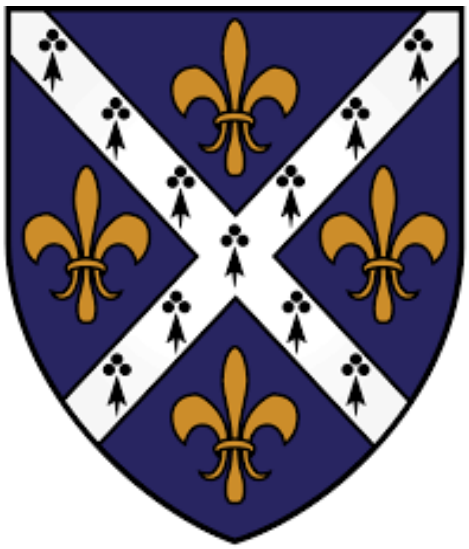
Mechanisms of disease perpetuation and pathogenesis in autoantibody-mediated epilepsies with a focus on LGI1- and CASPR2-related syndromes

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ABSTRACT

Background and Aims: This thesis explores the clinical and immunological aspects of LGI1- and CASPR2-autoantibody syndromes – rare, immunotherapy-responsive conditions mediated by autoantibodies against neuronal cell surface antigens. Two paradigms of autoantibody production exist: ongoing germinal centre (GC) reactions, and long-lived plasma cells (LLPCs). We aimed to expand the clinical phenotypes, and explore the underlying immunobiology using patient biological samples. We aimed to examine the concept of GC reactions through study of serum IgM antibodies, CXCL13 levels, and patient serum binding patterns to the two major LGI1 domains (LRR and EPTP), and to directly investigate the potential role of the bone marrow (BM) compartment by asking whether BM-derived antigen-specific LLPCs contribute to LGI1-autoantibody production.

Methodology: Detailed clinical phenotyping was undertaken for 107 LGI1-autoantibody patients. The CASPR2 IgM live cell-based assay (CBA) was developed, optimised, and tested in 120 sera including n=51 from 25 patients with CASPR2-IgG. LGI1 IgMs were tested by live CBA in 120 IgG-depleted sera (n=70 from patients with LGI1-IgG). End-titrations against full-length LGI1, and LRR and EPTP were quantified by live CBA for 102 sera and 11 CSF samples from 38 LGI1-autoantibody patients. A CXCL13 assay was developed and serum CXCL13 levels were determined in 543 samples from 398 subjects, including 161 sera from 52 LGI1 autoantibody patients. LLPCs, defined by flow cytometry as live, $CD3^-CD14^-CD20^-CD19^{-/low}CD138^+$ cells, were purified from fresh BM aspirate from n=2 patients with LGI1-autoantibody encephalitis. BM LLPC oligocultures were maintained in optimized culture conditions (IL-6, CXCL12, APRIL, and BAFF). After 7 days, culture supernatants were tested for LGI1-IgG by live CBA.

Results: Potential triggers including prodromal infection and vaccination were identified in ~20% of patients, at a median time of 3.5 weeks prior to symptom onset. 11 distinct subtypes of seizures were identified, and patients manifested up to 5 seizure semiologies. When compared to a pharmacoresistant epilepsy cohort (n=218), the proportion of patients with ≥ 3 seizure types was higher in the LGI1-autoantibody patient group (p=0.0039**). Relapses were common, (39/107; 36%), and 36% of these had multiple relapses. The most common precipitant of relapse was steroid weaning or cessation (80%). FBDS significantly associated with relapse, present in 77% relapsing versus 46% non-relapsing patients (p=0.002**). Novel clinical features including hoarding and obsessive-compulsive behaviours were found in 35%, and unusual manual stereotypies which we have termed

'complex motor behaviours' in 5%, which may represent either a novel seizure semiology or the sleep disorder agrypnia excitata.

IgMs were uncommon, detected in 16% of CASPR2- and 17% of LGI1-autoantibody patients. In LGI1-antibody patients, 7/8 (88%) positive samples were from relapsing patients, suggesting that IgM may be a marker of relapse. Almost all (97%) patients had serum autoantibodies to both LGI1 domains, and within individual samples EPTP and LRR end-titres showed strong correlations (Spearman r = 0.9257, p<0.0001****). Patients showed constant EPTP:LRR ratios across disease course, suggesting ongoing production of autoantibodies to both LGI1 domains, representing putative ongoing GC activity. LGI1-antibody patients had the highest serum CXCL13 levels (mean 386 pg/ml; LGI1 versus pharmacoresistant epilepsy, seizure-free epilepsy, and HCs p<0.0001****; LGI1 versus CASPR2 p=0.0015**). Serum CXCL13 levels were higher in LGI1-antibody patients with relapses compared to non-relapsing patients (p<0.0001****). LGI1-IgG autoantibodies were detected in the BM LLPC culture supernatant from one of the patients at a frequency of 1:4920 of total cultured BM LLPCs.

Conclusions: Herein, the clinical features of the largest UK cohort of patients with LGI1-autoantibodies are described, expanding the current clinical phenotypes with seizure and relapse characteristics, and novel descriptions including hoarding and 'complex motor behaviours'. We show that whilst IgMs are rare, LGI1-IgM may be a marker of relapse, and other GC activity markers including CXCL13 may be useful biomarkers in LGI1-autoantibody patients. We demonstrate the presence of LGI1-specific BM-derived LLPCs in a patient with autoimmune encephalitis by direct sampling of their BM. These proof-of-concept data demonstrate that the BM is a site for long-term autoantibody production and suggest a role for BM-derived LLPCs in autoimmune encephalitis. Collectively, these results suggest that both GC reactions and LLPC-driven mechanisms of autoantibody production appear to operate synergistically in patients with LGI1 autoantibodies. These paradigms may be applied to other autoantibody-mediated conditions. Overall, these findings expand the clinical phenotypes and provide novel insights into disease immunobiology which may inform use of more targeted immunotherapies to improve clinical outcomes in these patients.

