

IMMUNOLOGICAL FACTORS IN THE PATHOGENESIS OF EPILEPSY IN SUB-SAHARAN AFRICA

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Abstract

Over the last few years, antibodies to voltage gated potassium and calcium channels have been found in the serum of patients with, limbic encephalitis, cerebellar ataxia and epilepsy. It has been previously suggested that the immune system is involved in certain types of epilepsy, which may have an autoimmune nature and may trigger antibody formation against voltage gated ion channels. This may be particularly important in Africa, since the causes of epilepsy are often caused by infections, and people living in these tropical regions are exposed to a larger burden of infections. We therefore measured antibodies in Kenyan patients with epilepsy in order to determine if there were immunological factors that play a role in the pathogenesis of epilepsy and if this was related to prodromal infection. We analysed 52 plasma samples of patients with epilepsy and 29 age-matched controls (mean age cases=19.5 SD 17.8; mean age controls=19.6 SD 19.1). Radioimmunoassays were carried out on the samples, for autoantibodies to calcium and potassium voltage-gated channels and for glutamic acid decarboxylase (GAD); the main synthetic enzyme in the synthesis of the inhibitory neurotransmitter GABA. Additionally, using a novel technology recently developed in Oxford, whereby human cell lines are transiently transfected with fluorescently tagged ion-channels, we assayed samples for autoantibodies to the NMDA receptor. In patients with active epilepsy, 6 % had raised antibodies to potassium voltage-gated channels. Additionally, 8% of the patients were discovered to have anti-dendrotoxin antibodies as their sera was able to precipitate to the ¹²⁵I-dendrotoxin in the absence of VGKC-labelled brain extract. This is an interesting finding and suggests that cross reactivity may be involved. Autoantibodies to NMDA and GAD were also detected in a few of the patients with epilepsy. Further studies are warranted to elucidate fully the possible role of immune factors in epilepsy

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CHAPTER ONE: INTRODUCTION

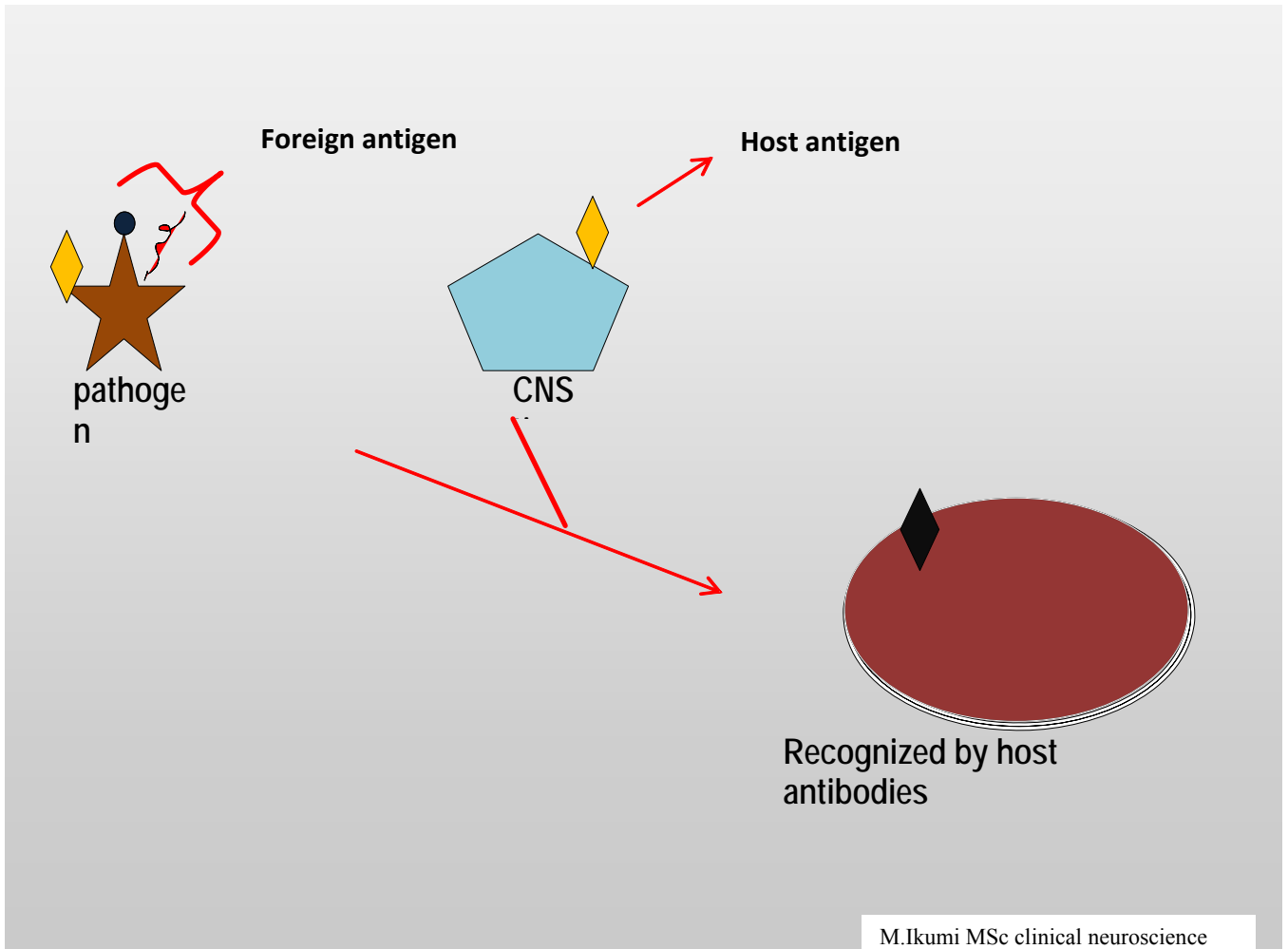
Epilepsy is the most common serious neurological condition worldwide. There are over 300,000 people with epilepsy in the UK and an estimated 2 million people with the condition in the USA. In the developed countries the annual incidence of epilepsy is estimated at about 50/100,000 people in population. There is, however, an even greater incidence of epilepsy in developing countries with up to 190 people per 100,000 developing epilepsy every year [1]. There are many different epilepsy syndromes, and the aetiology of many of these syndromes remain unknown. It has been hypothesized, however, that there may be an autoimmune link to some of these epilepsies [2]. The central nervous system (CNS) was initially thought to be immuno-privileged due to the blood brain barrier (BBB) protection, graft acceptance, and its low levels of circulating lymphocytes and monocytes [3]. It is becoming increasingly clear, however, that inflammatory reactions do occur in the CNS either due to damage to the BBB allowing infecting organisms or the associated mediators into the brain from the systemic circulation, or because of intrinsic inflammatory reactions from within the brain itself [3]. Animal studies have shown an increase in pro-inflammatory cytokine levels in the animal brains after ischemia [4]. It has also been shown in humans that these immune reactions are common features in several neurological diseases associated with epilepsy [5-7]. This “autoimmune link” has been highlighted in studies showing that autoantibodies to immunological proteins such as the glutamate N-methyl D-aspartate (NMDA) receptor may also contribute to the pathogenesis of epilepsy [5, 6, 8]. Most of the studies on autoimmunity in epileptic syndromes have been conducted on people with epilepsy of Caucasian origin. One UK study found that 20% of patients with epilepsy known to be associated with autoimmune disease, had autoantibodies to a particular ion channel, the voltage-gated potassium channel (VGKC) [9]. This study investigates the presence of anti-neuronal antibodies in African people that have been diagnosed with epilepsy and is one of the first studies to do so. We aim to study the prevalence of VGKC, VGCC, GAD and NMDA autoantibodies in the blood serum samples of an African cohort of epilepsy patients that has been exposed to numerous

infections including malaria, in order to determine if inflammatory reactions possibly caused by infection could contribute to the development of epilepsy.

1.1 What is autoimmunity?

The adaptive immune system in vertebrates is superior to the immune system found in invertebrates as it is specific in responding to infection and retains memory of previous events. Additionally, genes controlling the immune system are able to via genetic polymorphisms, enhance species survival against new infections by generating immunological diversity[10]. When fighting against infections, however, the immune system may sometimes turn against itself resulting in autoimmunity. An autoimmune response is where abnormal immune response target the antigenic components of an individual. There are two ways in which infections may cause an autoimmune response. The first is via antigen mimicry, where the host's antigens are similar to the infectious microbial molecules (figure 1). Secondly, when the host antigens immunogenicity is enhanced causing infection-related signals that sometimes even override the regulatory mechanisms that control autoimmune responses. This occurs when the infection causes the release of self-antigens that had been previously been sequestered, or by triggering a reaction from the innate immunity and thus causing self-antigen- expressing cells to induce autoimmunity [10].

Figure 1: Molecular mimicry where bacterial, viral or microbial antigen molecules resemble the host's antigen molecules which are recognised by the host. This has been shown in the Guillain-Barré syndrome where a liposaccharide molecule from the *Campylobacter jejuni* infectious agent is similar to the GM1 ganglioside on the host's nerve tissue[11].



CHAPTER TWO: NEUROLOGICAL CONDITIONS PRESENTING WITH EPILEPSY WITH AN AUTOIMMUNE BASIS:

2.1 Rasmussen's encephalitis (RE):

Rasmussen's encephalitis (RE) is an example of an inflammatory type of epileptic syndrome [3]. It is a rare progressive disorder that causes unilateral brain damage via inflammatory infiltrates and usually presents with seizures that are intractable to antiepileptic drugs although some benefits have been reported from the use of immunotherapy [12].

Experimentally, Rogers et al (1994) found that rabbits immunized with anti-glutamate receptor 3 (GluR3) proteins had cortical inflammation and seizures- features that resembled those of patients with RE [13]. They also found anti-GluR3 antibodies in the serum of patients with RE with the levels indicative of seizure frequency [13-16]. The frequency by which these antibodies are found in patients is, however, still controversial as one study did not find any antibodies in patients with RE [17]. Nevertheless, it has become recently more apparent, that anti-GluR3 antibodies can also be found in patients with other forms of epilepsy [2, 14, 15] which suggests a wider association of these antibodies with epilepsy.

