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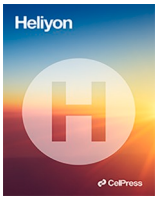

Research article

Analysis of the expression patterns of AVP, IGF-1, and TNF-α, APP, CD44, IFN-β IFN A β-6, α-syn, and NFL and CLU genes in generalized and focal seizures

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A B S T R A C T

Objective: The aim of our study was to investigate the relationship between clinical indicators and gene dysregulation in different types of epilepsy, while also seeking to identify a diagnostic model capable of distinguishing between focal and generalized seizures. This highlights the critical importance of understanding clinical indicators and gene dysregulation for targeted therapeutic interventions to effectively address the specific seizure types effectively.

Materials and methods: In this study, we conducted a comprehensive analysis of the peripheral blood of epilepsy patients (n = 100) and a control group (n = 51) to determine the differential gene expression. Our analysis involved a range of statistical approaches, including correlation analysis to establish the association between clinical indicators and gene dysregulation, and principal component analysis to highlight distinct disease group from control group. Furthermore, we developed diagnostic models using logistic regression to aid in the accurate diagnosis of epilepsy.

Results: Among several selected genes in this study such as *AVP* (AUC = 0.832, p < 0.0001), *IGF-1* (AUC = 0.658, p = 0.0015), *TNF-α* (AUC = 0.8970, p < 0.0001), *APP* (AUC = 0.742, p < 0.0001), *CD44* (AUC = 0.614, p = 0.021) and *NfL* (AUC = 0.937, p < 0.0001), and *CLU* (AUC = 0.923, p < 0.0001) have shown the outstanding discrimination. In addition to this, when all genes were included in the model, the overall diagnostic power increased significantly (AUC = 0.9968). A differential diagnostic model for focal and generalized seizures was established which discloses AUC = 0.7027, (95 % CL, 0.5765 to 0.8289, $p = 0.0019$).

Conclusion: The conclusions drawn from these findings represented that this is the first study to highlight the distinctive gene patterns of both focal and generalized seizures, implying that peripheral blood can serve as a diagnostic source to distinguish between these seizures types, aiding in the accurate classification of epilepsy. The findings from this study indicate a promising direction for investigating more targeted pharmacological interventions directed to address the distinct needs of both focal and generalized epilepsy, which offers advancements in treatment strategies for distinctive seizure types.

1. Introduction

Epilepsy is a non-communicable and chronic neurological disorder characterized by repeated seizures accompanied by a brief

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period of strange movements in a portion of the body, often the entire body, as well as loss of awareness and bowel and bladder abnormalities. Majorly, epilepsy is associated with stigma, psychological comorbidity, and substantial economic [1]. According to the World Health Organization's 2010 Global Burden of Disease research, epilepsy is the world's second-most burdensome neurologic illness in terms of disability-adjusted life years [2]. Because focal seizures originate in a specific area of the brain, they are also known as partial seizures. This might be due to focused damage caused by trauma, meningitis, or stroke [3]. When focal seizures progress to big occurrences, they begin to develop in another area of the brain and are transformed into tonic-clonic seizures [4]. Seizures with generalized onset help to raise neuronal discharges across the cerebral cortex. The most prevalent reason is an imbalance between inhibitory and excitatory synapses. It has been claimed that generalized seizures can arise because of an individual's heredity. A person who has a family member who has had generalized seizures is more likely to acquire them themselves, however, the degree of these seizures differs from person to person [5].

Around the world, almost 50 million people suffer from epilepsy [6]. Every year, an estimated 5 million people worldwide are diagnosed with epilepsy [7]. The incidence of epilepsy is thought to be greater in the youngest and oldest age groups. There was no difference in the point prevalence of active epilepsy or the cumulative incidence between high and low–income nations. The active yearly period emergence, on the other hand, was much greater in low–income nations. Surprisingly, more middle-income nations (14/50; 28 %) reported an active point prevalence of more than 10 per 1000 people than high-income countries (2/13; 15 %). The prevalence of epilepsy was unaffected by age, gender, or research quality. In low to middle-income nations, the active yearly period prevalence, lifetime emergence, and incidence rate of epilepsy were greater. The greatest documented occurrences were epilepsies of undetermined cause and generalized seizures [8].

Epileptogenesis is a set of chemical, physiological, and anatomical processes that are triggered by brain damage and which can result in spontaneous seizures. A variety of insults, including febrile seizures, traumatic brain injury (TBI), infection, prenatal or perinatal injuries, brain tumors, congenital abnormalities, autoimmune, or genetic disorders and genetic factors lead to the development of epileptogenesis [1,9]. Epilepsy is one of the most prevalent and debilitating neurological illnesses in the world. Although the causes of epilepsies vary, epilepsy appears to be a progressive illness, linked with cognitive impairment, deterioration of other neuropsychiatric comorbidities, and the development of pharmacoresistant [10–12].

Despite the need for substantial research to find a validated or marked link between focal or generalized epilepsy, the validation and clinical shreds of evidence remain unfulfilled. Therefore, this work employed gene expression profiles from patients diagnosed with epilepsy predominantly in 1–25 age group, to assess the distinguishing elements between the disease group and the healthy group. To achieve this goal, a selected gene panel which includes *Clusterin*, amyloid precursor protein (*APP*), neurofilament light (*NfL*), tumor necrosis factor-α (*TNF-α*), *Tau*, arginine vasopressin (*AVP*), Insulin-like growth factor-1 *(IGF-1)*, Presenilin1 (*PSEN1*), α-synuclein *(α-syn*), interferon-beta (*IFN-β*) and Cell-surface glycoprotein-44 (*CD44*) was used to identify new markers or previously established one in literature, in Pakistani population. In Pakistan, no similar research has ever been conducted.