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ABBREVIATIONS

ACE-R – Addenbrooke's Cognitive Examination–Revised

ASM – anti-seizure medication

BCM – B cell medium

BM – bone marrow

CASPR2 – contactin-associated protein-like 2

CBA – cell-based assay

CXCL13 – C–X–C motif chemokine ligand 13

CSF – cerebrospinal fluid

DC – disease control

DRE – drug-resistant epilepsy

EEG – electroencephalogram

EPTP – epitempin (LGI1 domain)

FBDS – faciobrachial dystonic seizures

eGFP – enhanced green fluorescent protein

GC – germinal centre

GTCS - generalised tonic clonic seizure

HADS – Hospital Anxiety and Depression Scale

HEK cell – human embryonic kidney cell

HC – healthy control

IgG – immunoglobulin

IT – immunotherapy

IVIG – intravenous immunoglobulin

IVMP – intravenous methylprednisolone

LE – limbic encephalitis

LRR – leucine-rich repeat (LGI1 domain)

LGI1 – leucine-rich glioma-inactivated 1

LLPC – long-lived plasma cell

MoS – Morvan's syndrome

MRS – Modified Rankin Scale

MS – multiple sclerosis

NCS – nerve conduction studies

NMT – neuromyotonia

NSAb – neuronal surface antibody

PC – plasma cell

PLEX – plasma exchange

VGKC – voltage-gated potassium channel

CHAPTER 1. INTRODUCTION:

LGI1-Autoantibody Syndromes: Clinical and Immunological Principles

1.1 Overview and Aims

This DPhil is focused on the ongoing clinical and immunological characterisation of two neuroimmunological conditions: LGI1- and CASPR2-autoantibody syndromes. Autoantibody-mediated neurological disorders are a relatively recently described entity in medical practice. Leucine-rich glioma-inactivated1 (LGI1)-directed autoantibodies cause limbic encephalitis with pathognomonic faciobrachial dystonic seizures (FBDS) (Irani et al. 2011), whilst autoantibodies against contactin-associated protein receptor-2 (CASPR2) are associated with encephalitis, neuromyotonia, or both (Morvan's syndrome) (Vincent et al. 2004; Shillito et al. 1995; Liguori et al. 2001; Irani et al. 2010; Lai et al. 2010). Both LGI1- and CASPR2-autoantibody associated syndromes display distinct HLA-associations (Binks, Varley, et al. 2018) and striking responses to immunotherapy, which can prevent permanent cognitive sequelae if administered early (Irani et al. 2011; Thompson et al. 2017). Clinical diagnosis and confirmatory autoantibody testing are paramount, and optimization of diagnostic tests and delineation of the mechanisms of autoantibody production have been the focus of several research groups over recent years. Within this thesis, we aimed to expand the clinical phenotypes and better characterise outcomes in a larger cohort of patients. Additionally, we examined various potential disease biomarkers, and several immune compartments, including the periphery, CSF, and bone marrow, to better understand the sites and cells responsible for autoantibody production, which would help to direct future therapies.

1.2 Autoantibody-Mediated Neurological Syndromes

Over the last decade or so, there has been rapid development within the fairly novel and fascinating field of Autoimmune Neurology, with description of a growing constellation of autoimmune encephalitic syndromes, each with highly distinctive clinical features, demographic, and association with a specific autoantibody (Irani et al. 2010; Lai et al. 2010; Irani et al. 2012; Dalmau et al. 2008). For example, psychiatric features followed by the onset of a movement disorder and seizures in a young female are typically indicative of NMDAR-autoantibody encephalitis (Dalmau et al. 2007; Dalmau et al. 2008), whilst confusion, memory disturbance, and faciobrachial dystonic seizures in an elderly male should raise suspicion of

LGI1-autoantibody encephalitis (Irani et al. 2010; Lai et al. 2010; Irani et al. 2011). NMDAR-autoantibody encephalitis is commonly associated with underlying ovarian teratoma, presence of which should be sought (Dalmau et al. 2007; Dalmau et al. 2008). Additionally, autoantibodies associate with other neurological syndromes, including peripheral nerve manifestations such as neuromyotonia (NMT) and pain, triggered by CASPR2-autoantibodies (Irani et al. 2010; Irani et al. 2012; Dawes et al. 2018; Ramanathan et al. 2021). Crucially, these syndromes represent a set of highly immunotherapy-responsive and thus potentially reversible conditions (Dalmau et al. 2008; Irani et al. 2010; Irani et al. 2011; Irani et al. 2012; Thompson et al. 2017), thus transforming the field of Neurology – once conceived to represent a specialty of largely progressive, untreatable conditions. Importantly, the incidence of autoimmune encephalitis is comparable to that of infectious encephalitis (~1 per 100, 000; Minnesota, USA in 2015), and many experts in the field believe that it is likely to exceed the frequency of infectious encephalitis, particularly with increasing awareness, recognition and diagnosis of autoimmune neurological syndromes over time (Dubey et al. 2018; Ramanathan et al. 2019).

1.2.1 History of Autoantibody-Mediated Neurological Syndromes

The earliest clinical descriptions of limbic encephalitis (LE) are from the 1960s, where Corsellis et al described three patients with amnestic syndromes, confusion, and new-onset seizures – all three were subsequently found to have bronchial carcinoma (Corsellis et al. 1968). The patients showed lymphocytic CSF with raised protein. Post-mortem examinations of the brain revealed macroscopic “brownish discolouration” of the hippocampi, and microscopically, extensive neuronal loss within the medial temporal lobes associated with astrocytic infiltrates and gliosis, particularly affecting the amygdala and hippocampi. Importantly, this contributed to the growing recognition of the now well recognised syndrome of LE, typically comprising the subacute onset of amnesia, encephalopathy with confusion, seizures, psychiatric features, and sleep disturbances (Irani et al. 2010; Irani, Gelfand, Al-Diwani, et al. 2014). The syndrome of LE is distinct to infective encephalitis, since over the last ten years, it has been associated with the identification of novel autoantibodies (Irani et al. 2010; Lai et al. 2010; Irani et al. 2012), hence giving rise to a novel subgroup of disorders termed ‘autoimmune encephalitis’.