2.2 Hashimoto's syndrome:

This is a syndrome that is characterized by persisting neurological deficits associated with high antithyroid antibody titre and can either be a progressive disease with continuous cognitive deterioration, or begin suddenly with tremor, agitation or seizures[3, 18]. Antithyroid antibodies are a main feature of the syndrome, however Hashimoto encephalopathy is usually unrelated to the function of the patient's thyroid. Due to the presence of intrathecal antibodies and a dramatic response to corticosteroid therapy it is suggested to be an autoimmune syndrome and that it may have a role in causing the neurological syndromes characterized by complex partial epilepsy, recurrent status epilepticus, cerebral oedema and disseminated encephalomyelitis [3, 18, 19].

2.3 Systemic Lupus Erythematosus (SLE):

SLE is an autoimmune disease affecting the central nervous system and is characterized by different circulating autoantibodies including antiphospholipid, anticardiolipin and antinuclear protein antibodies[8]. The neurological clinical presentation of SLE includes headaches, seizures and psychosis [20, 21]. Indeed studies have found the incidence of epilepsy in SLE patients, to be between 5-10% with particular prevalence in patients with high anticardiolipin antibodies [22, 23]. High titres of these antibodies have been reported to be particularly common in epilepsy[23]. Similarly seizures in SLE patients tend to respond to antiepileptic drugs[22].

2.4 Landau Kleffner syndrome:

This is an age-related childhood syndrome characterized by the autistic regression of otherwise normal development including aphasia, as well as neurological manifestations such as seizures and epilepsy [24, 25]. Immunomodulatory therapy has, however been shown to be both beneficial with a remarkable improvement in language function, cognitive abnormalities as well as seizure frequency [24]. Additionally, autoantibodies to the cell nucleus, as well as to central and peripheral myelin, and endothelial cells in the brain have been found in the sera and cerebrospinal fluid of patients suggesting an autoimmune aetiology [19, 24, 26]. Immunomodulatory therapy has been shown to be beneficial with a remarkable improvement in language function, cognitive abnormalities as well as seizure frequency [24].

CHAPTER THREE: IMMUNOLOGICAL FACTORS DUE TO INFECTIOUS AGENTS THAT ARE IMPLICATED IN SEIZURE DEVELOPMENT

Infections of the CNS are increasingly being associated with the development of seizures. The probability of developing epilepsy is usually dependent on the aetiology of the infection [27]. For instance, there is a higher probability of developing epilepsy in persons that survived *Streptococcus pneumoniae* meningitis (14.8%) than *Haemophilus influenzae* meningitis (6.1%) [28]. Similarly, patients who have once had bacterial meningitis are estimated to have a five-fold risk of developing epilepsy, with the likelihood increasing to ten-fold in patients with viral encephalitis. [1].

Viral infections such as human immunodeficiency virus (HIV), protozoan infections like malaria and toxoplasmosis, some fungal infections as well as helminthic infestation such as *Toxocara canis* and *Taenia solium* have been linked to epileptic seizures [29].

There are several suggestions implicating immunological mechanisms that lead to seizure formation, due to the physical presence of foci or CNS infections. It has been previously reported that pro inflammatory cytokines like interleukin1 and tumour necrosis factor, reduce the seizure threshold in the brain by interfering with glutamate or gamma-aminobutyric acid (GABA)-controlled neurotransmission thus causing acute symptomatic seizures. Cortical inflammation is another means by which seizures may occur due to the effect of infection on the immune system. This is reported to be the main seizure causing mechanism in bacterial meningitis and is reflected by the high protein versus the low leucocyte count in the cerebrospinal fluid (CSF) depicting systemic complications and sepsis which then lead to seizures [30, 31]. Viruses on the other hand, are able to cause direct damage to the CNS causing oedema and triggering inflammatory changes as well as phagocytic and demyelination processes [32]. In HIV for example, CNS injury is potentiated by viral toxins in the immune system that cause neuronal death through complicated interactions in macrophages, astrocytes, microglia and monocytes, via enhanced glutamate release.

These interactions between the neurons, macrophages and astrocytes, cause neurotoxic substances such as eicosanoids, cytokines, platelet activating factor and free radicals to form [33-35].

In neurocysticercosis, a helminthic infection that commonly causes epilepsy; the parasites form cystic lesions in the hosts' brain which could then trigger an inflammatory reaction leading to epileptic activity.

Finally, Protozoan infections, for example severe cerebral malaria, have also been shown to lead to epilepsy possibly by causing acidosis, granulomas and infarction thus affecting the brain [36, 37]

4.1 Search strategy and selection criteria

The references for this review were identified through a PubMed search for articles from 1964 to 2009. We also identified papers via Google scholar and similarly through searches of authors' files in the articles selected. The terms used for the search included but were not limited to, "autoimmune epilepsy", "antibodies in epilepsy", "infections and epilepsy", "antibodies", "neurological antibodies", "VGKC autoantibodies", "GAD autoantibodies", "NMDA-r autoantibodies" and "VGCC autoantibodies". I selected the most relevant studies, that described the techniques used to determine the antibodies in the different neurological conditions and that were published in English.

4.1 Evidence of autoantibodies in different neurological conditions

Over the years there have been studies suggesting that autoimmunity may be a contributing factor to several neurological conditions. Several different techniques have been used to conduct these studies, most of which have been conducted Europe or in the United States of America (table 1). In the early seventies the Patrick and Lindstrom group in the USA were able to induce an autoimmune effect on the acetylcholine receptors (AChR) in rabbits injected with purified acetylcholine from the electroplax of electric eel. Four out of the seven immunized rabbits developed muscle weakness and paralysis characteristic of myasthenia gravis[38]. This study which has been reproduced in other species [39], was one of the earlier studies that was able to show that there was an autoimmune component to the neurological condition of myasthenia gravis and the findings confirmed in other patients with myasthenia gravis [40, 41] Plyoplys *et al* (1987) then conducted a study that gave a general overview of the presence of anti-CNS antibodies in several neurological conditions including schizophrenia, mental retardation cerebral ataxia and epilepsy. Using the Western blotting technique, they found that 30% of their patients had immunoreactive bands, interestingly most patients only showed one major band (65%). They however, also found that there was no significance between the diagnosis of the

patients and the serum antibody activity as the control group was indistinguishable from the other studied groups[42]. This method could have compromised their results, as the Western blotting technique requires tissue homogenization using degradable solvents that denatures neuronal antigens especially those with lipid components. It can be used to detect antibodies to non-conformational linear peptides, but the solubilised proteins usually allow binding to non-functional epitopes in intracellular domains[43]. Thus, it may be possible that they were not able to detect all of the serum that was positive for anti-CNS immunoreactivity [42]

In 1994, a study using a cell based assay was able to show a possible link between anti-GluR3 antibodies and Rasmussen's encephalitis when two out of three rabbits immunized with GluR3 protein developed behaviour that was typical of epileptic seizures. This link was further confirmed in patients with RE in which they tested for antibodies to a GluR peptide –GluR3B[13]. This result was somewhat controversial, however, as it has not been reproducible in immunised mice[44] and similarly antibodies to the full length of the peptide GluR3 have not been found in humans [15, 17]. Furthermore, in the study by Wiendl et al 2001, they were unable to detect corresponding GluR3 titres in the CSF in patients with raised serum levels of GluR3[15].

Further findings have also been made on the voltage gated ion channels. Antibodies to the voltage gated calcium channel (VGCC) which has several subtypes in the α -subunit (P/Q, N, L, R, T), have been shown to be involved in the Lambert Eaton Myasthenic syndrome(LEMS) [39]. Using an immunoprecipitation technique, Sher et al (1989) were able to identify antibodies to VGCC in patients with LEMS by labeling the N-type VGCC from a human neuronal cell line using ^{125}I -conotoxin GVIA (^{125}I -CgTx) a neurotoxin from *Conus geographus*. This method proved to be reproducible with the patients' serum antibody concentration ratios remaining constant with repeated assays [45]. Motomura *et al* (1997) found that more than 90% of patients with LEMS had P/Q type VGCC antibodies compared to about 33% with anti-N type VGCC antibodies [46]. More recently, however, P/Q VGCC autoantibodies have been discovered in paraneoplastic cerebellar degeneration (paraneoplastic LEMS), in association with small-cell lung cancer [47-49].

Using immunoprecipitation of ^{125}I - α -dendrotoxin labelled VGKCs extracted from human frontal cortex, anti-VGKC antibodies were first identified in patients with acquired neuromyotonia- a disorder characterized by spontaneous muscle twitching, sweating and painful cramps [50]. This technique, however, was only able to detect antibodies in about 65% of patients. Thus using a new immunohistochemical technique that involves frozen oocytes that express the different cRNA subunits of VGKCs, Hart et al (1997) were able to detect antibodies in all patients with neuromyotonia. Similarly, VGKC autoantibodies have been reported in other neurological conditions such as Morvan's syndrome, (patients with neuromyotonia and marked central dysfunction, including, hallucinations, insomnia and amnesia) [51], as well as in Limbic encephalitis [52] and more recently in epilepsy and seizure related diseases [9, 53-55]. A radioimmunoassay study investigating the different types of antibodies present in the sera of a cohort of 139 patients with different types of epilepsy detected autoantibodies to VGKC in 16 patients, the ten patients with the highest titres were found in patients with epilepsy that was associated with immune mediated disorders compared to 6 patients, with lower titres, who had long-standing drug resistant epilepsy [9].

Autoimmunity to GAD an enzyme that is concentrated in neurons that secrete the neurotransmitter GABA, was initially reported in patients with stiff-person syndrome and insulin dependent diabetes mellitus[56]. However, there have been several more reports indicating that GAD autoantibodies are also prevalent in epilepsy [9, 57, 58] as well as other seizure related disorders, cerebellar ataxia [59] and limbic encephalitis [60].

Similarly antibodies to the NMDA receptor were earlier identified in patients with acute haemorrhagic stroke [61] but were later discovered in the systemic lupus erythematosus syndrome [62] and in epilepsy using enzyme linked immunosorbent assays [2, 63] . Similarly, Dalmau *et al* (2008) using the cell-based technique involving human embryonic kidney cells (HEK293) that expressed the NR1-NR2B heteromeric subunits of the NMDA receptor [64] .