In this study, we selected several genes that are known to be involved in specific aspects of neuronal disintegration (*NfL, α-syn, APP, PSEN1, and CLU*), neuroinflammation (*IL-6, IFN-β, TNF-α, and CD44*) and neuromodulatory effect (*IGF-1 and AVP*). Our hypothesis was that the expression levels of specific target genes could be correlated with clinical parameters, providing potential predictors for different types of epilepsies.

2. Methods

2.1. Study settings and design

We conducted a prospective study at the Punjab Institute of Neuroscience in Lahore, Pakistan. The diagnosis and classification of epilepsy were carried out following the criteria outlined in the 2017 International Classification of Epileptic Syndromes [5]. We employed a case-control study design to investigate the determinants of epilepsy among patients between January 2020 and June 2020. The study encompassed all patients meeting the study criteria, both epilepsy patients and control subjects, attending the hospital's outpatient, inpatient, and emergency departments during the designated study period.

This study's sampling techniques were carried out in accordance with the Ethical Review Committee's (ERC-70-2021) recommendations and were approved by the Institutional Review Board (IRB-307/07-2021-B) of Forman Christian College. All the subjects signed a written permission form. The research was conducted in the outcome of the Helsinki Declaration.

2.2. Study participant-recruiting criteria

Out of the one hundred epilepsy cases studied, a majority of ninety-four patients had experienced a seizure within the last 24 h, while a minority of six cases reported being seizure-free for either the past year or month. Notably, all patients included in the study had developed epileptic seizures before experiencing secondary complications at the time of sample collection.

The selection of patients for the research study was based on well-defined inclusion and exclusion criteria. Specifically, individuals aged 18 or above with complete clinical documentation available at the time of sample collection were chosen for participation. Patients diagnosed with epilepsy underwent a comprehensive evaluation, including a thorough medical history review, physical and neurological examinations, electroencephalograms (EEG), magnetic resonance imaging (MRI) brain (1.5 or 3 T) and computer tomography (CT-Scan). Conversely, individuals under the age of 18, individuals with other neurological or psychiatric conditions that could potentially confound study results and a history of individuals with significant head trauma unrelated to epilepsy were all excluded from the study.

The control group comprised healthy individuals of matching age who visited headache clinics for routine examinations. These individuals did not report current acute headache episodes or have a history of febrile seizures, epilepsy, or acquired demyelinating diseases of the central nervous system (CNS). The recruitment of healthy control subjects was conducted in accordance with the methodology described in our previous publication [13].

2.3. Blood sampling and RNA extraction

Vacutainer vials were used to collect peripheral venous blood. All the patients had seizures in 24 h during the time of sample collection; however only 6 samples did not reciprocated the same observation. Obtained samples were centrifuged at 1300 g for 10 min for serum collection. For serum collection, obtained samples were centrifuged at 1300 g for 10 min. Within 2 h, the serum was transferred to a polypropylene microtube and kept at −80 °C until further examination. For the processing of RNA, PBMCs were isolated using density gradient centrifugation (Sigma, Poole, UK) and then later stored in 500 μL Trizol reagent (MRC, Catalog No. RT111, USA) followed by 500 μL of Chloroform and 0.5 m Acetic acid. The tube was centrifuged at 15,000 rpm for 15 min at 4 °C. Following the removal of the top phase into a clean microfuge tube. To precipitate, an equivalent amount of absolute isopropanol was added and stored at −20 °C. The samples were then centrifuged at 4 °C for 10 min at 13,000 rpm. Clear pellets were collected and cleaned twice in 70 % ethanol. Finally, the pellets were air-dried and re-suspended in RNAse-free water.

2.4. qRT-PCR analysis

Quality of extracted RNA was assured using NanoDrop (Thermo Fisher, USA). The RNA quality was determined using a 0.7 % of agarose gel. A gel documentation equipment (Thermo Fisher, USA) was utilized to view the bands on a gel. Primers were synthesized using NCBI Blast (Table 1). The RevertAid First Strand cDNA Synthesis Kit (Catalog No. K1622, USA) from Thermo Fisher Scientific was used to synthesize cDNA from the total RNA. Before the qRT-PCR, the gradient thermocycler was used to adjust the conditions for each gene (Applied Biosystem, USA). The quantitative analysis was carried out using the (CFX96, Bio-Rad, USA) according to the instructions supplied. The SYBR green I qRT-PCR kit provides the reaction mixture (Thermo Fisher, USA). Melting curve analysis was done after each run to ensure that the primers were specific. The gene expression levels were standardized to the reference genes using the relative quantification approach. Data obtained were normalized using housekeeping genes HPRT1 and GAPDH. The following formulas were used to assess the change between calibrator and sample of interest: Fold change $= 2^{-\Delta\Delta Ct.}$ [14] and Logfold_2 change = Log (Fold change, 2) [15].

2.5. ELISA

In the present study, serum levels of AVP and IL-6 were quantified according to the given instructions provided by the manufacturer (Zokyo ELISA kits, Wuhan, China). The volume of 50 μL of the standard solution and samples of interest were added to the appropriate wells. The plate was sealed with a closure membrane and incubated for 60 min at 4 °C. Then HRP conjugate reagent 200 μL was added and was incubated for 60 min at 37 °C. Subsequently, the plate was uncovered; the liquid was discarded, and was washed thrice by washing buffer. A 50 μL of Chromogen solution A and 50 μL of Chromogen solution B was added to each well for 30 min in dark at 37 °C. In the end, 50 μL of stop solution was added. Finally, the plate was measured in a microplate reader at 450 nm absorbance

Table 1
Primers used in this study.