1.2.2 The Original VGKC-Antibody Assay

The initial clinical insights of an autoimmune basis to acquired neurological disease were originally described in Scotland in 1960 by Simpson, who hypothesised myasthenia gravis to be an autoimmune process likely involving an antibody against muscle end-plate protein {Simpson:1960gr}. Further observations were made 30 years later in Oxford from a patient with acquired NMT (Isaacs' syndrome), a form of peripheral nerve hyperexcitability, who exhibited severe disease, which was refractory to sodium channel blockers. Treatment with plasma exchange, however, prompted almost complete symptom resolution (Sinha et al. 1991). Additionally, purified IgG from this patient caused muscle hyperexcitability in phrenic nerve-diaphragm preparations. Together, these observations suggested an underlying autoantibody-driven mechanism.

At the time, voltage-gated potassium channels (VGKC) were hypothesised as a likely target. A VGKC-antibody assay was developed, which utilised alpha-dendrotoxin (α- DTX), a neurotoxin from green mamba snake venom which is known to label Kv1.1, 1.2, and 1.6 potassium channels. Radioiodinated α-DTX was used to label soluble, mammalian brain extracts, which detected precipitated serum or CSF autoantibodies, which were presumed to bind VGKCs (Shillito et al. 1995; Hart et al. 1997). Originally, VGKC-antibodies were identified in patients with Morvan's syndrome (NMT plus central nervous system manifestations) (Liguori et al. 2001) and LE (Pozo-Rosich et al. 2003; Vincent et al. 2004; Thieben et al. 2004), and these patients showed marked clinical improvements following immunotherapy. Hence, these early discoveries helped to describe and classify a potentially reversible set of autoimmune neurological conditions.

1.2.3 Identification of Neuronal Surface-Directed Autoantibodies: Discovery of LGI1 and CASPR2

Later studies in 2010 focused on biochemical interrogation of proteins complexed to VGKCs. This research led to the discovery that LGI1 and CASPR2 were the actual targets of autoantibodies in the immunotherapy-responsive syndromes LE, NMT and Morvan's

syndrome, rather than the previously presumed VGKCs (Irani et al. 2010; Lai et al. 2010). An important new concept in the field emerged that, crucially, the *extracellular*-exposed domains of these proteins were the direct antigenic targets. Additionally, a small subset of patients with these syndromes had contactin-2 autoantibodies (Irani et al. 2010). Importantly, these autoantibodies are detected in the serum and CSF of patients with these highly specific clinical syndromes.

Over the last decade, it has become apparent that autoantibodies to several neuronal cell surface proteins are found in patients with an increasing constellation of distinct neurological syndromes. Further, it is now clear that these autoantibodies are directly pathogenic by binding and disrupting their target antigens on the neuronal cell surface, the manifestations of which produce the distinct clinical syndrome **(Figure 1.1)**. In contrast, patients with paraneoplastic neurological syndromes harbour antibodies which recognize intracellular onconeural antigens including Hu, Ma2, Ri, Yo, CV2/CRMP5, typically with underlying associated tumours (Irani, Gelfand, Al-Diwani, et al. 2014; Leypoldt & Wandinger 2014). Paraneoplastic or 'onconeural' antibodies are not considered pathogenic as they are unable to access their targets *in vivo*, since they are intracellular. Rather, paraneoplastic neurological syndromes are thought to be predominantly T cell-mediated, with T-cell mediated cytotoxic neuronal damage, and likely resultant production of the intracellular antibodies as a secondary phenomenon. Paraneoplastic syndromes shall not be reviewed further, since this thesis shall examine conditions associated with pathogenic autoantibodies directed towards extracellular domains of the neuronal cell surface.

Table 1.1 summarises the multitude of common neuronal surface autoantibodies and clinical autoimmune encephalitic syndromes which have been described over the last decade. Characteristic demographics and clinical syndromes are helpful in distinguishing the autoimmune encephalitis subtype, for instance an elderly male with confusion and seizures is likely to have LGI1- or CASPR2-autoantibodies, whilst a child with a movement disorder is more likely to have autoantibodies against D2R or GluRD2. Additionally, some of the syndromes have strong HLA associations (LGI1- , CASPR2- and IgLON5-) and prominent tumour associations, which should be sought, since their excision and treatment often act as a form of immunotherapy, with good response of the neurological syndrome.

Table 1.1 Clinical and Paraclinical Features of Autoimmune Encephalitis Subtypes Caused by Neuronal Surface Autoantibodies

Neuronal Autoantibody	Median Onset Age (years)	M:F Sex Ratio	Clinical Features	Investigations	Immunotherapy Response/ Outcomes
NMDAR	21	1:4	Encephalitis with **flu-like prodrome** → **psychiatric phase** (psychosis and catatonia) → **neurological phase:** polymorphic movement disorder (90%; dystonia, orofacial dyskinesias), seizures (70%) → **critical illness stage:** autonomic instability, central apnoea and coma. Ovarian teratoma in 30-40%. NMDAR encephalitis can occur post HSV1 encephalitis.	MRI brain normal in 75% CSF 80% abnormal: lymphocytic pleiocytosis EEG 90% abnormal – slowing, epileptiform features, or extreme delta brush pattern.	~50% improve after first line IT ~50% require 2nd line IT. 80% MRS 0-2 at 2 years Relapse rate up to 30% Up to 15% mortality
LGI1	64	2:1	LE: frequent focal seizures including pathognomonic FBDS which precede cognitive impairment. >90% harbour HLA-DRB1*07:01.	MRI brain ~60% abnormal – increased signal+/-swelling in MTL CSF ~30% abnormal (lymphocytic pleiocytosis/ raised protein)	At 2 years 1/3 recovered, 1/3 independent but unable to work, 1/3 severely disabled or dead.