From these studies and from table 1, we find that a wide spectrum of neurological conditions, some of related to epilepsy, have an autoimmune basis. Table 1 show the studies and also allows comparison between the different techniques used to detect autoantibodies in these conditions.

Antibody detection techniques mainly fall into two broad categories:

1. Those that are cell- based and detect antibodies to extracellular domains of functionally active molecules using neuronal explants or transfected cells that are transfected with the protein of interest [58, 65]

2. Those that detect antibodies to linear sequences such as western blotting and ELISA [43].

Generally autoantibodies were measured using immunohistochemistry or immunofluorescence on the target tissue after which western blotting techniques were used to confirm findings [58, 65, 66] . However there was an increased use of the ELISA as a preferred means of antibody testing which was found to be particularly successful in testing for autoantibodies in patients with paraneoplastic conditions because the paraneoplastic antigens are mainly expressed on the inside of the cell rather than extracellularly [39, 66]. In contrast, autoantibodies to voltage gated ion channels and other functional membrane proteins, are able to be measured with a higher specificity and sensitivity by the immunoprecipitation of the target protein radioactively labelled with neurotoxins [39]. Thus the radioimmunoassay technique has become more popular as a diagnostic assay for detection of autoantibodies to ion channels[39]. This method, however, requires a specific high affinity ligand to work. Therefore the most optimum assays to date are the cell-based assays. This is because they do not require a ligand since all the required genes are already available and because you get conformationally correct channels expressed in the membranes and thus the antibodies are able to recognize the extracellular regions of the cells [39].

Few studies on autoimmunity have been conducted in other parts of the world where infections are very prevalent and may present a possible cause for some types of epilepsies. Diseases such as malaria and neurocysticercosis have been reported to be associated with seizure disorders and predispose the patient to epilepsy [67-69]. Using an electroimmunotransfer blot assay (EITB),

Correa *et al* (1999) found that 22% of the patients from a cysticercosis endemic area in Mexico, that had taeniasis (caused by *T. Solium* which also causes cysticercosis), had anti-cysticercal antibodies [70]. They also found that 22% of patients that had epilepsy but did not have taeniasis also had anti-cysticercal antibodies [70]. Similarly, autoantibodies to (P/Q and N type) VGCCs were detected in children with malaria in a study conducted on an African cohort. The increase in the titre of VGCC antibodies appeared to correlate with increasing severity of malaria. Additionally, patients were found who had autoantibodies to VGCCs and to GAD [71]. Although these infectious diseases may clearly have an autoimmune component, they only imply an association between this autoimmunity and the development of epilepsy and perhaps the antibodies found in these conditions may only be markers of the infection. The pathogenesis and mechanisms by which this may lead to seizures and indeed epilepsy is still to be elucidated and further research is needed in this area.

Table 1 showing some of the studies on antibodies in different neurological conditions conducted over the last twenty years.

Author	Total pop	Type of study	Type of neurological disorder	Number with condition	%prevalence of autoimmune activity (N)	Types of antibodies	Country of study
A.PLIOPLYS et al 1987	257	Human serum-Western Blots	1.Schizophrenia	66	28%	Anti-CNS	Canada
			2.Mental retardation	22	27%		
			3.Cerebellar ataxia	18	33%		
			4.Parkinson's disease	18	22%		
			5.Myasthenia gravis	18	22%		
			6.Epilepsy Other diagnoses	20	45%		
Sher et al 1989	32	Human serum: immunoprecipitation	Lambert eaton myesthenic syndrome,	12	92%	Anti-VGCC antibodies	Italy
			Myesthenia gravis, Small Cell Lung Cancer	7	0		
				6 (5LEMS/1 no LEMS)	100%		
J Newsom-Davis et al, 1991	2	Case study: radioimmunoassay (RIA)	Lambert Eaton myasthenic syndrome,	2	100%	Anti-AChR antibodies	UK
Rogers et al, 1994		Animal(Rabbit) protein immunoblot analysis (and Human)	Rasmussen's encephalitis(model)	2/3 rabbits		Anti-GluR3	USA
L. Gahring et al, 1995	7	Human samples:Western blots, immunohistochemistry, electrophysiology, cell transfection	Paraneoplastic disorders	7	71%	Antibodies to GluR1, GluR4, GluR5, and/or GluR6	USA
Motomura et al, 1997	72	Human serum samples; Immunoprecipitation	Lambert eaton myesthenic syndrome	72(24 with small cell lung cancer)	91.7%	Anti-VGCC	UK
M. Levite et al, 1999	20	Animal (mice) : ELISA assay	Rasmussen's Encephalitis	20 immunised with anti GluRB antibodies	All developed similar symptoms to RE but not epilepsy	Anti-GluR3B	Israel
Peltola et al 2000	235	Human samples: enzyme immunoassay irradiated polystyrene plates immunofluorescence	1.Localization-epilepsy	50	See types of antibodies	IgG (22) 14.5% and IgM (64) 44% class anticardiolipin, IgG (9) 6.4% and IgM(3) 2.1% anti-b2-glycoprotein I, Antinuclear (37) 24.3%	Finland
			2.Generalised Epilepsy	50			
			3. New-diagnosed seizures	52			
Peltola et al 2000	100	Human serum saples: radiobinding, immunohistochemistry, immunobloting	1.Localization-epilepsy	51	(8) 15.7%	Anti-GAD (2 pts stiff mann syndrome, 6 pts similllar to diabetesM1)	Finland
			2.Generalised Epilepsy	49	0		
Wiendl et al 2004	168	Human serum samples: ELISA	1.Rasmunsens encephalitis	8	(2)25%	Anti-GluR3	Germany
			2.Focal epilepsy	40	(13) 33%		
			3.Other neurological diseases	79	(6) 8%		

Watson et al 2004	79	Human serum: ELISA	1.Rasmunsens encephalitis 2.intractable epilepsy	30 49	5	_7-nAChR (2 patients) VGCC (1 ptnt) GAD (2 ptnts)	UK
Y. Ganor et al, 2004	133	Human Samples: ELIZA diagnostic assays	1.Rasmussen's Encephalitis 2."other non-RE Types of Epilepsy"	6(RE) 71 (n-RE)	83% 24%	anti-GluR3B Ab's,	Israel
McKnight et al, 2005	289	Human serum samples: radioimmunoassay	Epilepsy (including autoimmune, drug resistant, and referred groups)	139	(16)11.5% (5) 3.6%	Anti-VGKC Anti-GAD	UK
Husebye et al 2005	109	Human Serum; ELISA	Systemic lupus erythematous(SLE)	109	(66)31%	Anti NMDA	Israel
Majoie et al 2006	106	Human (female): radioimmunoassays	Long standing intractable epilepsy	7	5.7% 0.9%	Anti- VGKC Anti- VGCC	Netherlands
Tan et al, 2008	72	Human serum samples: ELISA assay	Autoimmune neurological conditions	42 with seizures	58% with seizures	Anti-VGKC	USA
Dalmau et al 2008	100	Human Serum; ELISA, Cell based assay	Encephalitis with NMDA(NR1/NR2B) antibodies	76 with seizures	100%	Anti-NMDA	USA

5.1 Hypothesis

The hypothesis of this study is that there is a higher prevalence of antibodies in patients with epilepsy compared to age matched controls and this is related to prodromal infection.

5.2 Aims

We have assessed the sera of a cohort of patients from a sub-Saharan African community in Kenya who were exposed to an increased risk of infection and checked their antibody status with the aim of establishing if VGCC, VGKC, GAD, and NMDA autoantibodies were present.

6.1 Patient Description:

We studied patient samples collected from Kilifi district at the coast of Kenya in Sub-Saharan Africa. This district is inhabited by 650,000 people and is a malaria endemic area. These were samples that had already been collected as part of a previous study (Wellcome Trust (070114/Z/02/Z): The causes and consequences of seizures associated with *Plasmodium falciparum* infection). Ethical approval was obtained from the relevant local authorities and individual consent was also given.

A qualified clinician determined the clinical characteristics after examination of the patients. This was a blinded study and the clinical history of the patients was not available during the investigations. We were later able to obtain the basic clinical information such as age, sex, if patients had previously been admitted, if they were on medication, if they had active epilepsy at time of testing (defined as) and the duration of their epilepsy.

In this pilot study, we randomly selected 52 epilepsy patients and 29 control samples that were collected from this malaria endemic area and measured the antibodies to neuronal components using the methods below. The number of samples for each assay was determined by the availability of the reagents. All tests were repeated several times and mean results for each sampled taken for analysis.

6.2 Reagents:

6.2.1 VGCC and VGKC assays:

- Solubilisation buffer: (25mM Tris-HCL, 5mM HEPES, 0.32M sucrose PH 7.2). Additionally, the following protease inhibitors are added to the SB just before the experiment; 1 μ M pepstatin, 2 μ M leupeptin and 0.1mM phenylmethanesulphonylfluoride.
- 1M NaCl SB:
- 4% digitonin (Calbiochem D1407)
- Incubation Buffer (IB): 0.1% digitonin /20 mM phosphate buffer (pH 7.4)
- Washing Buffer (WB): 0.1% Triton X-100 20 mM phosphate buffer pH 7.4.
- Neurotoxin: [¹²⁵I]- ω -conotoxin MVIIC (Perkin Elmer, UK 2000 Ci/mmol) and [¹²⁵I]- dendrotoxin (Perkin Elmer,UK. 2000 Ci/mmol).
Sheep anti-human IgG (Binding site, Birmingham, UK)

6.2.2 GAD Assay:

- Pre-prepared autoantibody assay kit from RSR Ltd.

