

## Complement system biomarkers in epilepsy

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### ABSTRACT

**Purpose:** To explore whether complement dysregulation occurs in a routinely recruited clinical cohort of epilepsy patients, and whether complement biomarkers have potential to be used as markers of disease severity and seizure control.

**Methods:** Plasma samples from 157 epilepsy cases (106 with focal seizures, 46 generalised seizures, 5 unclassified) and 54 controls were analysed. Concentrations of 10 complement analytes (C1q, C3, C4, factor B [FB], terminal complement complex [TCC], iC3b, factor H [FH], Clusterin [Clu], Properdin, C1 Inhibitor [C1Inh] plus C-reactive protein [CRP]) were measured using enzyme linked immunosorbent assay (ELISA). Univariate and multivariate statistical analysis were used to test whether combinations of complement analytes were predictive of epilepsy diagnoses and seizure occurrence. Correlation between number and type of anti-epileptic drugs (AED) and complement analytes was also performed.

**Results:** We found:

- 1) significant differences between all epilepsy patients and controls for TCC ( $p < 0.01$ ) and FH ( $p < 0.01$ ) after performing univariate analysis.
- 2) multivariate analysis combining six analytes (C3, C4, Properdin, FH, C1Inh, Clu) to give a predictive value (area under the curve) of 0.80 for differentiating epilepsy from controls.
- 3) significant differences in complement levels between patients with controlled seizures ( $n = 65$ ) in comparison with uncontrolled seizures ( $n = 87$ ). Levels of iC3b, Properdin and Clu were decreased and levels of C4 were increased in patients with uncontrolled seizures.
- 4) no correlation was found between the level of complement biomarkers and the number of AEDs taken, but an association between some analyte levels and drug therapy was seen in patients taking sodium valproate, clobazam, and perampanel.

**Conclusion:** This study adds to evidence implicating complement in pathogenesis of epilepsy and may allow the development of better therapeutics and prognostic markers in the future. Replication in a larger sample set is needed to validate the findings of the study.

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**Abbreviations:** FB, factor B; TCC, terminal complement complex; FH, factor H; Clu, clusterin; C1Inh, C1 inhibitor; CRP, C-reactive protein; ELISA, enzyme linked immunosorbent assay; AED, anti-epileptic drugs; CNS, central nervous system; WNRTB, Wales Neuroscience Research Tissue Bank; BSA, bovine serum albumin; PBS-T, phosphate-buffered saline containing 0.1% Tween; HRP, horseradish peroxidase; ROC, Receiver Operating Curve; AUC, area under the curve; MAC, membrane attack complex.

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## 1. Introduction

Epilepsy is a common disease; in England the prevalence of people with epilepsy who take anti-epileptic medication is 0.43–1.16% [1]. The 2017 International League Against Epilepsy consensus on epilepsy classification highlights the importance of defining aetiology, including immune causes [2]. Despite contemporary advances in neuroimaging and clinical genetics, the aetiology of epilepsy is still unknown in over a third of cases and a third of patients have seizures resistant to current antiepileptic drugs (AEDs) [3,4]. In these cases resective surgery is the best current option and can be

curative, particularly in temporal lobe epilepsy; however; seizure recurrence occurs in up to half of patients within 5 years of operation [5]. Thus far precision medicine in epilepsy has been limited to the realm of the genetic encephalopathies [6]. A better understanding of aetiology would enable more effective treatment, targeted towards underlying pathogenic mechanisms [7].

Increasing evidence from experimental animal models and resected human brain tissue supports a role of the immune system in epilepsy [8]. At the population level there are prevalence correlations between auto-immune disorders and epilepsy; the risk of epilepsy is 3.8 times greater in people with any one of 12 autoimmune disorders and even higher in children with autoimmunity [9]. Systemic autoimmune disorders, such as systemic lupus erythematosus, have a neurological phenotype that includes a predilection for seizures [2]. The most studied forms of immune epilepsy are Rasmussen's encephalitis, and the autoimmune encephalitis associated with circulating antibodies [10]. Currently, primary immune-mediated epilepsies are recognised as neural autoantibody disorders affecting both cell-surface expressed proteins such as LGI1 and *N*-methyl-D-aspartate (NMDA) receptor, and intracellular proteins such as GAD [11]. A study of neural auto-antibodies in epilepsies of apparent unknown aetiology suggested that immune activation may explain up to 20% of non-paraneoplastic cases [12].

While the usefulness of autoantibody measurements, where present, as diagnostic biomarkers and treatment outcome predictors is robust, the mechanistic nature of the relation between autoantibodies and disease has yet to be elucidated in most cases. Positive responses have been reported for B cell ablation therapy using rituximab in some cases, suggesting a direct role of the autoantibodies in pathogenicity [13,14]. Furthermore, an absence of neural autoantibodies does not rule out the success of immunotherapies, or exclude a diagnosis of limbic encephalitis [13,15]. This lack of consensus and growing evidence of an immune/inflammatory component in epilepsy development makes it necessary to enlarge diagnostic and prognostic assessment to include other immunological biomarkers [16]. In response to this need, the involvement of different immune pathways in epilepsy pathogenesis is increasingly investigated in animal models and in humans [17–19].