				EEG ~60% abnormal (epileptiform features/ diffuse slowing) Hyponatraemia in ~70%	Relapse rate up to 30% Mortality <5%.
CASPR2	66	9:1	3 main syndromes: Peripheral nerve hyperexcitability (NMT), LE, and Morvan's syndrome. HLA-DRB1*11:01 in ~50% Thymoma in 20%	MRI brain abnormal in 30% CSF abnormal in 30% EEG abnormal 70% EMG – hyperexcitability (myokymia)	Good or complete response to IT in 50%. Relapses in up to 30%. Mortality 10%.
$GABA_AR$	40	1:1	Encephalitis with frequent status epilepticus (SE), epilepsia partialis continua, hallucinations. ~20% thymoma, SCLC, non-Hodgkin lymphoma.	MRI brain >80% abnormal ("fluffy" cortical and subcortical abnormalities) CSF abnormal in ~50%. EEG >80% abnormal (encephalopathy)	IT-responsive but full recovery in 30%. Mortality up to 20% due to SE. Relapses in 10%.
$GABA_BR$	61	1.5:1	LE with seizures Tumours in ~50% (SCLC)	MRI brain abnormal 70% (increased signal MTLs) CSF ~80% lymphocytic pleiocytosis EEG abnormal 75%	~90% respond to IT. Mortality up to 40% (due to tumour).

AMPAR	55	1:2.5	LE with amnesia, seizures, movement disorder, and psychiatric features Tumour in ~70% (thymic, SCLC, breast, ovarian)	MRI brain ~85% abnormal (bilateral MTL changes) CSF 70% abnormal EEG 45% abnormal	Most improve: median MRS=1 (of survivors). ~15% mortality (due to malignancy).
DPPX	55	2.5:1	Encephalitis with confusion, myoclonus, tremors, hyperekplexia, hallucinations, seizures, sleep and bulbar dysfunction. Severe prodromal diarrhoea/ weight loss. B-cell neoplasm in 10% (GI follicular lymphoma/ leukaemia)	MRI brain normal/ non-specific CSF ~30% abnormal EEG ~70% focal or diffuse slowing	60-70% response to IT. Multiple relapses in ~25%. Mortality up to 20%.
GlyR	45	1:1	3 main syndromes: PERM (progressive encephalomyelitis with rigidity and myoclonus), Stiff-person syndrome (>10% cases), epilepsy. Thymoma in 15%; lymphoma, metastatic breast ca.	MRI typically normal: brain – TL inflammation (5%); spine – short cord lesions (15%), LETM (5%). CSF pleiocytosis ~40%, 20% OCBs. EEG abnormal in 70%. EMG: continuous motor unit activity, spontaneous/ stimulus-induced activity/ NMT in 60%.	Median MRS=1 at follow-up (up to 7 years). Relapse rate 15%. 10% mortality.

MOG	37	1:1	Optic neuritis, transverse myelitis, brainstem encephalitis, encephalitis.	MRI brain 75% bilateral subcortical lesions, 30% brainstem involvement. MRI cord 50% short-segment TM, LETM; frequent conus medullaris involvement. MRI orbit – extensive bilateral ON lesions; chiasmal involvement. CSF lymphocytic pleiocytosis in 60%; OCBs rare	75% good response to steroids. ~60% good or full recovery. Relapses common.
IgLON5	64	1:1	4 main syndromes: Sleep disorder (REM parasomnias, sleep apnoea), bulbar syndrome, PSP-like syndrome, cognitive syndrome+/-chorea. Insidious onset; severe and progressive.	MRI brain normal in 80%; 15% brainstem atrophy. CSF lymphocytosis in 30%; elevated protein in 50%. HLA-DRB1*10:01/ HLA-DQB*05:01 in 87%.	~60% response to second-line IT versus ~30% first-line IT. Mortality 70%.
Neurexin-3α	44	1:4	Marked overlap with NMDAR-autoantibody encephalitis: prodromal fever, headache, and GI symptoms → confusion, seizures, orofacial dyskinesias → central hypoventilation.	MRI brain MTL signal change in 20%. CSF typically abnormal with pleiocytosis; elevated Ig index.	Severe syndrome: 60% partial recovery; mortality 40% despite IT.

mGluR5	Late 20s	1.5:1	LE; Ophelia syndrome in Hodgkin's lymphoma	MRI brain normal in 50%	Responsive to lymphoma treatment and immunotherapy
D2R	Paediatric: 6 years (1-15); non-Caucasian	1:1	'Basal ganglia encephalitis': Parkinsonism, dystonia, chorea, OCD, psychosis.	MRI brain basal ganglia swelling, hyperintensity or enhancement in 50% acutely; atrophy/ gliosis (late)	Respond to immunotherapy. Relapse rate 25%.
GluRD2	Paediatric: 12-36 months	1:1.4	Opsoclonus, myoclonus, ataxia, cognitive impairment. Neuroblastoma in 50%	MRI brain – cerebellar and cortical atrophy (late)	Unknown