6.2.3 NMDA Assay:

- Human embryonic kidney cells (HEK)
- Poly-L-lysine solution (0.01% in PBS)
- Dulbecco's modified eagle's medium (DMEM) + Fetal Bovine Serum (FBS) + penicillin, streptomycin, amphotericin (PSA)
- 4 μ g DNA/30 μ l lipofectamine mixture (lipo). The cDNA encoding the 2 subunits- NR1-GFP(1.89 μ g/ μ l) and NR2B(0.85 μ g/ μ l) are used. These are diluted to total 4 μ g/250 μ l DMEM (without FBS or PSA) per well.
- DMEM +fetal calf serum (FCS) (no PSA) for lipo transfection
- DMEM +HEPES(5 μ l) + 1% BSA (2.3gHEPES/500ml DMEM)
- DMEM + HEPES(5 μ l)
- PBS
- 36% formaldehyde solution (diluted 1:9 in PBS= 4% used)
- Fluorescent-labeled goat anti-human IgG secondary antibody (Alexa Fluor 568)
- Mounting solution (including DAPI)

6.3 Immunoprecipitation antibody assays:

Radioimmunoprecipitation of [¹²⁵I]-neurotoxin-labeled voltage gated channels was performed to identify antibodies to VGCCs and VGKCs. The toxins used include the radioactive labels [¹²⁵I]- ω -conotoxin MVIIC, and [¹²⁵I]- ω -dendrotoxin which are channel blockers specific to the P/Q-type VGCC, and VGKCs respectively, within the CNS. The assays are performed in the following way.

6.3.1 Measurement of antibodies to VGCC:

VGCC cerebellum extract was previously prepared in the laboratory and was therefore already ready for antibody tests in our samples.

We performed antibody tests on 40 epilepsy case and 22 control samples and repeated each test several times for accuracy. We included 6 healthy Caucasian control samples as well as 2 known high titre VGCC positive controls and 2 known low titre VGCC positive controls.

Before each assay about 200 μ l of cerebellum extract that had previously been incubated for 2 hours at 4°C with [¹²⁵I]- ω -conotoxin MVIIC (5 fmoles) was spun at 13,000 rpm for 5 minutes. We diluted each sample 1: 10 in IB and then vortexed the diluted serum and spun the serum sample for 5 minutes at 13000rpm in order to remove any insoluble precipitants. To 25 μ l of the diluted serum, 50 μ l of the spun [¹²⁵I]- ω -CTX -labeled VGCC extract was added and the mixture incubated overnight at 4°C.

After incubation, 25 μ l of sheep anti-human IgG was added to the samples. This was then left to incubate at room temperature for 1 hour. 1 ml of PTX was the added to the mixture and spun for 5 minutes at 13,000g. The pellets were then gently washed twice with 500 μ l PTX after which they were counted using a gamma counter and results were expressed as counts per minute (cpm) of the ¹²⁵I- ω -CTX-labelled toxin.

6.3.2 Measurement of antibodies to VGKC

Whole rabbit brain extract was previously labeled by incubating in dendrotoxin buffer and [¹²⁵I]-dendrotoxin labeled toxin for at least 6 hours at 4°C, and then spun at 13000 rpm for 5 minutes before each assay.

We tested 51 epilepsy patient samples and 14 community controls as well as 4 positive and 4 healthy Caucasian controls. All samples were diluted 1:10 in IB. These were then spun at 13,000 rpm for 5 minutes and 50 μ l of the diluted sera taken out. To this, 50 μ l of spun [¹²⁵I]-dendrotoxin mixture was added and the samples incubated overnight at 4°C.

We then added 50 μ l of sheep antihuman IgG and incubated the mixture for 1 hour 30 minutes at room temperature. 500 μ l of PTX was then added and the samples spun at 13,000rpm for 5 minutes. The supernatant was removed and the pellet washed twice with 500 μ l PTX after which samples were counted for VGKC binding.

6.3.3 Measurement of antibodies to GAD:

This assay was conducted using the RSR prepared antibody assay kit for detection of antibodies to GAD.

6.4 Cell based Assay:

6.4.1 Culturing cells:

This assay is performed using pre-cultured human embryonic kidney (HEK) cells that are regularly subcultured in a growth medium containing penicillin, streptomycin and amphotericin (PSA), foetal bovine serum (FBS) and Dulbecco's modified eagle's medium (DMEM)-which is optimum for keeping cells healthy and actively growing in log-phase. We used cells from the laboratory, which has several cultured cells available for sub-cultivation. A 25cm Corning flask of actively growing cells that were 80-90% confluent was obtained. Using sterile techniques, the old medium was removed and after gently rinsing the monolayer of cells using PBS, about 1ml of trypsin (0.1%) was added. The culture was then incubated for about 1-2 minutes until the cells were loosened. After gently tapping the flask to detach the cells 5ml of cell culture medium is added and the cells vigorously washed to ensure the suspension contains at least 95% of single cells. The growth medium also aids in inactivating the trypsin action. The new flask with the diluted cells was then incubated in a high humidity incubator at 37°C.

These cells were then used for NMDA-r antibody assays.

6.4.2 Measurement of antibodies to NMDA-R:

This assay was performed in four days and repeated several times for accuracy. We measured antibodies in 10 epilepsy patients, 5 community controls, 2 positive and one healthy Caucasian control.

Day 1:

Using fine forceps we placed 3 cover slips (13mm in diameter) into each well of a six well plate. 2ml of poly-L-lysine solution (0.01%) was then added to each well and left for 15 minutes sure all the slips are completely covered. The poly-L- lysine was then taken off the wells the slips left to dry for at least an hour in the sterile hood. During this time, we seeded the HEK cells by adding trypsin to break the cell-cell bonds. The cells were then diluted in medium (1:10) and spun for 5 minutes at 1000rpm. 25 µl of cells were then added to 25 µl of trypan blue (1:1 dilution) and put onto a slide for counting and the number of cells needed calculated before adding into each well.

Day 2 Transfection:

After checking to see that the cells were healthy and well attached, 4 µg of DNA was diluted in 250 µl DMEM (without FCS/PSA). In a separate tube, 5 µl lipofectamine was also diluted using

254 μ l DMEM per well. The mixtures are then left to incubate for 5 minutes at room temperature. The 2 solutions are then mixed together and incubated for a further 15 minutes at room temperature. During this incubating period, the medium in the 6 well plate was changed to antibiotic free medium containing only DMEM and FCS. Finally, 500 μ l of the transfection mixture was added to each well and the incubated at 37°C overnight for a maximum of 14-18 hours.

Day 3:

After incubation, the medium in each well was changed to DMEM + FBS+PSA and 500 μ M ketamine was added. This is an NMDA-r antagonist, which inhibits the NMDA-induced increase of intracellular calcium.

Day 4:

The samples to be tested were diluted 1:20 using DMEM solution that contained HEPES and 1%BSA ensuring that the total volume of the diluted mixture was 250 μ l. After dilution, the samples were then transferred to a 24 well plate and one cover slip added per well with the cell side up. They were then left to incubate for one hour at room temperature.

The supernatant was then taken off and the cells gently washed twice in 250 μ l DMEM+ HEPES and once in 250 μ l PBS. To the wells, 250 μ l of 4% formaldehyde that had been diluted in PBS was added and the cells left to incubate for 10 minutes at room temperature.

The coverslips were washed three times in DMEM + HEPES before adding 250 μ l of the fluorescently labeled secondary IgG antibody (goat anti-human IgG, Alexa Fluor 568). This had been previously diluted 1: 750 in DMEM + HEPES + 1%BSA.

The wells were then left to incubate for about 45 minutes in the dark at room temperature after which the cells were washed 3 times in DMEM + HEPES and twice in PBS. The cells were left in PBS to avoid drying out.

The coverslips were then mounted onto well labeled microscope slides using fine forceps and DAPI mounting solution, and left to dry overnight in the dark.

6.5 Statistical analysis

I used the analysis programme STATA 9.2 to analyse the data (Stata Corporation, 2003, College Station, TX). Unpaired t-tests (for unequal variances) were used to make statistical comparisons between the means of the patient and control groups. Autoantibody assays are displayed using comparative scatterplots. A Fisher's exact test was used to compare the number of epilepsy patients that had anti-VGKC antibodies above and below the median. The Graphpad Prism statistical programme was used to give an output for dendrotoxin titrations. A two-tailed *P* value of < 0.05 was considered to be significant for all the tests.

Samples from African epilepsy patients (n= 52, 27 males) and healthy community controls (n=29, 13 males) as well as 14 Caucasian samples both from healthy controls and from patients with different neurological conditions were included and screened for antibodies to VGKC, VGCC, GAD and the glutamate receptor NMDA. The cohort of epilepsy patients had an age range of between 1-75 years (mean 19.5y, SD 17.8y), which was not significantly different than that of the controls (range = 1-79 years, mean 19.6y, SD 19.1y). All patients in the test group had active convulsive epilepsy, with many having had epilepsy for more than ten years (42%) (Table 1). Only 25/52 patients had received antiepileptic drugs (AEDs); 17 received only 1 AED, 7 patients had previously been administered 2 different drugs and one patient had been tried on three different drugs. The main anti-epileptic drugs that the patients were recorded to have used were phenobarbital, phenytoin or sodium valproate. This cohort was a part of a larger ongoing epidemiological study in which participants were recruited from the community and therefore some of the epilepsy patients would not have previously been admitted into hospital. Of the 52 (61%) patients, 32 had records of previous admission to the district hospital with most cases of admission being due to either febrile or afebrile convulsions (62.5%). Out of these patients admitted due to convulsions, 8 were shown to have developed epilepsy before admission. Additional causes of admission included malaria infection (15.6%), injury and burns (9.4%), anaemia (6.3%) as well as other diagnoses such as appendicitis and kidney problems (6.3%) (see table 2).

Table 2: patient characteristics and autoantibodies detected.

	Epilepsy patients		Controls		Detected autoantibodies				
	N=52 (%)		N=29(%)		VGKC N=3	Den N=4	GAD N=2	NMDA-r N=2	VGCC N=1
Age at serum collection									
1-10yrs	19	(37)	13	(45)	1	3			1
10-20yrs	18	(35)	6	(21)	2		1	1	
20-30yrs	6	(12)	5	(17)					
>30yrs	9	(17)	5	(17)		1	1	1	
mean	19.5		19.6						
sex									
male	27	(52)	13	(45)	2	2	2	1	1
female	25	(48)	16	(55)	1	2		1	
Previous admission	32	(62)	7	(24)					
Afebrile convulsions	14	(44)			3	2	1	1	
Febrile convulsions/fever	6	(19)	3*	(33)		1			
Malaria	5	(16)						1	
Injury/burns	3	(9)	1	(11)					
Anaemia	2	(6)							
Other (includes appendicitis bladder control, birth problems etc)	2	(6)	5	(56)					
Epilepsy duration									
<6 months	5	(10)	0						
1 year	1	(2)							
3 years	7	(13)				1	1		
5 years	6	(12)			1				
7 years	6	(12)				1			1
10 years	5	(10)						1	
>10 years	22	(42)			2	2	1	1	

*some controls had been previously admitted with fever. However all controls that were previously admitted did not have any record of convulsions. VGKC= voltage gated potassium channel, GAD= glutamic acid decarboxylase, NMDA-r = N-methyl-D aspartate receptor, VGCC= voltage gated calcium channel.