PRIMERS	GENES
F: TGTCCAAGATGCAGCAGAAC	*APP*
R: ACACCGATGGGTAGTGAAGC	
F: ACTTTTGCAGCTTCCTTCCA	*PSEN1*
R: TTGACCTCGTCCCTCAAATC	
F: CTTGATGCCCTTCTCTCCGT	CLU
R: GGTCATCGTCGCCTTCTCGT	
F: GTGCTCAGTTCCAATGTGCC	*α-syn*
R: CAGTGAAAGGGAAGCACCGA	
F: CACCAAGCAGTGCTGCATAC	AVP
R: TCGGGCAGTTCTGGAAGTAG	
F: GGCTGACCAAGCTGAAACTC	IGF-1
R: ATCGCTTAAACCCAGGAGGT	
F: GCCTCAAGGACAGGATGAAC	*IFN-β*
R: AGCCAGGAGGTTCTCAACAA	
F: AGCTGCCAAGGAAGAGTCTG	*NfL*
R: AAGGAAATGGGGGTTCAATC	
F:ATCAGGAGACCTGCTTGATG-R; TGGTGGCTTTGTCTGGATTC	IL-6
F:CTTCTCCTTCCTGATCGTGG	*TNF-α*
R:GCTGGTTATCTCTCAGCTCCA	
F; ACAACTGGTGATGGAGACTCATCC	CD44
R; GATTCCAGAGTGGCTTATCATCTTGG	

immediately.

2.6. Data analysis

Graph prism V9.0 was used to do statistical analysis (GraphPad Software Inc., San Diego, CA, USA). A comprehensive power analysis, performed using G*Power 3.1.9.7 software, confirmed that the study was adequately powered to detect significant differences. To compare the differences between the two groups, the Mann-Whitney *U* test was utilized. Continuous data are displayed with the mean and standard deviation (mean S.D.). To investigate the association between dysregulated gene expression and clinical outcomes, correlation matrices were generated using the Spearman rank correlation opted after finding the distribution of data using the Kolmogorov–Smirnov test of normality. Before Principle Component Analysis (PCA), the dataset underwent transformation, including Z-score normalization and mean centering, to optimize it for analysis. To study a single discriminating capacity of each gene in the disease and control groups, logistic regression was used to generate receiver operating characteristic (ROC) curves. Multiple logistic regression was also performed to assess the prediction potential of all differentially expressed genes combined. Finally, a differential diagnostic model was also constructed using logistic regression analysis to differentially diagnose focal seizure from generalized seizures. All statistical tests were two-tailed, with a p-value of 0.05 selected as the significance threshold.

3. Results

3.1. Patients characteristics

The demographic features of the patients and healthy individuals are shown in Table 2. One hundred epileptic patients and 51 control individuals were included in this study. The selected epileptic patients had an average age of 26.4 ± 11.0 (mean ± S.D). There were 42 female epileptic patients and 58 male epileptic patients in this research. The mean duration of illness was 7.0 ± 7.0 years (mean ± S.D) and the age of onset was 18.5 ± 13.3 years (mean ± S.D). Generalized seizures were observed in 68 patients meanwhile 26 patients were experiencing focal seizures. In 68 % of instances of generalized seizures, 55 patients had generalized tonic-clonic seizures, and 13 patients had generalized myoclonic seizures. There were 14 cases of Focal aware seizures and 12 cases of focal seizures. Only six individuals had no active seizures in the previous month Table .3. When looking into the disease-modifying treatment of recruited patients, the majority of the included participants were using anticonvulsants such as 81 % were taking Valproate, 17 % were taking Clonazepam, and 33 % were taking Phenytoin. Levetiracetam, Lamotrigine, and Valproic Acid were used by 53 %, 18 %, and 38 % of the participants respectively. Carbamazepine and Pregabalin, a neurotic pain reliever, were used by 37 % and 26 % of the enlisted people. Patients using anti-anxiety medications such as Topiramate and Diazepam were 55 percent and 61 percent. Different etiologies of recruited patients are described in Table .3. Total 25 cases had a history of Head injury/cranial trauma and 1 case had genetic etiologies with active focal seizures. Meanwhile, 37, 10 and 6 patients with generalized seizures had a etiologies such as family history, encephalopathies and encephalitis. On the other hand, 21 patients had idiopathic etiologies. Several recruited patients had developed other comorbidity within last 3 months such as 22 patients had stroke, and 10 patients developed vascular brain anomalies. In addition, one patient had glioma and other was case of pediatric acquired demyelinating disorders (MS). It was observed that all recruited patients had epileptic seizures before having secondary complications at the time of sample collection (Table 4).

3.2. Dysregulation in expression of selected gene

The peripheral blood mononuclear cells (PBMCs) mRNA expression data of chosen genes i.e. Clusterin (*CLU*), amyloid precursor protein (*APP*), neurofilament light (*NfL*), tumor necrosis factor-α (*TNF-α*), arginine vasopressin (*AVP*), Insulin-like growth factor-1 (*IGF-1*), Presenilin1 (*PSEN1*), α-synuclein (*α-syn*), interferon-beta (*IFN-β*) and Cell-surface glycoprotein-44 (*CD44*) of hundred patients with epileptic seizures and fifty-one control group with no history of neurological disorders were analyzed. The serum and PBMC levels of *AVP* and *IL-6* have shown increased expression (Fig. 1a). Both of these are well-studied biomarkers of epilepsy in literature.

