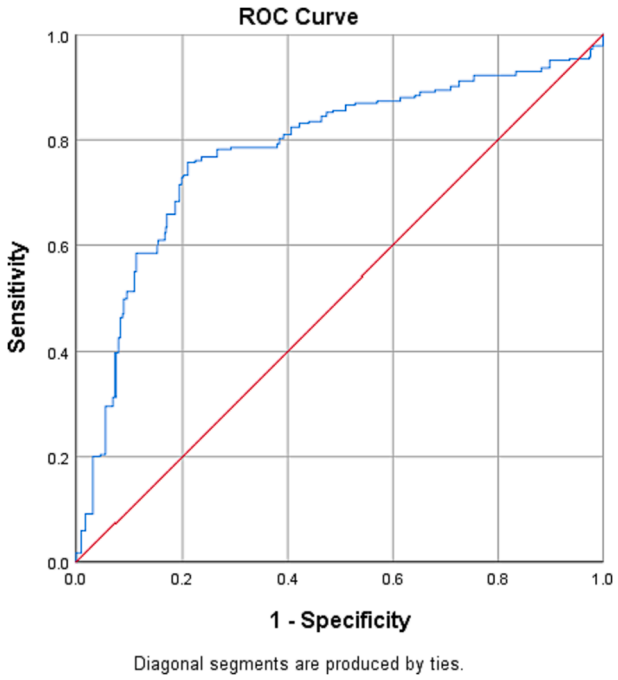

Fig. 1. ROC Analysis of cfDNA.

4. Discussion

The growing prevalence of stress-related disorders has imposed substantial social and economic burdens. It is crucial to identify reliable stress biomarkers to understand the connections between psychosocial stress and disease risk. Recent research suggests that increased levels of cell-free DNA, a potential biomarker for various pathological factors, exhibit a robust correlation with chronic stress (Hummel et al., 2018). The potential utility of cell-free DNA as a biomarker for diagnosing and managing stress-related disorders has been underscored by recent findings. Further investigation in this area is necessary in order to enhance our understanding and practical application of cell-free DNA as a biological marker for stress.

Psychiatric disorders exhibit varying levels of heritability, with anxiety and stress-related disorders, such as panic disorder and generalized anxiety disorder, displaying lower genetic influence (43 % and 32 %, respectively) (Docherty et al., 2016; Hettema et al., 2001). The etiology of these disorders is attributed to a complex interplay between genetic and environmental factors, with numerous gene variants across the human genome contributing to their risk (Pivac et al., 2009). Depression, on the other hand, demonstrates a genetic basis, with heritability estimates ranging from 25 to 45 % in the general population to 48–72 % in severe cases (Kendler et al., 2006; Muñoz et al., 2016; Uher, 2014; W. Wang et al., 2017). The presence of multiple brain-expressed genes suggests a polygenic nature, underscoring the importance of studying gene interactions in depression research (Mullins and Lewis, 2017). Kendler et al. (2018) found that both genetic factors and life experiences contribute equally to the risk of depression. Over 100 candidate gene studies have explored potential genetic links to depression, focusing on genes related to neurotransmission, stress regulation, neuroplasticity, inflammation, and the circadian system (e.g., *DRD3, DRD4, HTR1A, HTR2A, NR3C1, NR3C2, CRHR1, BDNF, IL1B, IL6,*

BMAL1, CLOCK). However, a meta-analysis of 183 studies revealed limited genetic polymorphisms associated with depression, including *APOE, GNB3, MTHFR, SLC6A4,* and *SLC6A3* López-León et al., 2008. Subsequent analyses confirmed associations with *5HTTLPR* and *MTHFR* C677T (Kiyohara and Yoshimasu, 2010; Wu et al., 2013).

The aim of the present investigation was to scrutinize the relationship between cell-free DNA (cfDNA) levels and stress, as well as to determine the correlation with several clinical and laboratory markers. The study's results yielded several significant revelations that enhance our understanding of the connection between cfDNA and stress. Initially, the study assessed the baseline demographic and clinical features. The case group, consisting of 285 individuals experiencing stress, was compared to a control group comprising 500 healthy individuals. The average age of the stressed individuals was 23 years, slightly older than the control group's mean age of 24 years ($p < 0.001$). However, there were no notable variations in weight or gender distribution between the two groups.