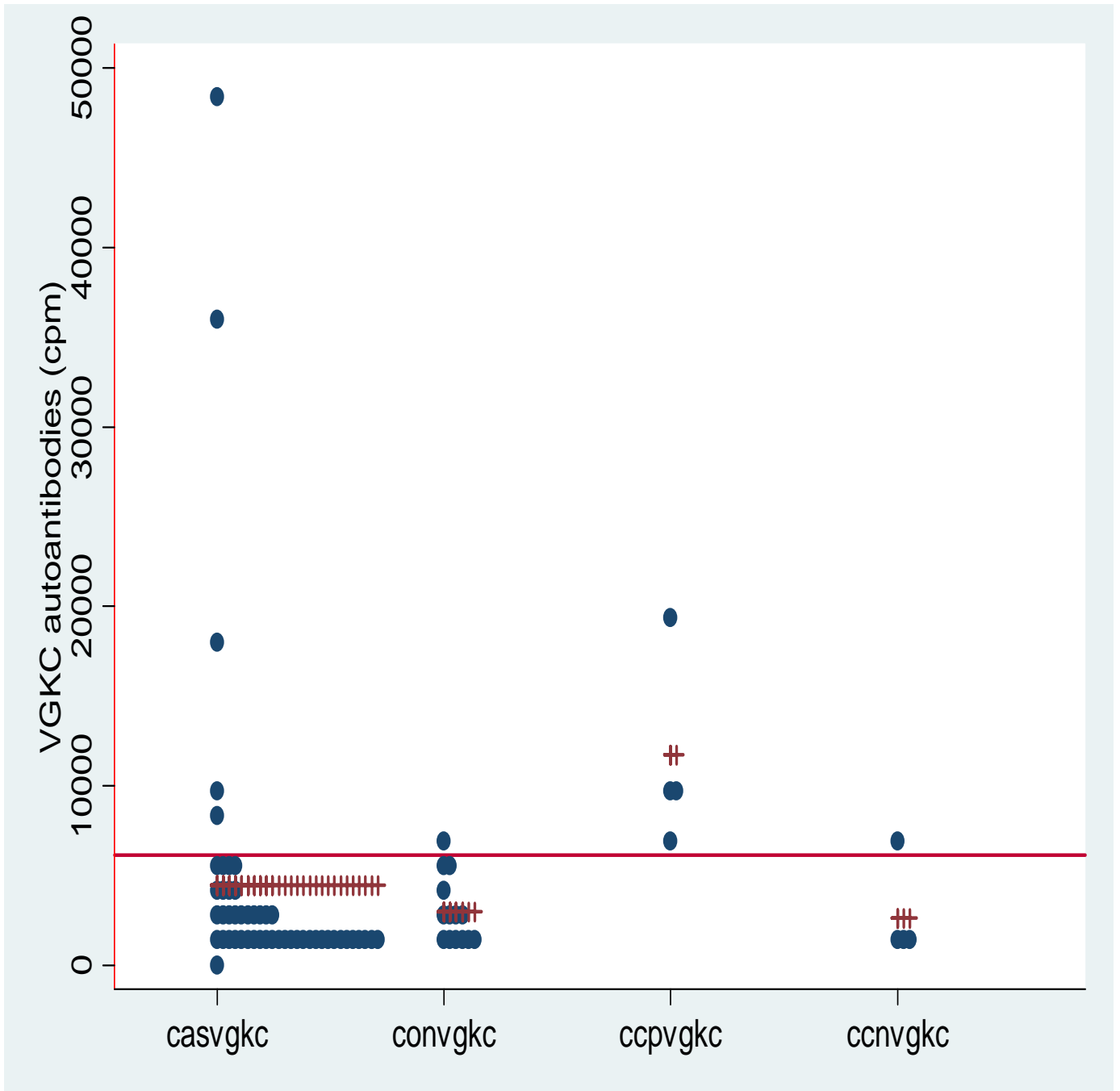
7.1 VGKC antibodies:

Fifty-one patients with epilepsy and 14 community controls were tested for anti-VGKC antibodies using the ¹²⁵I-dendrotoxin-rabbit brain VGKC radioimmunoassay. There was no significant difference between the mean of the epilepsy group compared to the controls (p= 0.26 table 3). However, the criteria for positivity in this assay was set as 2SD above the mean of the community African controls (6147.4) five (10%) of the samples tested were deemed positive (range 8623 cpm - 48914 cpm, figure 2). Additionally, if the Epilepsy patients were grouped into those that were above and those that were below the median, then the antibody levels in epilepsy patients positive for anti-VGKC antibodies, were found to be significantly greater than the median.(fishers exact test p=0.02).

Table 3: Comparison of mean values of VGKC antibody levels in African patients with epilepsy and healthy community controls (comparison using unpaired t-test for unequal variances, showed no significant difference p = 0.26)

Assay	total	Mean	Std. Dev.
Epilepsy patients	51	4440.2	8411.7
Controls	14	3005.5	1603.4

Figure 2: Anti-voltage gated potassium channel antibodies in Kenyan patients with epilepsy, healthy Kenyan community controls and in Caucasian positive and negative controls. casvgkc = Epilepsy cases tested for anti-VGKC antibodies, convgkc=community controls, ccpvgkc = positive Caucasian controls ccnvgkc = negative Caucasian controls,+++ = mean (— (line) shows mean of epilepsy patients +2SD= 6147.4 cpm).



7.1.1 Anti-dendrotoxin antibodies

Three of the samples were markedly higher than the maximum counts per minute (cpm) precipitable by commercial anti-VGKC antibodies (S.Irani, data not shown). This finding raised the suspicion that the antibodies detected were not to the ^{125}I -dendrotoxin-VGKC complex but to the ^{125}I -dendrotoxin alone. To test this hypothesis, six samples, five of which were anti-VGKC positive were incubated with ^{125}I -dendrotoxin alone (5pmoles/50ul, approximately 50,000cpm) and compared to controls. We found that four of the six samples were able to precipitate the ^{125}I -dendrotoxin, in the absence of brain extract, supporting the hypothesis that the antibodies were directed to the toxin itself (figure 3). Further titrations of the patient sera confirmed these results (figure 4).three of the four patients, who had had epilepsy for more than seven years, had previously been admitted into hospital for convulsions(table 2).Two of these patients (1 male) were 10 years of age while the third was a 40 year old man. Additionally two of these patients along with the fourth patient a seven-year old female (who had no record of previous admission), had been on the AED Phenobarbital to control their seizures. The 40-year old male was also found to have GAD antibodies.

Figure 3: Presence of anti-dendrotoxin antibodies in four patients with epilepsy compared to controls. Casden = Epilepsy cases tested for anti-dendrotoxin antibodies, convgkc = community controls, pos_con = positive controls, neg_con = negative controls, +++ = mean (line) shows mean of epilepsy patients +2SD= 6147.4 cpm).