Upon investigation, it was found that all the selected genes were upregulated in PBMCs of recruited patients. High dysregulation was recorded in *Clusterin* and *NfL* ($p > 0.0001$). However, α-synuclein showed a non-significant upregulation ($p = 0.3389$). Significant dysregulation was observed in *APP, IL-6, TNF-α, Tau, AVP, IGF-1, PSEN-1*, and *CD44* (Fig. 1b). After the establishment of the dysregulated profile, we also opted for another statistical approach called principal component analysis (PCA). Subsequently, combination resulted in two distinguishing clusters (Fig. 2a). This clustering was mainly attributed to *CLU, NfL, TNF-α APP,* and *AVP* (Fig. 2b).

Table 2
Demographic features of eplieptic patients.

Demographic attributes	Recruited patients (n = 100)	Control (n = 51)
Gender ratio	42F/58 M	14 M/37 M
Age at onset (mean ± S.D)	19.4 ± 12.8	–
Age (mean ± S.D)	26.47 ± 11.0	29.89 ± 8.6
Disease duration (mean ± S.D)	7.0 ± 7.0	–

Table 3
Clinical attributes of epileptic patients.

Clinical characteristics	Recruited patients (n = 100)
Age of onset	
1-5	10
6-10	17
11-15	18
16-20	24
21-25	12
26-30	4
31-35	5
36-40	6
41<	4
Types of seizures observed	
Generalized Seizures	68 %
Generalized Tonic Clonic Seizure	55 %
Generalized Myoclonic Seizure	13 %
Focal Seizures	26 %
Focal Aware Seizure	14 %
Focal Seizure	12 %
Seizure Free	6 %
Treatment administered	
Valporate	81 %
Clonazepam	17 %
Phenytoin	30 %
Carbamazepine	37 %
Levetiracetam	53 %
Valporic Acid	38 %
Pregabalin	26 %
Lamotrigine	18 %
Topiramate	55 %
Diazepam	61 %

Table 4
Different etiologies of recruited patients.

Etiology		Number of patients (%) (Type of seizures)
Symptomatic	Head injury/cranial trauma	25 % (FS*)
	Family history of epilepsy	38 % (1 FS, 37 GS**)
	Encephalitis	6 % (GS)
	Encephalopathies i.e. Dravet syndrome and Lennox-Gastaut syndrome	10 % (8 + 2, GS)
Idiopathic	Unknown	17 % (GS)
	Undocumented	4 % (GS)
Later developed other comorbidity		
Stroke		22 %
Glioma		1 %
Vascular brain anomalies		10 %
Acquired demyelinating disorders (MS)		1 %

Focal Seizures*.
Generalized Seizures**.

3.3. Correlational analysis of genes and clinical attributes

After describing the different levels of expression, we looked at the interconnectedness of the genes with clinical characteristics. Upon investigation, it was found that only *IFN-β* and *α-syn* had shown correlation with clinical features. Negative correlation was found between *α-syn* and disease duration ($r = -0.141$, $p = 0.1641$) but it was non-significant (Fig. 3a). Non-significant positive correlation was found between *IFN-β* and disease duration ($r = 0.151$, $p = 0.1232$) (Fig. 3 b). As well, seizure type and disease duration had shown notable negative association ($r = -0.2168$, $p = 0.030$) (Fig. 3c). Finally, a significant negative correlation was established between *IFN-β* and age of onset ($r = -0.1964$, $p = 0.050$) (Fig. 3d). Several investigated genes have shown remarkable association with each other. For an instance, *AVP* has shown significant association with *IFN-β* ($r = 0.3351$, $p = 0.0010$), *α-syn* ($r = 0.284$, $p = 0.004$), *TNF-α* ($r = 0.255$, $p < 0.001$) and *CD44* ($r = 0.260$, $p = 0.009$). In addition, *IGF-1* has shown notable correlation with *CLU* ($r = 0.428$, $p < 0.0001$), *α-syn* ($r = 0.463$, $p < 0.0001$), *IL-6* ($r = 0.280$, $p = 0.005$), *PSEN1* ($r = 0.288$, $p = 0.004$), and *IFN-β* ($r = 0.196$, $p = 0.05$). Moreover, *α-syn* was also found to have indicating the significant positive correlation with *TNF-α* ($r = 0.301$, $p = 0.002$), *IL-6* ($r = 0.311$, $p = 0.002$), *APP* ($r = 0.353$, $p < 0.0001$), *PSEN1* ($r = 0.198$, $p = 0.049$), *IFN-β* ($r = 0.304$, $p = 0.002$), and *CD44* ($r = 0.303$, $p = 0.002$). Likewise, *IL-6* and *PSEN1* ($r = 0.242$, $p = 0.015$), *IL-6* and *IFN-β* ($r = 0.262$, $p = 0.008$), *IL-6* and *CD44* ($r = 0.376$, $p = 0.005$), *APP* and