One such pathway is the complement system, a major effector of innate immunity and an adjuvant of adaptive immunity. Complement comprises around 30 plasma and cell-surface proteins that interact with one another to induce a series of inflammatory responses involved in defence against infection [20]. Complement activation in the CNS is increasingly recognised to be associated with exacerbation and progression of tissue injury in degenerative and inflammatory diseases [21,22]. Dysregulation of the complement system in epilepsy has been observed both in human and animal studies [23–27]. For example, sequential infusion of individual proteins of the membrane attack pathway (C5b6, C7, C8, and C9) into the hippocampus of awake, freely moving rats induced both behavioural and electrographic seizures as well as neurotoxicity, suggesting a direct role for the complement system in epileptogenesis [28].

The aim of this study was to identify whether changes in the complement system occurred in the plasma of patients with epilepsy, and investigate whether plasma complement biomarkers could be used in diagnosis or stratification related to epilepsy syndrome and seizure control.

## 2. Materials and methods

### 2.1. Hospital records and samples

Patients were prospectively recruited through i) a secondary care adult epilepsy clinic, or ii) attendance for video telemetry as part of

**Table 1**

Distribution of diagnosis and aetiology of epilepsy for the study patients. For three patients more than one aetiology was indicated.

| Diagnosis (syndrome)                                  | Number of patients, n (%) |
|---|---------------------------|
| Temporal lobe epilepsy                                | 61 (38%)                  |
| Juvenile myoclonic epilepsy.                          | 29 (18%)                  |
| Frontal lobe epilepsy                                 | 21 (13%)                  |
| Focal epilepsy – not localised                        | 23 (14%)                  |
| Idiopathic generalised epilepsy                       | 8 (5%)                    |
| Epilepsy with generalised tonic clonic seizures alone | 5 (3%)                    |
| Juvenile absence epilepsy                             | 4 (2%)                    |
| Occipital lobe epilepsy                               | 1 (0.64%)                 |
| Single epileptic seizure                              | 1 (0.64%)                 |
| Unclassified epileptic seizures                       | 4 (2%)                    |
| Aetiology   | Number of patients, n (%) |
| Hippocampal sclerosis                                 | 30 (19%)                  |
| Focal cortical dysplasia                              | 6 (3%)                    |
| Cerebral arteriovenous malformation                   | 3 (1.9%)                  |
| Closed injury of head                                 | 3 (1.9%)                  |
| Dysembryoplastic neuroepithelial tumour               | 3 (1.9%)                  |
| Other structural abnormalities                        | 18 (11%)                  |
| Idiopathic (presumed genetic) or Unknown              | 97 (61.78%)               |

pre-surgical evaluation. The diagnosis of epilepsy was confirmed and classified according to current criteria [5], and presented in Table 1. No restriction in terms of epilepsy syndrome was made as part of inclusion criteria. All cases and controls included in the study gave informed consent. Ethical approval was granted through the Wales Neuroscience Research Tissue Bank (WNRTB). Detailed electro-clinical phenotyping, brain imaging, medication and seizure type and frequency at the time of sample collection, and for one year prior, were obtained from hospital records for all patients. Controlled epilepsy was defined as no seizure of any type in the past year. Relevant clinical variables and the results of investigations were entered into, and then extracted from, a customised clinical database (PatientCare) [29]. Plasma samples (157) were acquired from patients with epilepsy (106 focal epilepsy, 46 generalised epilepsy, 5 unclassified or single seizure) and tested alongside those from 54 healthy non-neurological disease controls sourced via the WNRTB. The controls included mostly staff or students consented for research and stored in the facility (WNRTB ethics REC# 14/WA/0073). The presence of autoantibodies was tested as part of routine clinical practice in cases where there was clinically assessed suspicion of autoimmunity; 19 were tested for anti-NMDA (one positive, uncertain significance), 21 were tested for anti-VGKC (none positive) and 4 were tested for anti-GAD (none positive). Five cases had co-existing thyroid disease; there were no other autoimmune conditions in the cohort.

### 2.2. Immunoassays

Eleven complement analytes were selected for this study, guided by reference to previous studies of complement biomarkers in epilepsy which have described increased serum levels or gene expression of C3, C4, C1q, iC3b and terminal complement complex (TCC), and availability of reagents and in-house assays [23,24,26,28,30]. The concentrations of nine analytes: iC3b, C1q, C3, C4, Properdin, Factor B (FB), Factor H (FH), C1 inhibitor (C1inh), and TCC were measured using established in-house enzyme-linked immunosorbent assays (ELISA) (Table 2). The marker set was chosen to interrogate classical (C1q, iC3b, C3, C4), alternative (Properdin, FB, FH, iC3b) and terminal (TCC) activation pathways. The remaining two analytes Clusterin (Clu), a complement cascade regulator and C-reactive protein (CRP), a benchmark of