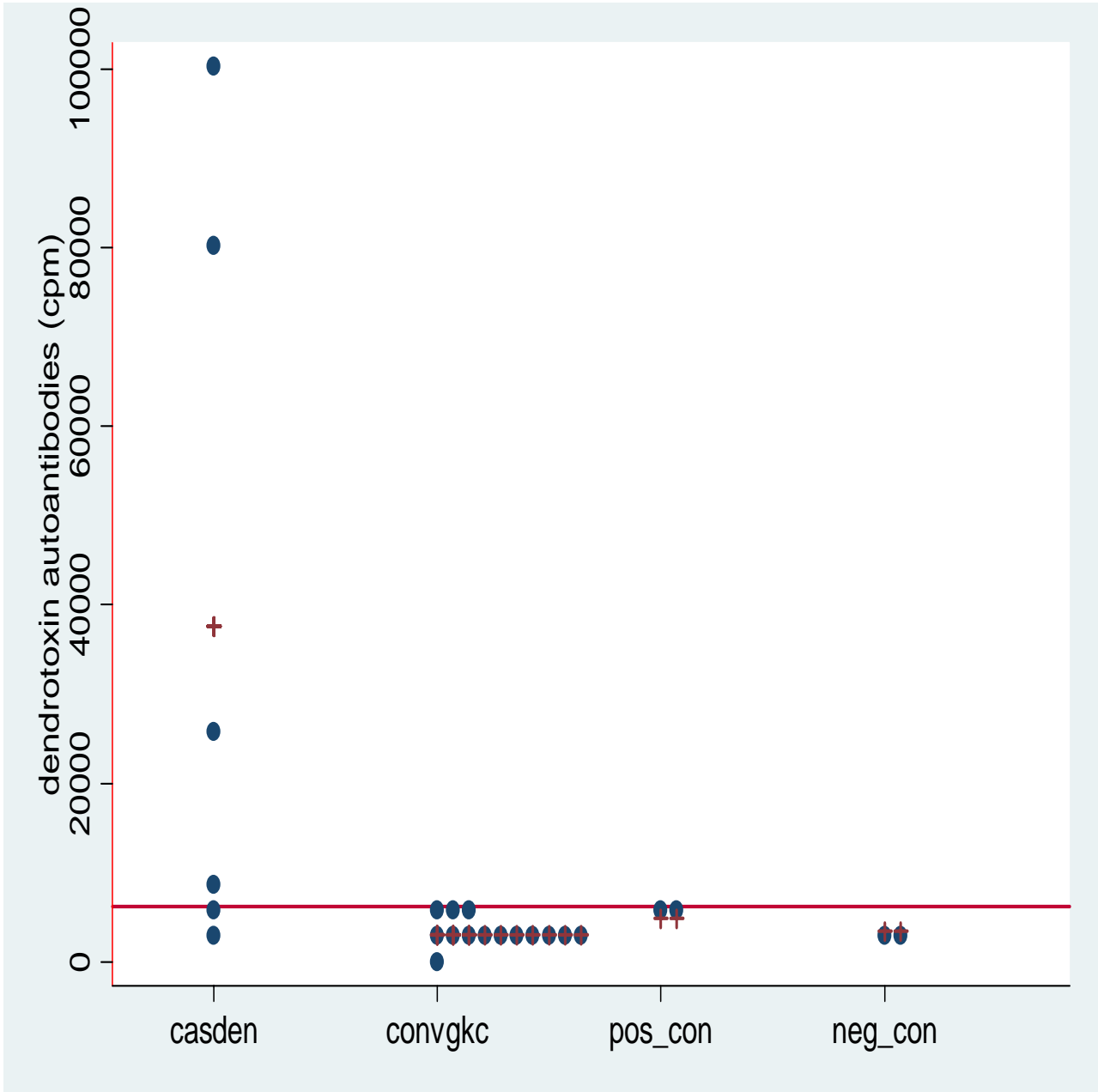
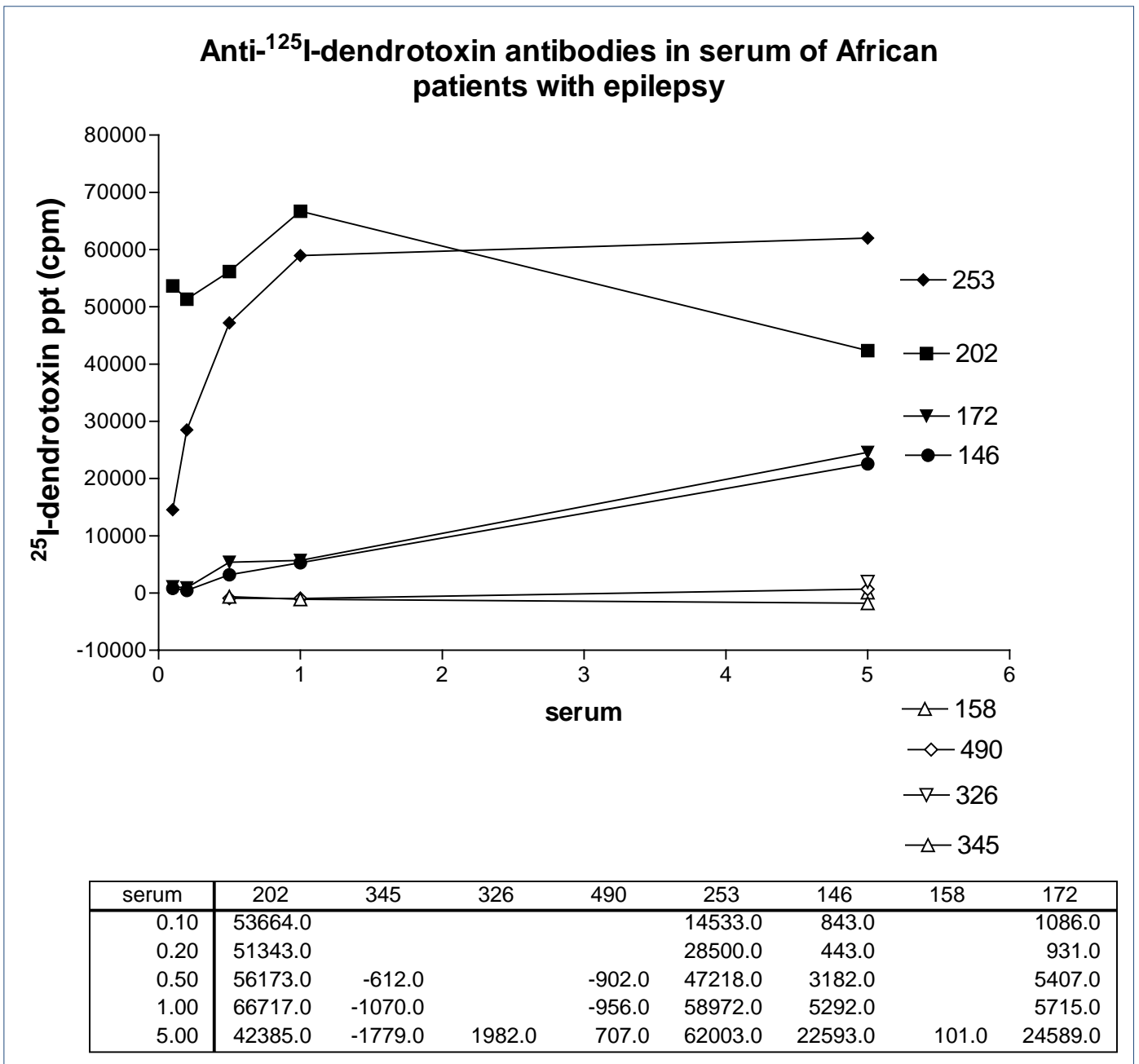


Figure 4: Anti- dendrotoxin antibody titres showing 4 epilepsy patients with anti-¹²⁵I-dendrotoxin antibodies. Shaded icons 253,202,172 and 146 = positive epilepsy patients, clear icons, 158, 490, 326, 345= negative epilepsy patients. The embedded table shows the antibody cpm of the samples as the serum concentration is increased (Graphpad prism output , B. Lang 2009).



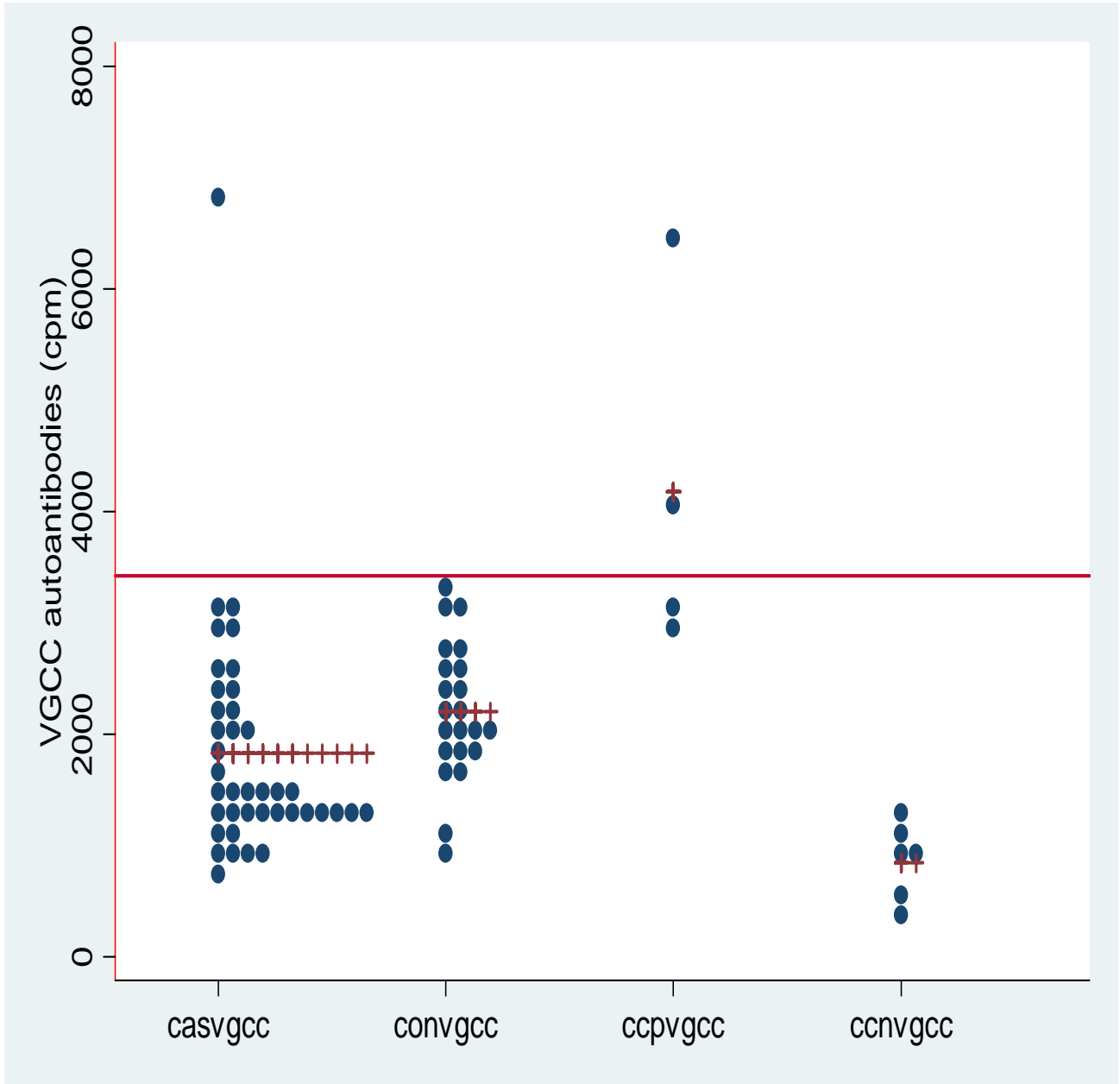
7.2 VGCC antibodies:

In the VGCC antibody assays, we found that the mean of the community controls (mean 2205+ SD 623) was considerably higher than the levels found in Caucasian controls (mean 847 + SD 343), and was partially attributed to the hyperimmunity of the African sera.. However we did not find a significant difference in the mean values between the patients and controls tested for anti-VGCC antibodies (p=0.08, table 4). Only one patient- a seven year old boy with active epilepsy- was shown to have elevated antibody levels measuring up to 6817cpm (figure 5), which was three times greater than the levels of community control.