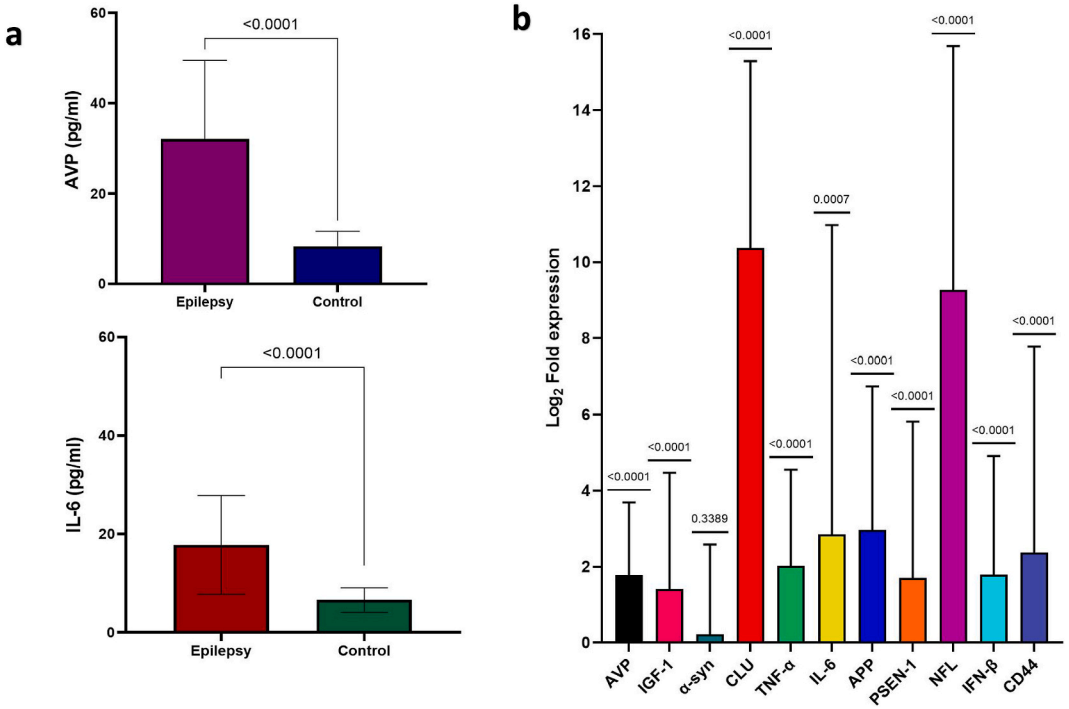

Fig. 1. Establishment of distinct gene expression profiles. Profiles of differentially expressed genes **(a)** depicts serum levels of AVP and IL-6 in epileptic patients and control group. Different patterns of dysregulated genes are revealed in **(b)** Boxplots with Log_2fold values. Individual plot is depicted in.

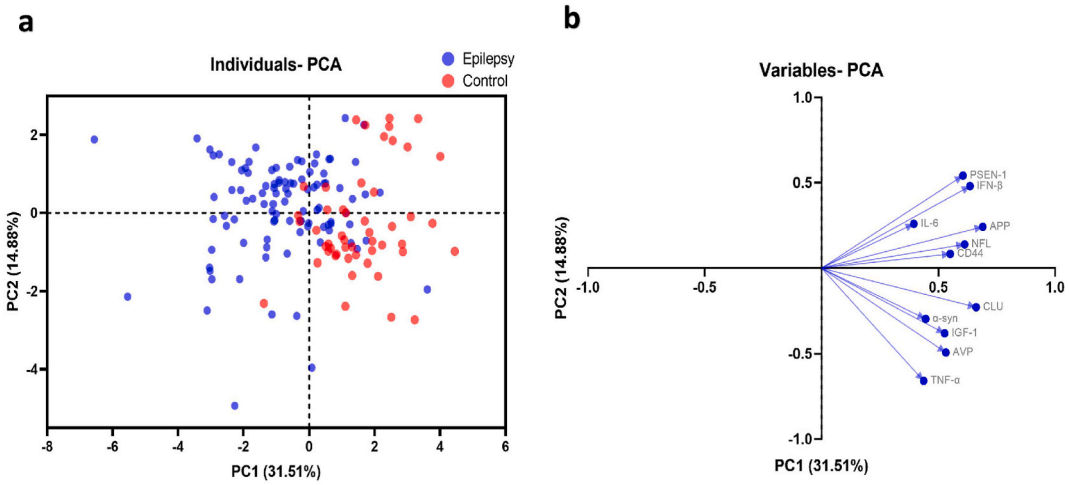

Fig. 2. Principal Component Analysis. Distinct clusters were formed **(a)**, successfully distinguishing illness samples from control samples. Dots represent samples, and each colour represents a group of people who were recruited. Panel **(b)** depicts a variable plot. Each contributing gene is represented visually by an arrow. The longer the arrow, the greater its influence on the variance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PSEN1 ($r = 0.430, p < 0.0001$), *APP* and *IFN-β* ($r = 0.436, p < 0.0001$), *APP* and *NfL* ($r = 0.201, p = 0.045$), *PSEN1* and *IFN-β* ($r = 0.398, p < 0.0001$), *PSEN1* and *CLU* ($r = 0.298, p = 0.003$), *NfL* and *CD44* ($r = 0.297, p = 0.005$), and *IFN-β* and *CD44* ($r = 0.202, p = 0.044$) have shown significant correlation with each other. However, non-significant association was found between *IGF-1* and *APP* ($r = 0.183, p = 0.069$), *α-syn* and *Clusterin* ($r = 0.154, p = 0.127$), *IL-6* and *APP* ($r = 0.187, p = 0.063$), *NfL* and *IFN-β* ($r = 0.185, p = 0.065$) and *TNF-α* and *CD44* ($r = 0.190, p = 0.058$).