inflammatory state, were measured using commercial kits (CRP and Clusterin DuoSet ELISAs, R&D Systems, Abingdon, UK). The samples available for analysis comprised plasma aliquots that had been separated promptly, stored at  $-80^{\circ}\text{C}$  and not subjected to freeze-thaw. For ELISA, Maxisorp (Nunc, Loughborough, UK) plates were coated with affinity-purified capture antibody for 1 h at room temperature, and blocked (1 h at RT) with 1% bovine serum albumin (BSA) in phosphate-buffered saline containing 0.1% Tween 20 (Sigma Aldrich) (PBS-T). After washing wells in PBS-T, purified protein standards or serum samples optimally diluted in 0.1% BSA in PBS-T, were added in duplicate and incubated for 1.5 h at  $37^{\circ}\text{C}$ . Different sample dilutions were used for different assays (Table 2). Wells were washed 3x with PBS-T then incubated (1 h) at RT with detection antibody (unlabelled or labelled with horseradish peroxidase (HRP)) and washed 3x with PBS-T. For assays using unlabelled detection antibodies, HRP labelled secondary antibody (anti-mouse or anti-rabbit IgG as appropriate) was added to wells, incubated and washed as above. Signals were detected using *o*-Phenylenediamine dihydrochloride (OPD, SIGMAFAST™, Sigma-Aldrich) and absorbance (492 nm) was measured. Standards were included on each plate and samples from controls and cases were randomly assigned to eliminate assay bias. A nonlinear regression model was used to fit standard curves generated by ELISA. Total protein concentration ( $\mu\text{g}/\text{ml}$ ) was automatically calculated by reference to the standard curve using GraphPad Prism version 5 (La Jolla, CA, USA). Detection limits, working ranges and assay performance were determined as described [31], using serum from local healthy controls.

### 2.3. Statistical analysis

Univariate statistical tests were performed using the Kruskal-Wallis test for comparison between healthy controls, focal epilepsy and generalised epilepsy, for each analyte. Mann-Whitney *U* test was used for comparison between focal epilepsy and generalised epilepsy for each analyte. Stepwise logistic regression models were tested, including the measured analytes together with co-variables sex and age to adjust for their impact on measured analytes in the cohort. The selected models were then tested using Receiver Operating Curve (ROC) analyses, with leave-one-out cross-validation. Differences in complement levels between patients with controlled seizures in comparison with uncontrolled seizures were tested using the Mann-Whitney *U* test, while correlation between complement analytes and frequency of seizures, and between antiepileptic drugs and concentrations of complement analytes, was investigated using the Spearman test. Differences in

complement concentrations between patients taking or not taking a given drug were tested using the Mann-Whitney *U* test. Due to the conservative nature of multiple testing procedures and the interdependence of our assays, the *p*-values derived from the univariate analyses were not corrected for multiple comparisons. Power calculations were performed, to evaluate how many samples would be needed to observe significant differences between the two groups. To do so, for each of the assays we generated simulated data based on the distributions observed in our study, separately analysing cases and controls. Using a guideline formula calculated as described by Peduzzi [32], the predicted minimum number of samples per group was 140. All tests and analyses were performed using the statistical software R, including the pROC packages [33].

## 3. Results

### 3.1. Plasma levels of FH and TCC distinguish epileptic patients from controls

Of eleven complement analytes measured, two (FH and TCC) were individually significantly different between the focal and generalised epilepsy population in comparison with healthy controls (Table 3). Both FH and TCC plasma levels were significantly increased in cases (for FH, controls  $235.80\ \mu\text{g}/\text{ml}$ ; focal epilepsy  $301.29\ \mu\text{g}/\text{ml}$ ,

**Table 3**

Complement analyte differences between epilepsy patients and controls. Significance of differences was tested using the Kruskal-Wallis test. Variables significant at  $p = 0.05$  are in bold and underlined. TCC – terminal complement complex; C1Inh – C1 inhibitor; FB – Factor B; FH – Factor H; Clu – clusterin; CRP – C-reactive protein. Post-hoc pairwise comparisons, performed using the Dunn test with Bonferroni correction, showed that TCC was significantly reduced in controls compared to both focal ( $p = 0.0011$ ) and generalised cases ( $p = 0.0077$ ), and FH was also significantly different in both comparisons ( $p < 0.001$  for both).

| Assay     | Control |        | Focal   |        | Generalised |        | p-value          |
|-----------|---------|--------|---------|--------|-------------|--------|------------------|
|           | Mean    | StDev  | Mean    | StDev  | Mean        | StDev  |                  |
| TCC       | 15.99   | 6.54   | 20.66   | 10.2   | 19.21       | 6.55   | <b>0.002</b>     |
| iC3b      | 56.06   | 51.22  | 53.43   | 32.06  | 54.52       | 31.49  | <b>0.22</b>      |
| C1q       | 114.46  | 54.54  | 114.16  | 61.6   | 108.94      | 45.9   | 0.91             |
| C3        | 1651.78 | 371.16 | 1716.54 | 317.54 | 1662.12     | 308.74 | 0.36             |
| C4        | 401.16  | 94.81  | 396.44  | 89.34  | 386.6       | 95.9   | 0.42             |
| Properdin | 10.569  | 2.233  | 10.33   | 2.801  | 10.178      | 2.545  | 0.59             |
| FB        | 117.67  | 35.02  | 126.72  | 52.3   | 120.26      | 48.01  | 0.63             |
| FH        | 235.8   | 62.11  | 301.29  | 67.1   | 294.03      | 61.42  | <b>&lt;0.001</b> |
| C1inh     | 160.62  | 34.51  | 172.98  | 34.93  | 164.56      | 30.04  | <b>0.12</b>      |
| CRP       | 1.83    | 6.028  | 1.61    | 1.92   | 1.17        | 1.48   | 0.3              |
| Clu       | 475.37  | 221.05 | 434.293 | 178.08 | 446.09      | 166.97 | 0.76             |