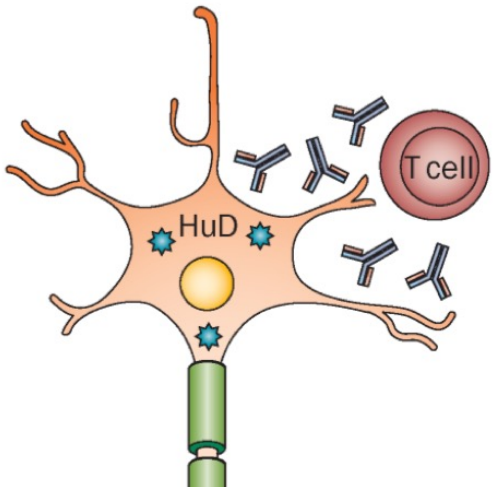
Characteristics of autoimmune encephalitis syndromes caused by various neuronal surface autoantibodies, with comparison of autoantibody, demographics, and clinical/ paraclinical features. Data summarised from Ramanathan et al, 2019, and Uy et al, 2021 (Ramanathan et al. 2019; Uy et al. 2021)

IT, immunotherapy; MRS, modified Rankin scale; HSV1, herpes simplex virus type 1; LE, limbic encephalitis; HLA, human leukocyte antigen; MTL, medial temporal lobe; SCLC, small cell lung cancer; GI, gastrointestinal; LETM, longitudinally extensive transverse myelitis; OCBs, oligoclonal bands; ON, optic nerve; REM, rapid eye movement; PSP, progressive supranuclear palsy; OCD, obsessive compulsive disorder.

NMDAR, N-Methyl-D-Aspartate Receptor; LGI1, leucine-rich glioma-inactivated 1; CASPR2, contactin-associated protein-like 2; GABAR, gamma-aminobutyric acid receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; DPPX, dipeptidyl-peptidase-like protein 6; GlyR, glycine receptor; MOG, myelin oligodendrocyte glycoprotein; IgLON5, immunoglobulin-like cell adhesion molecule 5; mGluR5, metabotropic glutamate receptor 5; D2R, dopamine 2 receptor; GluRD2, glutamate

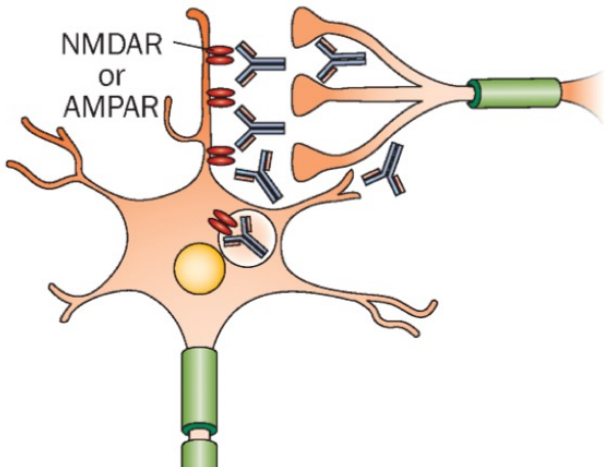
Figure 1.1 Autoantibodies to Intracellular and Extracellular Neuronal Antigens

A **Autoantibody against intracellular antigen**



Non-pathogenic

B **Autoantibody to extracellular, neuronal surface antigen**



Pathogenic

(A) Autoantibodies directed against intracellular neuronal antigens (such as HuD, a nuclear protein), are not considered to be directly pathogenic, since they are unable to access their intracellular antigenic targets (nuclear or cytoplasmic proteins). These antibodies are likely markers of T-cell mediated, irreversible cytotoxic neuronal damage, and are typically found in patients with underlying malignancies, with poor responses to immunotherapy. **(B)** Autoantibodies against extracellular, neuronal surface proteins (such as NMDAR or AMPAR) are directly pathogenic as they functionally disrupt their antigen by various mechanisms, including internalisation of antigen, or receptor cross-linking, causing manifestation of the clinical syndrome, including seizures.
Adapted from Lancaster and Dalmau, 2012 (Lancaster & Dalmau 2012).

NMDAR, N-methyl-D-aspartate receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid receptor.

1.3 LGI1- and CASPR2-Autoantibodies and Comparison to VGKC-Antibodies

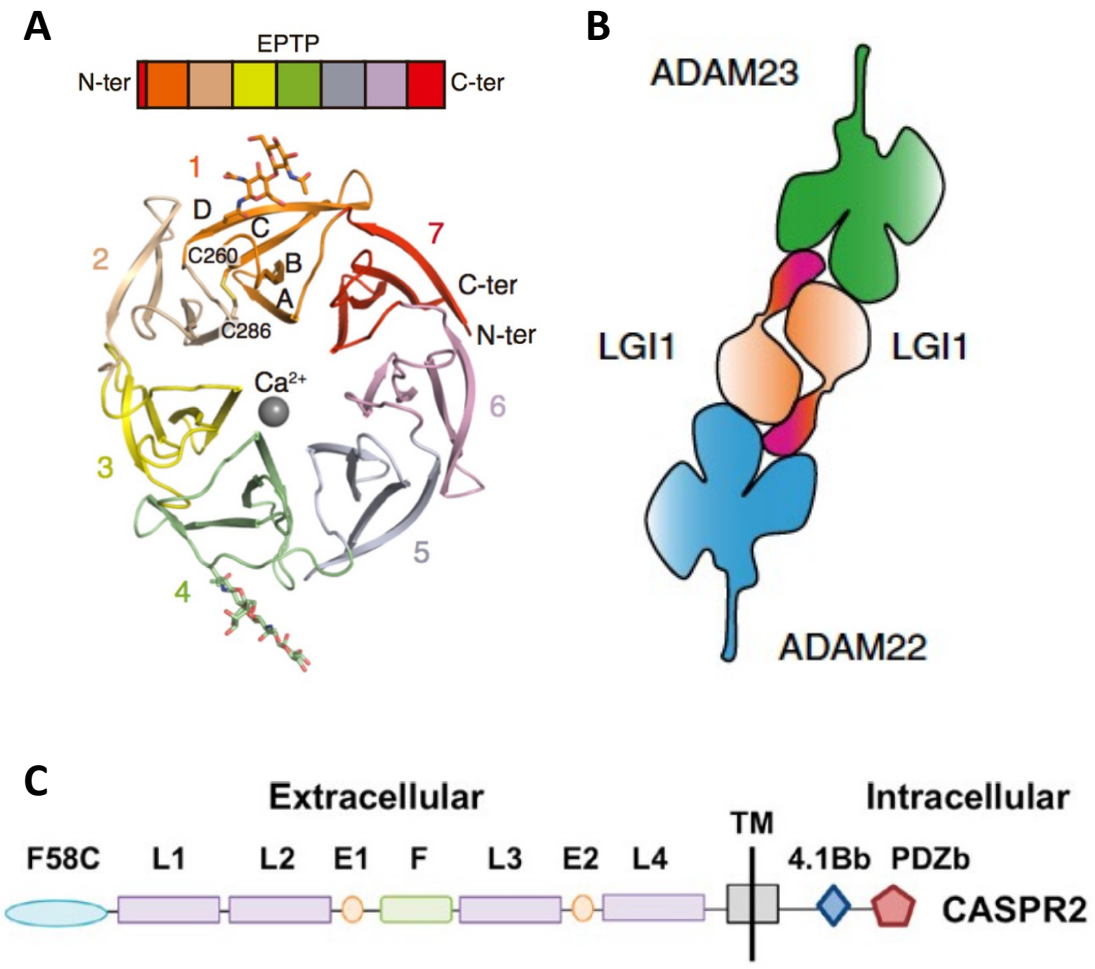
1.3.1 LGI1 Molecular Structure, Epitopes, Function, and Interactions