The evaluation of haematological parameters revealed significantly higher levels of white blood cell count, RBC count, Hemoglobin levels, hematocrit levels, MCH, MCV, and MCHC levels in stressed individuals compared to controls ($p < 0.001$). Conversely, platelet count was lower in the stressed group ($p < 0.001$). These results suggest that stress may have an impact on haematological variables and cause physiological alterations. Our investigation into the impact of stress on haematological markers aligns with the research conducted by Jern et al. (1989) which demonstrated increases in haematocrit, haemoglobin concentration, and blood cell count in response to emotional stress. Although leukocyte and platelet counts increased more than haemoglobin concentration, these findings provide valuable insights into the physiological responses triggered by emotional stress and the haematological changes that result from these responses (Jern et al., 1989).

In the present study, significant disparities were observed in the lipid profile analyses between the stressed and control groups. The stressed individuals demonstrated reductions in HDL cholesterol levels and elevated triglycerides, glucose, LDL and VLDL cholesterol levels (all p values < 0.001). Moreover, stressed individuals exhibited higher overall cholesterol levels. These results indicate a correlation between stress and dyslipidemia, a condition characterized by abnormal cholesterol levels that enhances the risk of cardiovascular diseases. The findings are consistent with a previous study that investigated the effects of physical activity and psychological stress on blood lipid profiles and revealed similar outcomes. The study highlighted how psychological stress increases the likelihood of lipid disorders, particularly larger ranges of low-density lipoprotein and triglycerides, and decreased levels of HDL cholesterol. Engaging in appropriate physical activity can attenuate the detrimental impact of stress on lipid profiles. These results underscore the importance of stress management and physical fitness in preventing lipid-related diseases in the general population (Assadi, 2017). The investigation focused on the assessment of stress, depression, and anxiety in Allied Health Sciences students, with a particular emphasis on the prevalence of mental health difficulties and altered lipid profiles in obese individuals. The study underscores the pressing necessity for mental health support in educational contexts (Haider et al., 2023).

The present investigation discovered significantly elevated levels of cfDNA in the stressed group relative to the control group ($p < 0.001$), which indicates its potential as a biomarker for stress-related conditions. This finding is consistent with a prior study that focused on acute psychological stress and observed a notable increase in cfDNA levels, suggesting its utility as a marker of neuroendocrine-immune activation (Herhaus et al., 2023).

The prior study's outcomes, which explored the influence of stress on blood cell parameters and lipid profile, align with our findings. They discovered that exam stress elevated triglycerides, total cholesterol, and very low-density lipoprotein levels, revealing a detrimental impact on lipid profile. Moreover, haematological measurements, including neutrophil count, platelet count, packed cell volume, lymphocyte count, and mean cell volume, were also affected by exam stress. These results underscore the impact of stress on both lipid and haematological profile markers (Subramanian et al., 2012). In this research endeavour, significant positive correlations were discovered between circulating cell-free DNA (cfDNA) levels and various haematological indices, including white blood cell (WBC) count, hemoglobin (Hb) levels, hematocrit (HCT) levels, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and platelet count ($p < 0.05$). Moreover, the levels of cfDNA were found to be substantially associated with cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides, while exhibiting a negative correlation with high-density lipoprotein (HDL) cholesterol (p value < 0.001). These findings suggest a connection between cfDNA levels and stress-induced physiological modifications and metabolic fluctuations.

The receiver operating characteristic (ROC) analysis of circulating cell-free DNA (cfDNA) demonstrated a moderate ability to differentiate between individuals experiencing stress and those who are not (area under the curve [AUC] = 0.727). The optimal cfDNA concentration cutoff of 599.15 ng/ul achieved a sensitivity of 0.757, successfully identifying 75.7 % of stressed individuals. The high Youden Index (0.981) indicates strong discriminatory abilities. The statistically significant p-value (<0.001) underscores the notable variation in cfDNA levels between stressed and non-stressed individuals. In light of these findings, cfDNA appears to hold potential as a non-invasive biomarker for stress, although further research is required to evaluate its therapeutic implications and enhance stress assessment and management.

5. Conclusion

The current findings conclude a significant association between cfDNA levels and a variety of clinical data, including haematological and biochemical markers in individuals under stress. Additionally, the ROC analysis of cfDNA indicated its limited ability to discriminate between stressed and non-stressed individuals. These findings suggest that cfDNA may be a useful and non-invasive method for screening stress and could serve as a potential diagnostic and prognostic biomarker for stress-related disorders.

Author contributions

WI performed the experimental procedures, WI and SK wrote the initial manuscript, SK and AI performed the statistical analysis and finalized the result. MUK and MAS supervised the whole research and critically finalized the manuscript. Final manuscript received approval from all the authors.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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