**Table 2**

The table lists the antibody pairs used in the multiplex sets, the sources of the antibodies and the standards, dilutions and assay working range. MM – mouse monoclonal antibody, RP – rabbit polyclonal antibody, HRP – horseradish peroxidase (antibodies labelled in-house), TCC – terminal complement complex; C1Inh – C1 inhibitor; FB – Factor B; FH – Factor H; Clu – clusterin; CRP – C-reactive protein. A kind gift from SRdC – Prof S. Rodriguez de Cordoba, Madrid. ECACC: European Collection of Authenticated Cell Cultures, Hycult: <http://www.hycultbiotech.com/>; CompTech: <http://www.complementtech.com/>.

| Assay     | Capture Antibody                        | Detection Antibody                                  | Standard                        | Working range (ng/mL) | Sample dilution |
|-----------|---|---|---------------------------------|-----------------------|-----------------|
| C1q       | MM anti-C1q mAb (WL02, Hycult)          | MM anti-C1q (DJ01, Hycult)-HRP                      | C1q (in-house purified)         | 32–1000               | 1:1000          |
| C3        | RP anti Human C3 (in-house)             | RP anti-C3 (in-house)-HRP                           | C3 (CompTech)                   | 32–1000               | 1:16,000        |
| C4        | RP anti-C4 (in-house)                   | RP anti-C4 (in-house)-HRP                           | C4 (CompTech)                   | 8–500                 | 1:4000          |
| Factor B  | MM anti-FB (JC1; in house)              | MM anti-FB (MBI-5; in-house)-HRP                    | FB (in-house purified)          | 64–1000               | 1:500           |
| Factor H  | MM anti-FH (OX24; ECACC)                | MM anti-FH (35H9; in-house)-HRP                     | FH (in-house purified)          | 16–1000               | 1:3000          |
| C1inh     | MM anti-C1inh (in-house)                | RP anti-C1inh (in-house)-HRP                        | C1 inhibitor (Cinryze drug)     | 4–100                 | 1:16,000        |
| Properdin | MM anti-Properdin (1.1.1; Gift of SRdC) | MM anti-Properdin (12–14-2; gift of SRdC)-HRP       | Properdin (CompTech)            | 7–100                 | 1:400           |
| TCC       | MM anti-TCC (aE11, Hycult)              | MM anti C8 (E2, in-house)-HRP                       | TCC (in-house purified)         | 60–1000               | 1:50            |
| iC3b      | MM anti-iC3b (Clone 9; in house)        | MM anti-iC3b (bH6; in house)-HRP                    | iC3b (CompTech)                 | 32–1000               | 1:50            |
| Clusterin | MM anti-clusterin (R&D Systems; DuoSet) | Biotinylated MM anti-clusterin-Biotin (R&D; DuoSet) | Clusterin (R&D Systems; DuoSet) | 3–50                  | 1:16,000        |
| CRP       | MM anti-CRP (R&D Systems; DuoSet)       | MM anti-CRP-Biotin/Avidin-HRP (R&D Systems; DuoSet) | CRP (R&D Systems; DuoSet)       | 0.8–50                | 1:600           |

generalised epilepsy 294.03  $\mu\text{g/ml}$ ,  $p < 0.001$ ; for TCC, controls 15.99  $\mu\text{g/ml}$ ; focal epilepsy 20.66  $\mu\text{g/ml}$ , generalised epilepsy 19.21  $\mu\text{g/ml}$ ,  $p = 0.002$ ). TCC and FH were strongly co-correlated (Spearman correlation coefficient 0.48).

### 3.2. Analysis of difference in complement levels between epilepsy types

The above results show significant differences in concentrations of two complement analytes between all epilepsy patients and healthy controls. We then tested whether differences in complement levels occur between epilepsies with different aetiologies. The results presented in Table 4 show that there was no statistical difference between the focal and generalised epilepsy groups in regards to the complement analyte concentrations.

### 3.3. Logistic regression and receiver operating characteristics (ROC) analysis

To find the set of analytes and demographics that best distinguished epilepsy patients and controls, different combinations

**Table 4**

Complement analyte differences between focal epilepsy patients and generalised epilepsy patients. Significance of differences was tested using the Mann-Whitney U test. TCC – terminal complement complex; C1Inh – C1 inhibitor; FB – Factor B; FH – Factor H; Clu – clusterin; CRP – C-reactive protein.