LGI1 is a secreted neuronal protein which associates with Kv1.1 potassium channels, comprised of an N-terminal leucine-rich repeat (LRR) domain, and a C-terminal epitempin-repeat (EPTP) domain. LGI1 is known to link the pre- and post-synaptic regions via ADAM proteins, akin to a molecular scaffold (Schulte et al. 2006; Yamagata et al. 2018). The crystal structure of the LGI1-ADAM22 complex has been recently determined (Yamagata et al. 2018); **(Figure 1.2)**. LGI1-autoantibodies block LGI1-ADAM22/ADAM23 interactions in rat hippocampal neurons, and reduce synaptic α-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid (AMPA)-receptors in a dose-dependent manner (Ohkawa et al. 2013), likely mediating seizures through hyperexcitability caused by inhibition of inhibitory interneurons. Recent studies corroborated these findings in vivo, with direct infusion of patient-derived IgG into ventricles of mice causing reduced hippocampal synaptic Kv1.1 and AMPA-Rs, and functional studies showing neuronal hyperexcitability with impaired synaptic plasticity correlating with severe memory deficits (Petit-Pedrol et al. 2018). Patients have been shown to possess antibodies to both the LRR and EPTP domains of LGI1 in equal ratios over the course of their disease (Ramberger et al. 2020). Further, more precise mechanisms of the individual LGI1-directed autoantibodies have been delineated. The LRR-specific autoantibodies have been shown to induce internalisation of the LGI1-ADAM22/23 complex, whilst EPTP-directed autoantibodies appear to inhibit docking of LGI1 to its receptors ADAM22/23 (Ramberger et al. 2020). These findings confirm dichotomous mechanisms of pathogenicity of the two LGI1-directed autoantibodies.

CASPR2 is a neurexin family protein which is co-expressed with membrane VGKCs at the juxtaparanodes of myelinated axons at the node of Ranvier (Saint-Martin et al. 2018). It is also expressed at synapses, and at the axon initial segment (Saint-Martin et al. 2018). CASPR2 boasts a large extracellular domain, a transmembrane domain, and an intracellular C-terminal domain **(Figure 1.2)**. There is evidence to support that CASPR2 autoantibodies are also pathogenic, since they can disrupt CASPR2 interactions with contactin-2 (Saint-Martin et al.

2018), and rodent passive transfer of human CASPR2 IgG reproduces human pain manifestations, directly mediated through reduction of Kv1 channel expression (Dawes et al. 2018). Together, these observations affirm that antibodies to the extracellular VGKC-complex domains LGI1 and CASPR2 are directly pathogenic, mediating their effects through directly binding to their targets. The characteristics of these molecules and their involvement in genetic syndromes are described in **(Table 1.2)**. The main focus of this thesis shall be on LGI1 autoantibody-mediated syndromes.

Figure 1.2 Molecular Characteristics of LGI1 and CASPR2



(A) The EPTP domain of LGI1 is a β-propeller structure, comprised of seven blades, each a β-sheet made of four strands (A-D). **(B) LGI1-ADAM22/23 interactions**: ADAM22-LGI1-ADAM23 2:2 heterodimer. EPTP (orange) binds to either of its ADAM22 and ADAM23 receptors – metalloproteases located on excitatory post- and pre-synaptic receptors, respectively – through essential hydrophobic interactions. The two LGI1-ADAM22 and LGI1-ADAM23 heterodimer complexes are linked head-to-head by interaction of the LRR domain (red) of each LGI1 molecule to the EPTP domain of the other, forming a trans-synaptic ADAM22-LGI1-ADAM23 bridge. Taken from Yamagata et al, 2018 (Yamagata et al. 2018). **(C)** CASPR2 has a large extracellular domain comprised of an N-terminal discoidin-like domain (F58C), 4 laminin domains (L1-4), 2 epidermal growth factor-like (EGF) domains (E1-2), and a fibrinogen-like domain (F). CASPR2 also has a transmembrane domain and an intracellular C-terminal domain, which includes 4.1B and PDZ binding motifs. It is thought that CASPR2 is a flexible protein, which can adopt either a vertical or horizontal structure. Taken from Saint-Martin et al, 2018 (Saint-Martin et al. 2018).