Table 4: Mean levels of VGCC antibodies in Patients with epilepsy and community controls
Comparison of means showed no significant different (p= 0.08, unpaired two sample t-test ; for unequal variances)

Assay	total	Mean	Std. Dev.
Epilepsy patients	40	1828.4	1038.5
Controls	22	2204.9	623.4

Figure 5: Anti-voltage gated calcium channel antibodies in African patients with epilepsy, healthy African community controls and in Caucasian positive and negative controls. Low positive Caucasian samples had similar amount of antibodies as African community controls. casvgcc= Epilepsy cases, convgcc=community controls, ccpvgcc=positive Caucasian controls, ccnvgcc=negative Caucasian controls, +++ = mean ($\bar{}$ (line) shows mean of epilepsy patients +2SD= 3426 cpm).



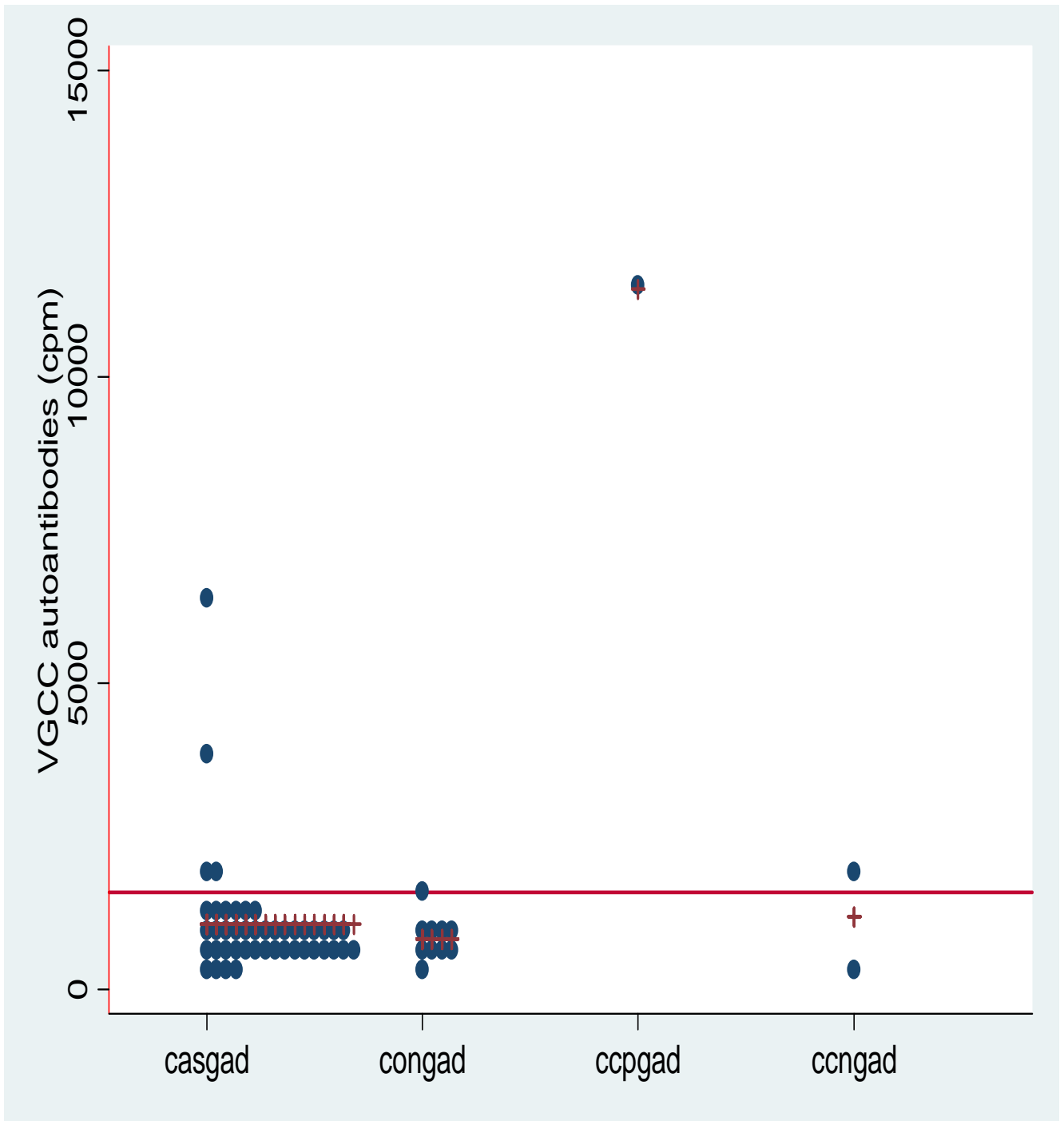
7.3 GAD Antibodies:

There was no significant difference between the level of GAD antibodies in patients with epilepsy and control groups (table 5). Two of the patients had slightly raised antibody levels-3760 cpm and 6300cpm, which equates to levels less than 10 units/ml, (calibration curves not shown). These values were more synchronous with levels found in diabetic patients or relatives of diabetic patients, rather than in epilepsy patients ($>100\text{U/ml}$) [58] (figure 6). We did not however have any clinical information showing a diabetic background for either of these two patients. One of the patients (6300 cpm) however, a forty year old male, was also found to have anti-dendrotoxin antibodies. He had previously been admitted for convulsions and had been on Phenobarbital. The second male epilepsy patient (13 years) had no previous record of admission but had had epilepsy for a duration of 3 years and also been on the AED Phenobarbital.

Table 5: Mean levels of anti-GAD antibodies in patients with epilepsy and controls ($p=0.2$, unpaired two sample t-test with unequal variances)

Assay	Total	Mean	Std. Dev.
Epilepsy patients	45	1057.7	964.1
Controls	10	822.8	387.4

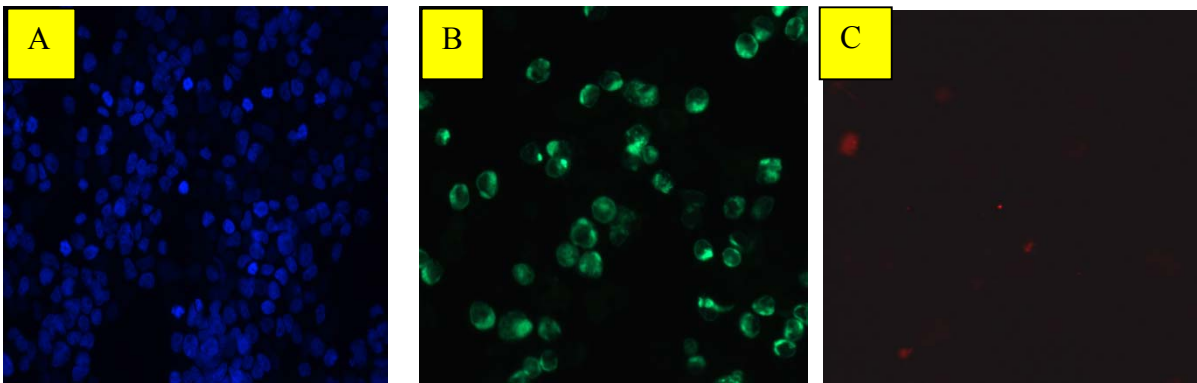
Figure 6: GAD antibody levels in African Epilepsy patients and controls. casgad= Epilepsy cases tested for anti-GAD antibodies, congad=community controls, posgad= commercially prepared positive GAD control (300U/ml = 11,434 cpm), neggad=one commercially prepared low positive control (10U/ml =2016 cpm) and one healthy community control (= 351 cpm), +++ = mean (— (line) shows mean of epilepsy patients +2SD= 1582.1 cpm).



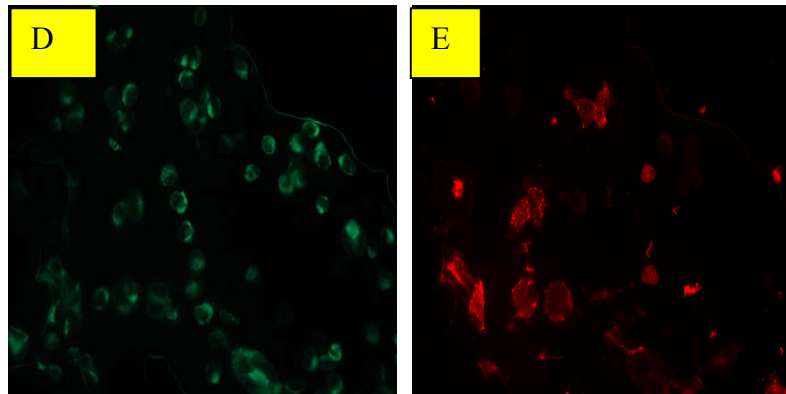
7.4 NMDA -receptor antibodies:

Ten patients with epilepsy were tested for antibodies to NMDA receptors using a cell based assay in HEK cells transfected with DNA encoding GFP-tagged NR1 and untagged NR2B NMDA receptor subunits. After cells had been incubated with patient sera (12.5µl) for an hour at room temperature, surface binding was demonstrated using secondary goat antihuman IgG-Alexafluor568. Cell nuclear staining was demonstrated by counter staining with DAPI (4',6-Diamidino-2'-phenylindole dihydrochloride) (figure 7). Two of the ten patients tested for anti-NMDA-r antibodies were found to be positive both with a score of 1.5. Both were shown to have had epilepsy for more than 10 years. One was a 16-year-old male patient that had previously been admitted for febrile convulsions and had been on the AED carbamazepine and had had epilepsy for 15 years. The second patient was a female aged 33 years who had a record of admission into hospital with malaria in her early teens. She had similarly been taking carbamazepine as well as phenobarbital and had had epilepsy for about almost ten years (9 years 11 months).

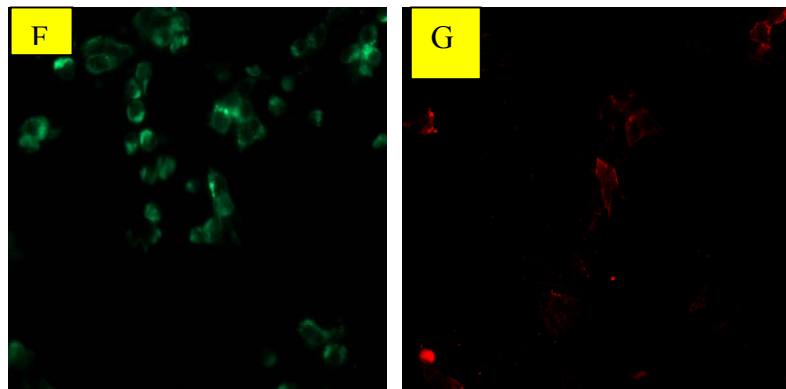
Figure 7: HEK cells transfected with the NR1-GFP tagged and NR2B subunits of the NMDA glutamate receptor.