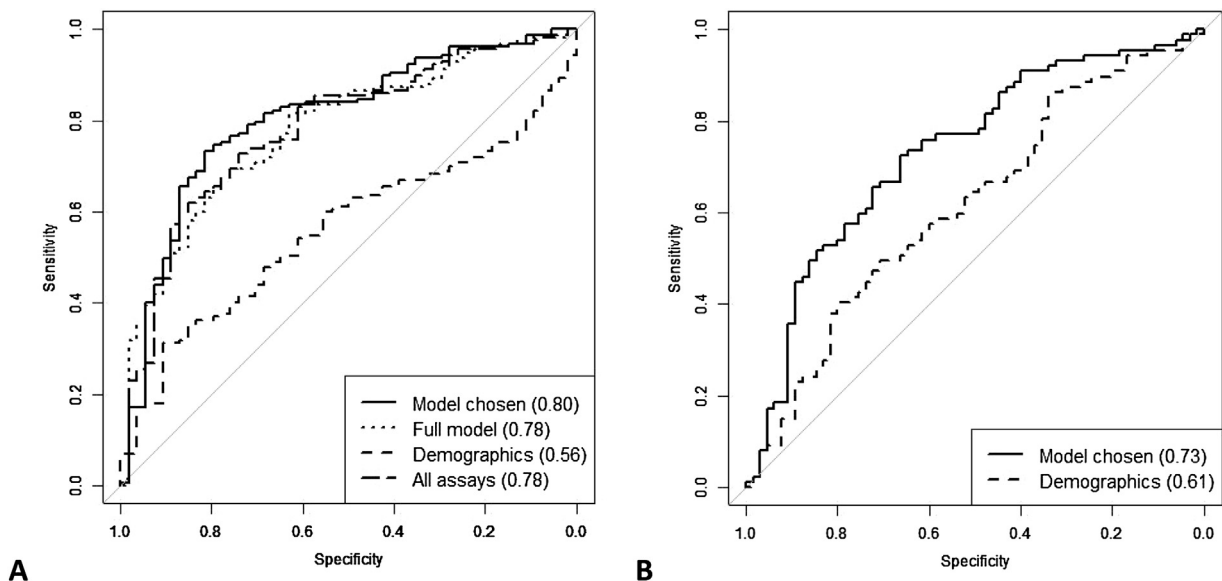
| Assay     | Focal   |        | Generalised |        | p-value |
|-----------|---------|--------|-------------|--------|---------|
|           | Mean    | StDev  | Mean        | StDev  |         |
| TCC       | 20.66   | 10.2   | 19.21       | 6.55   | 0.9     |
| iC3b      | 53.43   | 32.06  | 54.52       | 31.49  | 0.79    |
| C1q       | 114.16  | 61.6   | 108.94      | 45.9   | 0.68    |
| C3        | 1716.54 | 317.54 | 1662.12     | 308.74 | 0.28    |
| C4        | 396.44  | 89.34  | 386.6       | 95.9   | 0.28    |
| Properdin | 10.33   | 2.801  | 10.178      | 2.545  | 0.59    |
| FB        | 126.72  | 52.3   | 120.26      | 48.01  | 0.44    |
| FH        | 301.29  | 67.1   | 294.03      | 61.42  | 0.72    |
| C1inh     | 172.98  | 34.93  | 164.56      | 30.04  | 0.18    |
| CRP       | 1.61    | 1.92   | 1.17        | 1.48   | 0.21    |
| Clu       | 434.293 | 178.08 | 446.09      | 166.97 | 0.66    |

were tested in multiple logistic regression models. The resulting stepwise-selected model comprised six analytes: C3, C4, Properdin, FH, C1Inh and Clu. Although TCC was highly significant in the univariate analysis, it is not included in this multivariate model; this might be due to the fact, noted above, that it is highly correlated with FH, and also with C3 and C4. In the model, the predictive information carried by TCC is already carried by FH (and perhaps C3 and C4), and thus TCC does not add more information to the model. Clusterin, despite not being significant in the univariate tests (significant at  $p < 0.1$  but not  $p < 0.05$ ), was included in the model because it improved performance.

To study the accuracy and performance of the selected model, we performed ROC curve analysis. We computed and compared the AUC for four different models: the stepwise selected model (C3, C4, Properdin, FH, C1Inh and Clu); the full model with all the variables (eleven complement analytes and demographics); the model with only age and sex (demographics); and the model with only the eleven complement analytes. The different ROC curves are provided in Fig. 1A. All four ROC curves have been computed after leave-one-out cross-validation. The most accurate model was the stepwise selected model (AUC = 0.80), whereas the full model and the model including the eleven analytes only were slightly less accurate (AUC = 0.78). Finally, the model with only the demographics provided poor performance (AUC = 0.56). As the full model and assay model were equally accurate and varied only in the absence or presence of the demographics information, we can conclude that there is no significant age or sex effect for epilepsy in the samples analysed.

### 3.4. There are differences in complement analyte levels in patients with uncontrolled seizures

We next investigated whether complement levels differed with the level of seizure control. We identified significant differences in complement levels between patients with controlled seizures compared to those with uncontrolled seizures (Table 5). Levels of iC3b ( $p = 0.02$ ), Properdin ( $p = 0.05$ ) and Clu ( $p = 0.02$ ) were significantly decreased and levels of C4 ( $p = 0.02$ ) were



**Fig. 1.** A. Receiver operating characteristic (ROC) curves to predict the probability of epilepsy (combined focal and generalised) compared to control subjects. Four different models are presented: the stepwise selected model (model chosen); the full model with all the variables; the model with only age and gender (demographics); and the model with only the eleven complement assays. The most accurate model is the stepwise selected model (AUC = 0.80), followed by the full model and the model including the eleven assays only (AUC = 0.78). The model with only the demographics provides poor performance (AUC = 0.56). B. ROC curves for controlled versus uncontrolled cases: Two models are presented, the selected model (age alongside iC3b, C3, C4, Properdin, CRP) and the model with only the demographics variables. The selected model is significantly more accurate (AUC = 0.73) than the model with demographics alone (AUC = 0.61).