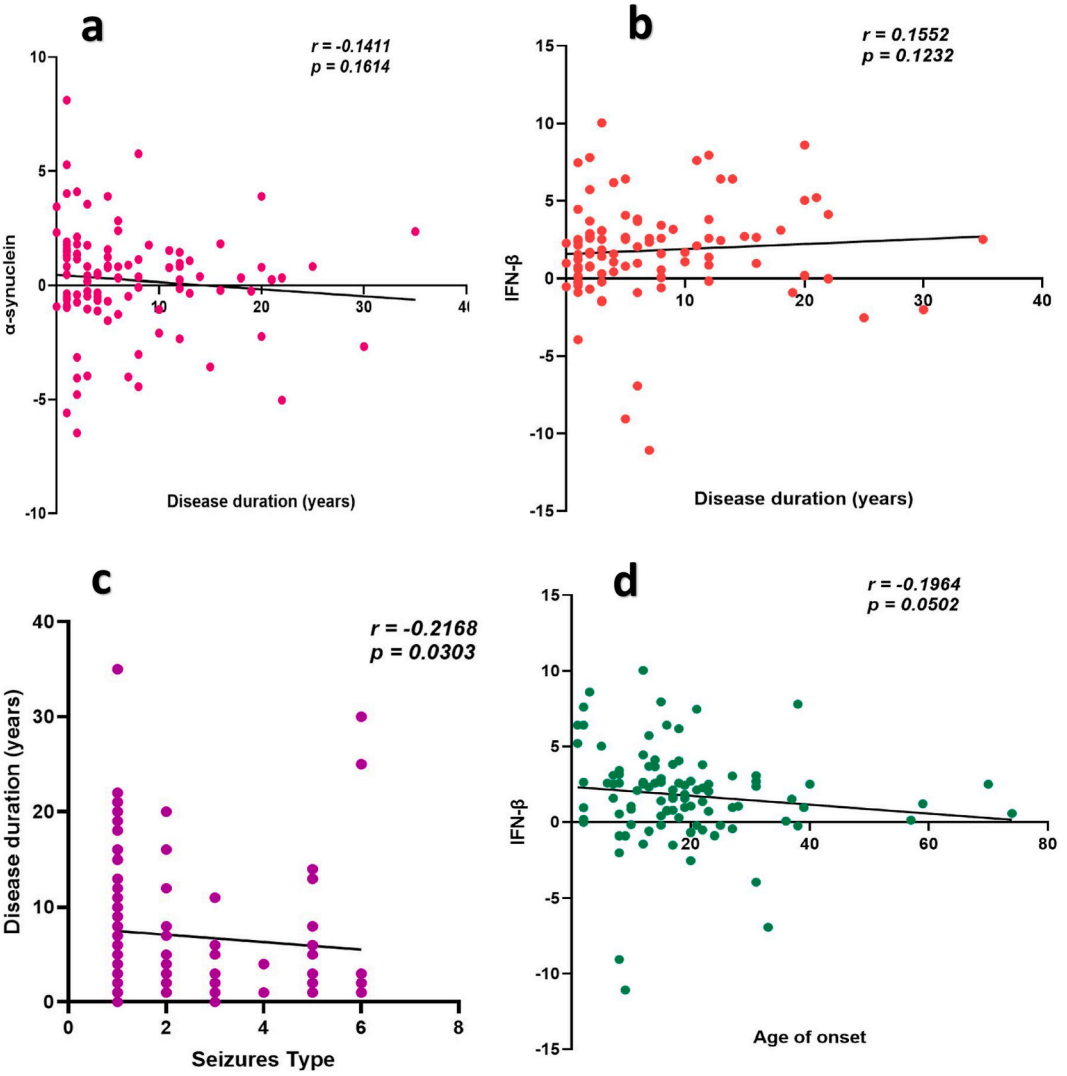

Fig. 3. Correlational analysis between differentially expressed genes and clinical variables. A non-significant (**a**) Negative correlation was found between *α-syn* and disease duration ($r = -0.141$, $p = 0.1641$). Non-significant positive correlation was found between *IFN-β* and disease duration ($r = 0.151$, $p = 0.1232$) (Fig. 3 b). As well, seizure type and disease duration had shown notable negative association ($r = -0.2168$, $p = 0.030$) (Fig. 3c). Finally, a significant negative correlation was established between *IFN-β* and age of onset ($r = -0.1964$, $p = 0.050$) (Fig. 3d).

3.4. Potential discriminatory markers

The area under the curve (AUC) of Receiver operating curve using logistic regression analysis was used to evaluate the discriminating between disease group and control group. The AUC is used to measure the diagnostic test quality. The AUC was used to assess each predictor's ability in distinguishing between groups of participants. Several genes had shown a distinguishing discriminatory *AVP* (AUC = 0.832, $p < 0.0001$), *IGF-1* (AUC = 0.658, $p = 0.0015$), *TNF-α* (AUC = 0.8970, $p < 0.0001$), *APP* (AUC = 0.742, $p < 0.0001$), *CD44* (AUC = 0.614, $p = 0.021$), and *IFN-β* (AUC = 0.668, $p = 0.001$). Most outstanding discrimination was observed in *NfL* (AUC = 0.937, $p < 0.0001$), and *CLU* (AUC = 0.923, $p < 0.0001$) (Table .5). Combining all genes raised the AUC values to 0.9968, thus strengthening the diagnostic power (Fig. 4).

3.5. Differential diagnostic model

By integrating the dysregulated expression of eleven genes, including Clusterin (*CLU*), Amyloid Precursor Protein (*APP*), Neurofilament Light (*NfL*), Interleukin-6 (*IL-6*), Tumor Necrosis Factor-α (*TNF-α*), Insulin-like Growth Factor-1 *(IGF-1*), α-Synuclein, Presenilin-1 (*PSEN-1*), Cell-surface Glycoprotein-44 (*CD44*), Interferon-Beta (*IFN-β*), and Arginine Vasopressin (*AVP*), we established differential diagnostic models for patients with generalized epilepsy and focal epilepsy. Utilizing logistic regression, our analysis

Table 5
Parameters tested to determine the diagnostic values.