(A) NR1/NR2B transfected HEK cells after incubation with healthy control serum. The cells have been stained with nuclear stain DAPI and bright blue cells indicate healthy cells. (B) Green fluorescent protein expressing in this sample, 40 % of cells were successfully transfected with NR1-GFP. (C) shows absence of binding by anti human IgG-Alexa fluor568(red) after incubation of HEK cells with healthy control serum



In contrast (D) shows the NR1-GFP/NR2B transfected HEK cells (green) of a positive Caucasian control while (F) the transfected cells of an African Epilepsy patient. (E) shows surface binding of patient sera (red) with a score of 3.5 and (G) shows the surface binding of secondary antihuman IgG to the epilepsy patient cells. Score 1.5.



CHAPTER EIGHT: DISCUSSION

CNS infections have been linked to epilepsy formation, although it is still unclear if this is due to the involvement of autoantibodies to neuronal components such as voltage gated ion channels. The main causes of epilepsy in sub-Saharan Africa are infections that may or may not be of CNS origin [72, 73]. An autoimmune cause has been suggested for certain subgroups of Caucasian patients with epilepsy, but currently, there are no studies on autoimmune epilepsy that have been conducted in an African cohort.

In this pilot study we were able to screen patients with epilepsy and controls from an African community living in Kilifi, a malaria endemic area in coastal Kenya. Using the standardized radioimmunoassay diagnostic technique [74] we were able to demonstrate elevated levels of antibodies to VGKC in 6% of patients with epilepsy compared to community controls. These findings are comparable to the UK studies by McKnight *et al* (2005) where they found that 3% (2/67 patients) of their drug resistant epilepsy cohort had VGKC autoantibodies and Majoie *et al* (2006) where they also reported 6% of patients with long standing epilepsy had VGKC antibodies [9, 53]. This cohort is similar to our study cohort as all the patients in our cohort had active epilepsy. Similarly, at the time of testing two of the 3 patients with antibodies to VGKC were on AEDs while all 3 patients had had epilepsy for more than 3 years.

The method used to identify the VGKC autoantibodies employs the use of [¹²⁵I]- ω -dendrotoxin, which is a protease inhibitor homologue that has been shown to potently and specifically bind to the K⁺ channel subunits Kv 1.1, 1.2 and 1.6, in the brain [53, 75, 76]. For this reason while looking for autoantibodies to VGKC we discovered 4 patients (8%) had antibodies that bound to the labeled neurotoxin rather than to the K⁺ channel itself. This is the first time that **anti-dendrotoxin** antibodies have been detected in the sera of patients with epilepsy. It has been previously reported that neurotoxic poisoning for example from a snake bite or botulism, could cause muscle weakness or hyperactivity due to interference with ion channels such as those expressed in the neuromuscular junction [39]. Not many studies showing the effect of dendrotoxin have been conducted in humans however, probably due to a limited number of patients with venom poisoning. Similarly it is highly unlikely that the two patients with epilepsy in this study had both

developed antibodies as a result of a snake bite. We therefore hypothesise that the anti-dendrotoxin antibodies we are detecting are raised to a different protein, possibly another protease inhibitor molecule, and these antibodies are capable of cross-reacting with the dendrotoxin. Antibodies against sections of the sequence of the K⁺ channel 1 (mKv1.1) in the mouse brain, have been reported to be able to cross-react with dendrotoxin-binding proteins [77, 78]. Similarly, cross-reactivity has also been reported in the neurological condition Guillain-Barre syndrome (GBS). A liposaccharide extracted from the *Campylobacter jejuni* pathogen, in a patient with GBS, was shown to mimic the sugar components of the anti-GMI antibody in the nerve tissue of the patient [79].

We were also able to detect that 4% of the epilepsy patients, had autoantibodies to GAD which is the enzyme responsible for catalysing the decarboxylation of glutamate into gamma-aminobutyric acid (GABA)-the main inhibitory neurotransmitter in the brain [56, 80]. Anti-GAD antibodies can be found in up to 80% of new-onset diabetes Mellitus type 1 (DM1) patients as well as in the neurological condition stiff-person's syndrome a CNS disorder characterized by muscle rigidity and occasionally associated with insulin-dependent diabetes and epilepsy [56, 81]. However the autoantibody titres in stiff person's syndrome and other neurological disorders are usually between 10 and 1000 times higher than those usually seen in DM1 [82, 83]. More recently however there have been more reports on the presence of GAD autoantibodies in patients with both DM1 and epilepsy [58, 84, 85]. Our results show that the two epilepsy patients with GAD antibodies had levels that were similar to those associated with DM1. However we did not have the clinical information that could confirm this. One of the patients, a 40 year old man, had had epilepsy for more than 10 years and was on the antiepileptic drug phenobarbital while the 13 year old boy was on both phenobarbital and phenytoin. Phenobarbital is known to act on GABA-A receptors and has previously been shown in rats to decrease the mRNA expression of GAD65 and 67 after prolonged use [86]. This interference of Phenobarbital with the GABA synthesizing enzyme could therefore possibly imply a decreasing antibody level in the two patients using the drug over time.

Two patients out of the ten tested for anti-NMDA-r antibodies were found to be positive. The NMDA-r is a neuronal ionotropic excitatory glutamate receptor that is mainly involved in neuronal pattern formation, synaptic plasticity and long term potentiation. It is a heteromeric complex that consists of two types of subunits[87, 88]: The NR1 subunit which binds to glycine [64], is widely distributed in the brain during development[89] and whose main function is controlling agonist binding and ion selectivity of the NMDA-r[90] ; and additionally different NR2 subunits (NR2A, NR2B, NR2C, and NR2D) which bind to glutamate[64] and are involved in the regulation of channel gating and Mg⁺ dependency [90]and display a distinct spatio-temporal expression pattern in the brain[89]. The NR1-NR2B receptors are usually expressed during the development of the fetus and have characteristically higher peak ionic currents than other receptors [91] and a six-fold slower inactivation constant resulting in an increased influx of calcium which acts as a second messenger in signaling plasticity[90, 92]. The NR1 subunit was combined with the NR2B subunit in our assay in order to accommodate the functional diversity, pharmacological and electrophysiological properties of this unique receptor [64, 90]. Previous studies have shown the involvement of the NMDA-r in neurological conditions such as systemic lupus erythematosus (SLE) and anti-NMDA receptor encephalitis that may be paraneoplastic and where most of the patients were women [64, 93]. Furthermore, there have been several reports on the expression of NR2B subunit expression tissue from the hippocampus and neocortex of patients with drug resistant epilepsy [94-96]. In this study the two patients that were positive for anti-NMDA antibodies both had long standing epilepsy for more than 10 years. However, further studies need to be done however to confirm these findings.

We did not find a significant difference between the anti-VGCC antibody levels in African patients with epilepsy and community controls. VGCC autoantibodies have previously been found in African children with malaria which is an infectious condition that is often associated with seizure development[71]. Our results show that in comparison to Caucasian patients positive for anti-VGCC antibodies, the baseline antibodies in African epilepsy patients as well as controls are as high as low positive Caucasian patient. This is probably due to the fact that the frequency of $\gamma\delta$

(delta gamma) T-cells in healthy Africans is usually higher than in Caucasians [97]. In fact the mean IgG plasma levels in healthy Africans is reported to be twice (19g/l) (or even higher, -up to 64g/l), than the levels of IgG in healthy Caucasians.

This is hypothesized to be due to exposure to environmental antigens or infectious agents that are known to stimulate the expansion of $\gamma\delta$ T cells such as *Plasmodium falciparum* malaria and Epstein-Barr virus [97]. In our cohort, three patients had previously been admitted with malaria one of whom displayed antibodies to the NMDA-r. However elevated VGCC levels were detected in a seven year old boy on the antiepileptic drug carbamazepine, who had not previously been admitted for malaria but who had had epilepsy from birth. This is an interesting finding as VGCC have not been reported in epilepsy before and have usually been associated with a limited number of neurological autoimmune disorders including Lambert Eaton myasthenia syndrome (LEMS) and paraneoplastic cerebellar ataxia [47]. It is not clear from the clinical history of the patient if he may have any other underlying conditions and follow up as well as electroencephalographic diagnosis needs to be conducted in order to understand his condition fully.

8.1 Study shortcomings:

The present study had a small number of subjects, and this may have influenced the significance in the results. Over 1000 test and control samples have been collected, however, and further analysis of these samples could therefore increase the significance of the results. Similarly, due to time constraints we were unable to obtain all the clinical information for the patients as well as perform analysis for other antibodies that have been previously been shown to play a role in other neurological disorders and autoimmune epilepsy syndromes such as those to the glutamate receptor (GluR3), the nicotinic acetylcholine receptor (AChR), the glycine receptor and aquaporin-4 (a water channel associated with multiple sclerosis[98]). Nevertheless the study was able to identify 3 different types of antibodies in a subset of African patients with active epilepsy.

CHAPTER NINE: CONCLUSION

Epilepsy patients from sub-Saharan Africa with long standing active epilepsy have a similar prevalence of autoantibodies to VGKC, NMDA-r and GAD as Caucasian patients with either long-standing or drug resistant epilepsy. A high level of autoantibodies has also been found in patients with acute or subacute epilepsy. It would therefore be interesting to look at a similar cohort of patients from SSA in particular those who have onset seizures following a known infection. The relationship between infections and epilepsy formation, is still unclear however further study is needed to determine if molecular mimicry in the case of antibodies to the [¹²⁵I] dendrotoxin could elucidate the pathogenic role of infectious agents. Infections that interfere with immunity and that are endemic in this area, such as malaria, neurocysticercosis and HIV, have been shown on several occasions to lead to seizures and epilepsy formation. It would be worth identifying whether any of the other autontibodies including GluR3, AChR, and aquaporin-4, previously described in other neurological conditions may be involved in the subsequent development of epilepsy and to investigate their pathogenicity.

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