**Table 5**

Complement analyte differences between controlled and uncontrolled seizure epilepsy. Significance of differences was tested using the Mann-Whitney *U* test. Variables significant at  $p=0.05$  are in bold and underlined. TCC – terminal complement complex; C1Inh – C1 inhibitor; FB – Factor B; FH – Factor H; Clu – clusterin; CRP – C-reactive protein.

| Assay     | Controlled |        | Uncontrolled |        | p-value     |
|-----------|------------|--------|--------------|--------|-------------|
|           | Mean       | StDev  | Mean         | StDev  |             |
| TCC       | 20.47      | 10.33  | 20.03        | 8.41   | 0.74        |
| iC3b      | 61.06      | 37.16  | 48.3         | 26     | <b>0.02</b> |
| C1q       | 109.48     | 71.66  | 114.9        | 43.72  | 0.07        |
| C3        | 1747.22    | 333.3  | 1664.84      | 297.46 | 0.19        |
| C4        | 372.86     | 84.97  | 408.86       | 93.06  | <b>0.02</b> |
| Properdin | 10.695     | 2.695  | 9.977        | 2.711  | <b>0.05</b> |
| FB        | 123.34     | 44.35  | 125.83       | 55.63  | 0.81        |
| FH        | 300.23     | 69.33  | 298.24       | 62.54  | 0.83        |
| C1inh     | 170.26     | 31.63  | 170.56       | 35.27  | 0.85        |
| CRP       | 1.28       | 1.42   | 1.62         | 2.02   | 0.49        |
| Clu       | 472.37     | 184.45 | 412.07       | 162.69 | <b>0.02</b> |

significantly increased in patients with uncontrolled seizures. There was a trend, although not statistically significant, to increased level of C1q ( $p=0.07$ ) in cases with uncontrolled seizures. A stepwise logistic regression model was computed to find a set of analytes that best distinguished the two groups. The resulting model comprised age (patients with uncontrolled seizures were older) alongside iC3b, C3, C4, Properdin and CRP. Despite its significance in the univariate analysis, Clu is not included, likely because it highly correlated with Properdin. A ROC curve analysis, with leave-one-out cross-validation, was performed as above; the AUC for the model was 0.73, whereas demographic variables (age and gender) alone gave an AUC of 0.61 (Fig. 1B). In those with uncontrolled seizures, there was no significant correlation between complement levels and type or frequency of seizures, although group sizes in this analysis were small.

### 3.5. Associations between anti-epileptic drug therapy and complement analytes

Several studies describe the effects of antiepileptic drugs (AEDs) on the immune system [34,35]. We investigated the correlation between 1) the number of AEDs taken, and 2) different AEDs (for those groups where we had greater than 15 patients taking any particular drug) and the plasma levels of complement analytes. We tested this in patients taking the following drugs (within parenthesis the number of patients taking the given drug): levetiracetam (67), lamotrigine (55), sodium valproate (36), clobazam (36), zonisamide (21), carbamazepine (18), eslicarbazepine (17), perampanel (16), topiramate (15). Spearman correlation analysis showed no correlation between the levels of complement biomarkers and the total number of AEDs taken. An association between levels of some analytes and drug therapy was seen in patients taking sodium valproate (elevated iC3b,  $p=0.0076$ ), clobazam (elevated CRP,  $p=0.09$ ), and perampanel (elevated TCC,  $p=0.001$  and Properdin,  $p=0.001$ ). No significant differences in complement analyte concentrations were observed for the other AEDs tested.

## 4. Discussion

To our knowledge, this is the first study investigating plasma concentrations of a panel of complement analytes in epilepsy. We chose a set of analytes that included markers of classical, alternative and terminal activation pathways. We report an association of higher FH and TCC in adults with epilepsy compared to controls and present a highly predictive model

(AUC 0.8 in ROC analysis) comprising 6 complement analytes (C3, C4, Properdin, FH, C1Inh and Clu) that distinguish between epilepsy cases and controls. We show that complement biomarkers also distinguish patients with well-controlled epilepsy from those with poorly controlled disease; a model comprising five analytes (iC3b, C3, C4, Properdin and CRP) gave an AUC of 0.73. We also tested the influence of specific AEDs on complement analytes.

C3 and C4 are the two most abundant complement proteins, C4 a key component of the classical activation pathway and C3 occupying a cornerstone position where all activation pathways converge; both are important sources of opsonic activity. All of the other analytes in the marker set distinguishing cases from controls are complement regulators, C1inh the sole plasma regulator of classical pathway initiation, FH and properdin controlling the alternative pathway amplification loop, and Clu regulating the terminal pathway. FH regulates complement both in the fluid phase and on self cells; capture of FH on self cells is important in defence against damage, while pathogen capture of FH reduces complement activation aiding pathogen survival. Dysregulation of the alternative pathway has been reported in other neurological and neuropsychiatric disorders, including schizophrenia [36], and altered plasma FH levels are a marker of multiple sclerosis [37]. TCC levels reflect activation of the terminal pathway that deposits the lytic membrane attack complex (MAC) on pathogens; MAC can also damage or activate self cells. A direct role in epilepsy was suggested by the demonstration that infusion of individual MAC proteins into the hippocampus of rats induced cytotoxicity and seizures [28].

Complement has recently emerged as a key player in brain development; C1q and downstream classical pathway products mark synapses for elimination during post-natal brain remodelling, a process that is essential for brain development, maturation, and optimal function [37]. This physiological process has also been implicated in pathological synapse elimination in the context of schizophrenia and dementia [37–39]. Increased levels of C1q and iC3b (classical complement pathway markers) were reported in human brain samples in focal cortical dysplasia [31], suggesting that aberrant complement activation occurs in patients with drug resistant seizures. Our current study suggests that dysregulation of the classical (C1inh, C4), alternative (FH, properdin, C3) and terminal (TCC) pathways also contribute to epilepsy pathogenesis.