Gene	[a]AUC	*p*-value	95 % Cl	[b]PPV%	[c]NPP%
AVP	**0.832**	**<0.0001**	**0.7619–0.9032**	**80.00**	**77.61**
IGF-1	**0.658**	**0.0015**	**0.5698–0.7475**	**14.29**	**65.28**
TNF-α	**0.8970**	**<0.0001**	**0.8383–0.9557**	**90.24**	**87.27**
APP	**0.742**	**<0.0001**	**0.6610–0.8241**	**75.00**	**74.07**
PSEN1	0.543	0.328	0.4494 to 0.6377	–	66.23
CD44	**0.614**	**0.021**	**0.5528 to 0.7042**	**45.45**	**67.14**
IFN-β	**0.668**	**0.001**	**0.5765–0.7596**	**75.00**	**71.11**
NfL	**0.937**	**<0.0001**	**0.8996–0.9757**	**84.78**	**88.57**
CLU	**0.923**	**<0.0001**	**0.8830–0.9644**	**78.00**	**88.12**
α-syn	0.536	0.465	0.4417–0.6311	–	66.23
IL-6	0.594	0.498	0.5054–0.6828	20.00	66.21
All-gene combination	**0.9968**	**<0.0001**	**0.9906–1.000**	**98.00**	**99.00**

*Significant results of independent variables are in bold.
[a] Area under the curve.
[b] Positive predicative value.
[c] Negative predictive value.

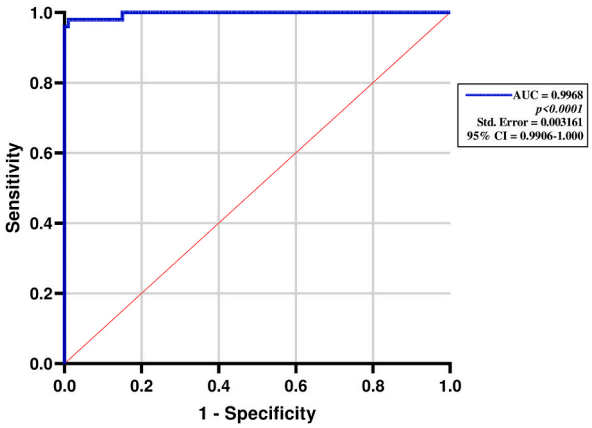

Fig. 4. ROC curve evaluation to discriminate between disease group and control group. Logistic regression of all predictors (genes) was performed to assess the discriminating power between the disease group and the control group.

revealed that the differentiation of generalized epilepsy from focal epilepsy was dependent on the expression profiles of these genes, with each gene acting as an independent variable. The calculated odds ratio for the model was 0.2773 (95 % CI, 0.06003 to 1.158), suggesting a reduced likelihood of generalized epilepsy compared to focal epilepsy. However, the wide confidence interval indicates a

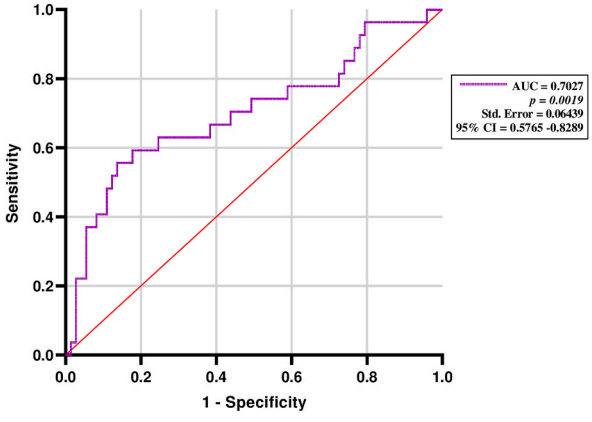

Fig. 5. ROC curve analysis
Selected 11 genes differentially diagnosing between focal and generalized epilepsy.

level of uncertainty regarding the strength and direction of this relationship. Moreover, our model exhibited a positive predictive power of 60.0 % and a negative predictive power of 74.0 %. The area under the curve (AUC) was statistically significant at $p = 0.0019$ (95 % CI, 0.5765 to 0.8289) with a standard error associated with the odds ratio. It is important to note that the number of samples used in this analysis significantly influences the generalizability of these findings and should be considered in the interpretation (Fig. 5).

4. Discussion

Status epilepticus causes an increase in *α-synuclein* expression in the dentate gyrus of pilocarpine-induced epilepsy animal models and in the hippocampus of patients with mesial temporal lobe epilepsy [15–17]. Levels of *α-synuclein, TNF-α, IFN-β,* and *IL-6* were found to be higher in epilepsy. Another study conducted by Ref. [18] found similar results, demonstrating upregulated α-synuclein levels in children with epilepsy and acquired demyelinating disorder. A-synuclein is one of the most abundant proteins within neurons of brains, which regulates the release of neurotransmitters and vesicle transport. When its oligomers started from its increased synaptic transmission and long-term potentiation, leading to cognitive problems and neurological problems [19]. Therefore, higher levels of α-synuclein in epilepsy explain the synaptic dysfunction. Furthermore, the investigation of CD44 levels, which play an integral role in alternative splicing and post-translational modifications [20], revealed upregulation in all recruited patients of this study. CD44 overexpression is linked to considerable brain infiltration of blood-borne inflammatory myeloid cells as well as memory $CD4^+$ and $CD8^+$ T lymphocytes. Furthermore, peripherally derived innate and adaptive proinflammatory immune responses have a substantial pathogenic role in the pathogenesis of intractable epilepsy [21].