Table 1.2 Molecular Characteristics of LGI1, CASPR2, and Associated Proteins

Molecular Evidence	LGI1	CASPR2	ADAM22/23	Contactin-2
Genetic Mutations	Autosomal dominant partial epilepsy with auditory features (Kalachikov et al. 2002)	Homozygous mutations cause epilepsy, intellectual disability and autism (Rodenas-Cuadrado et al. 2016)	Heterozygous mutations cause early infantile epileptic encephalopathy with cortical atrophy (Muona et al. 2016)	Homozygous mutations cause autosomal recessive familial cortical myoclonic tremor and epilepsy (Stogmann et al. 2013)
Neural Expression	CNS: Strongly expressed within mossy fibre CA3 layer of hippocampus and cerebellum PNS: more weakly expressed (Irani et al. 2010)	CNS: stratum radiatum of CA3 layer of hippocampus and cerebellum PNS: juxtaparanodes of myelinated axons (Irani et al. 2010)	CNS: ADAM22 predominantly within the cerebellum, hippocampus, amygdala, and cerebral cortex; ADAM 23 hypothalamus and thalamus (Sagane et al. 1998). Both are also expressed in the PNS.	CNS: cerebellum, CA1 and CA3 hippocampal pyramidal cells, olfactory bulb, white matter tracts (Savvaki et al. 2008). PNS: Juxtaparanodes of myelinated axons (Poliak et al. 2003).
Interacting Proteins	Binds to presynaptic ADAM23 and postsynaptic ADAM22 (Fukata et al. 2010), with some evidence for ADAM11 (Sagane et al. 2008). Associates closely with Kv1.1 channels presynaptically and AMPARs post-synaptically (Schulte	Contactin-2 (Irani et al. 2010; Poliak et al. 2003), via EGF2 and laminin G4 domains of CASPR2 (Saint-Martin et al. 2018)	LGI1 (Fukata et al. 2010), and closely related LGI1-family molecules	CASPR2 (Irani et al. 2010; Poliak et al. 2003) via immunoglobulin (Ig) and fibronectin (Fn) domains of contactin-2 (Saint-Martin et al. 2018)

	et al. 2006; Petit-Pedrol et al. 2018; Ramberger et al. 2020)			

Summary of the characteristics of LGI1, CASPR2, ADAM22/23, and Contactin-2, their expression profiles, interactions, and associated genetic syndromes. Taken from Michael et al, 2020 (Michael et al. 2020).

ADAM, a disintegrin and metalloprotease; AMPARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; CASPR2, contactin-associated protein-like 2; EGF2, epidermal growth factor-2; LGI1, leucine-rich glioma-inactivated 1.

1.3.2 Highly Distinctive Clinical Associations of LGI1- and CASPR2-Autoantibodies

The clinical phenotypes associated with presence of these autoantibodies are distinct and well recognised. Patients with LGI1-autoantibodies show a characteristic demographic, since they are typically elderly males in their sixth or seventh decade, and almost exclusively White European. Whilst LGI1-autoantibodies are most commonly associated with LE, they are found in many patients with Morvan's syndrome, and a few with NMT (Irani et al. 2010; Lai et al. 2010; Irani et al. 2012). Additionally, faciobrachial dystonic seizures (FBDS), first described by Irani et al in 2008 (Irani et al. 2008), are pathognomonic for LGI1-autoantibody encephalitis (Irani et al. 2011). FBDS are a highly distinctive seizure semiology comprising brief, stereotyped dystonic movements of the ipsilateral face, arm, and often leg, over a few (typically <10) seconds. They occur at characteristically high frequencies of up to several hundred times daily (6-960; median frequency ~30-40); (Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015; Navarro et al. 2016; Aurangzeb et al. 2017). Importantly, FBDS typically manifest during the prodromal phase of LE, prior to the onset of amnesia. Whilst FBDS are largely resistant to anti-seizure medications (ASMs), they are highly responsive to immunotherapies, often with striking, rapid responses over several days (Irani et al. 2011; Irani et al. 2013; Thompson et al. 2017). Thus, FBDS represent an excellent example of a distinctive phenotype with a robust response to immunotherapies (Irani et al. 2011; Thompson et al. 2017).

Patients with LGI1 autoantibody-associated encephalitis commonly manifest several seizure semiologies; 89-93% have been reported to have at least one or more seizure types (van Sonderen, Thijs, et al. 2016; Navarro et al. 2016; Gadoth et al. 2017). Focal seizures, frequently of temporal lobe onset, occur in 66-75% at high frequency of up to 2/ hour, and may comprise motor, sensory, and autonomic subtypes (van Sonderen, Thijs, et al. 2016; Navarro et al. 2016; Aurangzeb et al. 2017). Sensory semiologies may include thermal sensations and less commonly ictal piloerection or 'goosebumps', seen in up to 20% (Irani et al. 2011; Aurangzeb et al. 2017; Gadoth et al. 2018; Finke et al. 2017). Autonomic seizures include the neurocardiac syndromes of ictal tachycardia (Aurangzeb et al. 2017) or bradycardia (Naasan et al. 2014), both with reported rates of up to one fifth. Other manifestations of focal temporal lobe seizures include sensation of fear, déjà vu, ascending epigastric sensations, or falling sensation, and the majority (~70%) associate with loss of awareness (Navarro et al. 2016). Automatisms suggestive of temporal lobe origin are present in around one third and include gestures, orofacial automatisms, cough, and speech automatisms including coprolalia (Navarro et al. 2016). Further, 'paroxysmal dizziness spells' (PDS), are a recently described semiology found in 14% (Gadoth et al. 2017). Around 50-60% of patients develop generalised tonic clonic seizures (GTCS) and generalised status epilepticus occurs in ~20% (Irani et al. 2011; van Sonderen, Thijs, et al. 2016; Navarro et al. 2016). Whilst only 6-30% of FBDS show electrographic ictal epileptiform activity (Irani et al. 2011; Irani et al. 2013; Aurangzeb et al. 2017), almost all (70-94%) non-FBDS seizures, including focal motor seizures, demonstrate electrographic correlate on EEG (van Sonderen, Thijs, et al. 2016; Navarro et al. 2016; Aurangzeb et al. 2017). Frequent multifocal seizures are seen to arise from the medial temporal, frontal, and parietal lobes (Aurangzeb et al. 2017), and motor cortex (Navarro et al. 2016). Other features of patients with LGI1-autoantibodies include sleep disturbances ranging from 7-93% (Irani et al. 2010; Irani et al. 2011; Cornelius et al. 2011; Klein et al. 2013; Navarro et al. 2016; Finke et al. 2017; Gadoth et al. 2017; Blattner 2019), peripheral nerve involvement, pain and dysautonomia (Irani et al. 2011; Navarro et al. 2016; Gadoth et al. 2017; Binks, Klein, et al. 2018; Ramanathan et al. 2021).