Our data suggest that dysregulation of the classical pathway may be a feature of poor seizure control; there was a significant increase in plasma C4 and trending increased C1q in patients with uncontrolled seizures compared to those with controlled seizures. It is, however, unknown whether the observed changes are a driver or consequence of seizures, the latter perhaps reflecting a response to brain or peripheral tissue injury. Indeed, it is possible that changes in analyte levels occurred as a consequence of recent seizures. We had an accurate record of seizure frequency from the clinic records at the time of sampling; however, this did not always include the precise interval between the most recent seizure and the sample. We were therefore unable to correlate our measures with seizure recency; however, we were able to test against seizure frequency (or type) and did not find a significant correlation between these and analyte levels. Furthermore, the majority of our cases were recruited and sampled at routine outpatient visits, making it unlikely that our findings were a direct consequence of recent seizures.

A limitation of our study is the heterogeneity of the epilepsy sample in regards to the disease aetiology and drug treatment. We decided on an “all comers” data driven approach as an initial analysis in this area. We examined the effects of disease severity (seizure frequency) and specific drug therapies. Some AEDs,

including valproate and carbamazepine, are reported to have direct effects on the immune system [35]. In our study, sodium valproate, clobazam, and perampanel were associated with changes in levels of individual complement analytes; however, groups of patients taking individual drugs were too small (sodium valproate  $n = 36$ , clobazam  $n = 36$ , perampanel  $n = 16$ ) for statistical analysis to be conclusive. A further limitation is that this is a study of circulating plasma biomarkers and we are using these to probe what may be happening within the CNS. However, it has long been established that the blood-brain barrier in epilepsy is dysfunctional and leaky [40,41], thus the use of plasma biomarkers, which are much more easily accessible and measurable, is well-founded. Moreover, the dysregulation of complement described in our study closely mirrors changes identified in epilepsy brain tissue in humans and in animal models [26,28].

In order to confirm our findings and to tease out the effect of AEDs on complement biomarker levels, a replication set, focussing on the informative analytes, is needed. We need in future to access samples from early diagnosis patients, ideally recruiting patients promptly after their first seizure. A prospective study with frequent measurements of plasma complement levels would be beneficial for identification of dynamic changes in complement and would provide a more reliable analysis of the impact of complement dysregulation on seizure frequency and type.

## 5. Conclusion

Significant differences were found in a number of complement analytes between patients with epilepsy and controls, controlled and uncontrolled epilepsy, and certain AEDs. Multivariate analyses identified highly predictive models for distinguishing cases from controls and well-controlled from uncontrolled cases. These data adds further evidence to the role of complement dysregulation in the pathogenesis of epilepsy and may allow the development of better prognostic markers and therapeutics in the future.

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## Declaration of competing interests

Thomas RH has received consultancy and speaker fees from Eisai, Sanofi and UCB Pharma. Hamandi K has received consultancy and speaker fees from UCB Pharma, Eisai and GlaxoSmithKline.