Neurofilament light chain protein *(NfL)* is part of the neuronal cytoskeleton. NfL is released by neurons particularly when axonal damage occurs, in several neurodegenerative diseases [22]. In this study, levels of *NfL* were found to be significantly upregulated in patients with all epilepsy etiologies. The findings of this study were consistent with the results of another study [23]. Evidence has indicated that increased serum levels of *NfL* were highly correlated with prolonged febrile seizures. Findings of this study indicated that there was significant link between seizure occurrence and the degree of neuronal damage in epileptic patients [23]. On the contrary, another report when explored the serum *NfL* levels in children with febrile seizures found that after the convulsion, *NfL* levels were not increased, nevertheless, they did note the link between recurrent seizures and an increased risk of expressive language delay at the age of 2.5 years [24].

IGF-1 plays a constitutive role in promoting neuronal survival by suppressing apoptosis and enhancing lymphocyte production [25]. In this study, the expression levels of *IGF-1* were found to be substantially higher in all patients. These results were consistent with the findings of one study [26] while inconsistent with findings of another study [27], which disclosed the low *IGF-1* levels were because of severe intractable epilepsy. Another study elucidated the dualistic nature of *IGF-1*. They concluded that *IGF-1* was behaving as a neuroprotector after brain injury and its long-term activation had shown a pro-epileptic effect by causing neurotoxicity. They elaborated that prolonged *IGF-1* exposure increases an ictal event-mediated cell death [26].

Neuropeptides are reported which have a prominent role in altering neuronal excitability and act as an endogenous in different kinds of epilepsies [28]. Among them, *AVP* is considered to increase the susceptibility to seizures supported by several evidence [29&30]. In this study, AVP levels were investigated and found to be significantly higher in epileptic patients of all etiologies. The results produced were consistent with the findings of two other studies conducted independently [24&31]. *Clusterin* is a chaperone protein that majorly regulates cell apoptosis [32]. *Clusterin (CLU)* is a well-known prognostic and diagnostic biomarker of Alzheimer's disease [33,34]. In our study, epileptic patients have shown upregulated levels of *CLU*. The role of *clusterin* is still under investigation. Only one report, conducted by Yu et al. [35], investigated the expressions of CLU in epileptic patients and found that serum CLU levels were significantly lower in the drug-resistant group compared to the drug-responsive group. *CLU* is also known for its role in inhibiting the aggregation of amyloid-β (Aβ) peptides formed by Amyloid β precursor protein (*APP*). *APP* is a larger membrane protein that has an essential role in neuronal growth and repair. *A*PP proteolysis, catalyzed by enzymes such as PSEN-1 (β-secretase) and PSEN-2 (γ-secretase), leads to the formation of clusters of amyloid-β (Aβ) peptides in Alzheimer's disease [36]. PSEN1 is the multi-transmembrane protein that regulates immune cells differentiation. It helps to leave the larger peptides of Amyloid β into smaller ones [37]. The levels of *APP* and *PSEN1* in epileptic patients are found to be upregulated. Similar results were reported by two independent studies. They reported the enhanced accumulation of Amyloid β precursor proteins in the brain, which later on leads to the altered neuronal membrane and hyperexcitability of pyramidal cell activity [38&39]. An animal model evidence has shown the relation between Amyloid β burdens and multiple seizure activity. Amyloid β peptides acts as a pathogenic entity that controls the altering of neuronal membranes and thus suggesting the hyperexcitability further culminating into epileptogenesis [39]. There was no previously published evidence, which had quantified *APP* and *PSEN1* levels in blood levels of epileptic patients. Therefore, further evidence in large cohort or animal model is required.

Because of epilepsy heterogeneity, it causes impediments in timely diagnosis by displaying varied symptoms and phenotypes [7]. While idiopathic epilepsy exhibited significant differential gene expression compared to other classes of epilepsy, no adjustments were made to differentiate between different types of seizures [7]. In our dataset, the gene expression patterns of generalized epilepsy significantly differed from those of focal epilepsy. However, the heterogeneity of the disease often limits the use of blood differential gene expression patterns for differential diagnosis in clinical settings. Moreover, the varied gene expression between these two seizures raises the possibility of differentiated pharmacological strategies or more specialized therapy for both focal and generalized seizures.

However, our research has some limitations. Our study employs a cross-sectional design, thereby restricting our ability to establish causal relationships between variables. Acknowledging the necessity of longitudinal studies to elucidate temporal sequence and causality. Large enough sample sizes for all type of etiology will be required in future expression investigations in the blood to verify

the robustness of current findings. In addition, the severity, intractable epilepsy and seizure recurrences must be assessed during the period between follow-ups. In addition, quantification of these in animal models is a good approach for further investigation.

In conclusion, our research has shown the diagnostic power of a single gene and with all genes combined and their correlation with clinical characteristics. Furthermore, the establishment of an 11-gene differential diagnostic model could provide a new avenue for researchers to explore the analysis of various biological fluids, offering potential clinical advantages for front-line workers. Differential diagnosis of two different epilepsy types, such as focal epilepsy and generalized epilepsy, using peripheral blood will aid in their respective classification and the development of specialized pharmacological approaches.

Standard protocol approvals and patient consent

This study's sampling techniques were carried out in accordance with the Ethical Review Committee's (ERC-70-2021) recommendations and were approved by the Institutional Review Board (IRB-307/07-2021-B) of Forman Christian College (A subjects signed a written permission form. The research was conducted in the outcome of the Helsinki Declaration.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CRediT authorship contribution statement

Rabat Razia: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fazeel Majeed:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Rehab Amin:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Mariam Nisar Ayub:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Shahid Mukhtar:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Khalid Mahmood:** Writing – review & editing, Writing – original draft, Data curation. **Hamza R. Shabbir:** Writing – review & editing, Writing – original draft, Data curation. **Shahid Bashir:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Deeba Noreen Baig:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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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