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## References

- Steer S, Pickrell WO, Kerr MP, Thomas RH. Epilepsy prevalence and socioeconomic deprivation in England. *Epilepsia* 2014;55:1634–41.
- Scheffer IE, Berkovic S, Capovilla G, Connolly MB, French J, Guilhoto L, et al. ILAE classification of the epilepsies: position paper of the ILAE Commission for Classification and Terminology. *Epilepsia* 2017;58:512–21.
- Thomas RH, Berkovic SF, Bamagous GA, Norrie JD, wan PK. The hidden genetics of epilepsy—a clinically important new paradigm. *Nat Rev Neurol* 2014;10:283–92.
- Brodie MJ, Barry SJE, Bamagous GA, Norrie JD, Kwan P. Patterns of treatment response in newly diagnosed epilepsy. *Neurology* 2012;78:1548–54.
- de Tisi J, Bell GS, Peacock JL, McEvoy AW, Harkness WF, Sander JW, et al. The long-term outcome of adult epilepsy surgery, patterns of seizure remission, and relapse: a cohort study. *Lancet (London, England)* 2011;378:1388–95.
- EpiPM Consortium. A roadmap for precision medicine in the epilepsies. *Lancet Neurol* 2015;14:1219–28.
- Vigevano F, Arzimanoglou A, Plouin P, Specchio N. Therapeutic approach to epileptic encephalopathies. *Epilepsia* 2013;54(Suppl. 8):45–50.
- Walker L, Sillis GJ. Inflammation and epilepsy: the foundations for a new therapeutic approach in epilepsy. *Epilepsy Curr* 2012;12:8–12.
- Ong M-S, Kohane IS, Cai T, Gorman MP, Mandl KM. Population-level evidence for an autoimmune etiology of epilepsy. *JAMA Neurol* 2014;71:569–74.
- Varadkar S, Bien CG, Kruse CA, Jensen FE, Bauer J, Pardo CA, et al. Rasmussen’s encephalitis: clinical features, pathobiology, and treatment advances. *Lancet Neurol* 2015;14:195–205.
- Toledano M, Pittock SJ. Autoimmune epilepsy. *Semin Neurol* 2015;35:245–58.
- Dubey D, Alqallaf A, Hays R, Freeman M, Chen K, Ding K, et al. Neurological autoantibody prevalence in epilepsy of unknown etiology. *JAMA Neurol* 2017;74:397–402.
- Toledano M, Britton JW, McKeon A, Shin C, Lennon VA, Quek AM, et al. Utility of an immunotherapy trial in evaluating patients with presumed autoimmune epilepsy. *Neurology* 2014;82:1578–86.
- Bakpa OD, Reuber M, Irani SR. Antibody-associated epilepsies: clinical features, evidence for immunotherapies and future research questions. *Seizure* 2016;41:26–41.
- von Rhein B, Wagner J, Widman G, Malter MP, Elger CE, Helmstaedter C. Suspected antibody negative autoimmune limbic encephalitis: outcome of immunotherapy. *Acta Neurol Scand* 2017;135:134–41.
- de Vries EE, van den Munckhof B, Braun KPJ, van Royen-Kerkhof A, de Jager W, Jansen FE. Inflammatory mediators in human epilepsy: a systematic review and meta-analysis. *Neurosci Biobehav Rev* 2016;63:177–90.
- Alapirtti T, Waris M, Fallah M, Soilu-Hänninen M, Mäkinen R, Kharazmi E, et al. C-reactive protein and seizures in focal epilepsy: a video-electroencephalographic study. *Epilepsia* 2012;53:790–6.
- Marchi N, Granata T, Janigro D. Inflammatory pathways of seizure disorders. *Trends Neurosci* 2014;37:55–65.
- Tan C-C, Zhang J-G, Tan M-S, Chen H, Meng DW, Jiang T, et al. NLRP1 inflammasome is activated in patients with medial temporal lobe epilepsy and contributes to neuronal pyroptosis in amygdala kindling-induced rat model. *J Neuroinflammation* 2015;12:18.
- Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 2010;11:785–97.
- Bellander BM, Singhrao SK, Ohlsson M, Mattsson P, Svensson M. Complement activation in the human brain after traumatic head injury. *J Neurotrauma* 2001;18:1295–311.
- Bonifati DM, Kishore U. Role of complement in neurodegeneration and neuroinflammation. *Mol Immunol* 2007;44:999–1010.
- Başaran N, Hincal F, Kansu E, Ciger A. Humoral and cellular immune parameters in untreated and phenytoin-or carbamazepine-treated epileptic patients. *Int J Immunopharmacol* 1994;16:1071–7.
- Jamali S, Salzmann A, Perroud N, Ponsolle-Lenfant M, Cillario J, Roll P, et al. unctonal variant in complement C3 gene promoter and genetic susceptibility to temporal lobe epilepsy and febrile seizures. *PLoS One* 2010;5:e12740.
- Lukasiuk K, Kontula L, Pitkänen A. cDNA profiling of epileptogenesis in the rat brain. *Eur J Neurosci* 2003;17:271–9.
- Aronica E, Boer K, van Vliet EA, Redeker S, Baayen JC, Spliet WG, et al. Complement activation in experimental and human temporal lobe epilepsy. *Neurobiol Dis* 2007;26:497–511.
- Libbey JE, Kirkman NJ, Wilcox KS, et al. Role for complement in the development of seizures following acute viral infection. *J Virol* 2010;84:6452–60.
- Xiong Z-Q, Qian W, Suzuki K, McNamara JO. Formation of complement membrane attack complex in mammalian cerebral cortex evokes seizures and neurodegeneration. *J Neurosci* 2003;23:955–60.
- Wardle M, Sadler M. How to set up a clinical database. *Pract Neurol* 2016;16:70–4.
- Wyatt SK, Witt T, Barbaro NM, Cohen-Gadol AA, Brewster AL. Enhanced classical complement pathway activation and altered phagocytosis signaling molecules in human epilepsy. *Exp Neurol* 2017;295:184–93.
- Ingram G, Hakobyan S, Hirst CL, et al. Systemic complement profiling in multiple sclerosis as a biomarker of disease state. *Mult Scler* 2012;18:1401–11.
- Peduzzi P, Concato J, Kemper E, Holford TR, Feinstein AR, Harris CL, et al. A simulation study of the number of events per variable in logistic regression analysis. *J Clin Epidemiol* 1996;49:1373–9.
- R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2016. <https://www.R-project.org/>.
- Başaran N, Kansu E, Hincal F. Serum immunoglobulins, complement levels and lymphocyte subpopulations in phenytoin-treated epileptic patients. *Immunopharmacol Immunotoxicol* 1989;11:335–46.
- Beghi E, Shorvon S. Antiepileptic drugs and the immune system. *Epilepsia* 2011;52(Suppl. 3):40–4.

- [36] Boyajyan A, Ghazaryan H, Stepanyan A, Sim RB. Genetic polymorphisms of complement factor H in schizophrenia and ischemic stroke. *Mol. Immunol* 2013;56:294.
- [37] Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, et al. The classical complement cascade mediates CNS synapse elimination. *Cell* 2007;131:1164–78.
- [38] Stephan AH, Barres BA, Stevens B. The complement system: an unexpected role in synaptic pruning during development and disease. *Annu Rev Neurosci* 2012;35:369–89.
- [39] Hong S, Beja-Glasser VF, Nfonoyim BM, Frouin A, Li S, Ramakrishnan S, et al. Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science* 2016;352:712–6.
- [40] Oby E, Janigro D. The blood-brain barrier and epilepsy. *Epilepsia* 2006;47:1761–74.
- [41] van Vliet EA, Aronica E, Gorter JA. Blood-brain barrier dysfunction, seizures and epilepsy. *Semin Cell Dev Biol* 2015;38:26–34.