Patients with CASPR2 autoantibodies are typically elderly males, akin to those with LGI1-autoantibodies, although they show a greater male predilection (8:1 versus 2:1 male: female

ratio, respectively). Their clinical syndromes typically fall into one of three subtypes: central nervous system (CNS) manifestations with LE, peripheral nervous system (PNS) manifestations such as NMT, or Morvan's syndrome, comprising both CNS LE and PNS NMT. Importantly, all syndromes are characteristically immunotherapy-responsive. Whilst LGI1-autoantibody syndromes are not known to be paraneoplastic, a proportion of patients with CASPR2 autoantibodies harbour associated tumours – typically thymomas – especially those with NMT and Morvan's syndrome (Irani et al. 2010; Lai et al. 2010; Irani et al. 2012). Importantly, LGI1- or CASPR2-specificties are rare outside of these syndromes. Taken together, LGI1- and CASPR2-autoantibodies show strong and specific clinical associations with an unequivocal clinical value.

1.3.3 Frequency and Heterogeneous Clinical Features of 'Double-Negative' VGKC-Antibodies

The original findings of the association of VGKC-antibodies with immunotherapy-responsive syndromes were rapidly disseminated through the Neurology community. To avoid missing a potentially reversible condition, VGKC-antibodies were requested in patients with a wider spectrum of features. This led to a large number of positive VGKC-antibody results, in broad-ranging phenotypes. Many of these syndromes were non-distinctive, and did not appear immunotherapy-responsive or, intuitively, immune-mediated. Recently, data from several groups have clarified the clinical relevance of *double-negative* VGKC-antibodies: that is, VGKC-antibodies *without* LGI1 or CASPR2 reactivities (van Sonderen, Schreurs, et al. 2016; Olberg et al. 2013; Lang et al. 2017). The largest series by Gadoth et al reviewed testing of ~100,000 samples from 2008-2016, which yielded 3910 (3.8%) samples with VGKC-antibodies. Only 256 of these 3910 (6.5%) showed concomitant LGI1- or CASPR2-reactivities (Gadoth et al. 2017). Other studies report comparable rates of LGI1- and/or CASPR2-positive VGKC-antibodies: ~20% (Klein et al. 2013) and, in children, <5% (Chiriboga et al. 2018). Hence, double-negative VGKC-complex antibodies appear to account for ~85% of routine VGKC-antibody requests. It is important to review whether there is any clinical utility in detecting these common antibodies.

Firstly, in healthy controls, double-negative VGKC-antibodies are found at rates of ~5% (Shillito et al. 1995; Vincent et al. 2004; van Sonderen, Schreurs, et al. 2016; Lang et al. 2017). Following basic principles, the disease control rate must be equal to or greater than the healthy control rate. Next, and in marked contrast to the well-defined clinical phenotypes associated with LGI1- and CASPR2-autoantibodies **(Figure 1.3; Table 1.3)**, *double-negative* VGKC antibodies associate with a constellation of syndromes without age or gender predilection. These range from epilepsies, non-neuropathic pain, Alzheimer's disease, peripheral neuropathy and headache, to primary psychiatric syndromes and leptomeningeal metastasis (van Sonderen, Schreurs, et al. 2016; Olberg et al. 2013; Lang et al. 2017; Binks, Varley, et al. 2018). Thus, it appears that double-negative VGKC antibodies show limited clinical specificity. Thirdly, the VGKC-antibody titre is not a clinically reliable measure: double-negative VGKC antibodies can be found at very high-titres (e.g. 400-3000 pM), whilst VGKC antibodies from patients with additional LGI1 or CASPR2 reactivity can be of low titre. Hence, the titre per se is an unhelpful discriminator for LGI1 or CASPR2 specificity (van Sonderen, Schreurs, et al. 2016; Olberg et al. 2013; Gadoth et al. 2017; Klein et al. 2013; Paterson et al. 2014). Worryingly, the concept of a 'clinically-relevant' VGKC-antibody titre has created a number of misdiagnoses over time, often where the presence of the antibody has overruled the clinical diagnosis (Paterson et al. 2014; Huda et al. 2014; Jammoul et al. 2016; Tan et al. 2008).

Interestingly, VGKC-antibodies have been frequently detected in healthy swine abattoir workers (Meeusen et al. 2012), suggesting autoimmunisation by inhaled aerosolized brain tissue constituents containing the antigen. This likely provides explanation for the generation of these antibodies in these control subjects. Additionally, their occurrence in patients with prion disease (Jones et al. 2014; Rossi et al. 2015) and Alzheimer's disease (Lang et al. 2017) indicates that VGKC antibodies may occur as an epiphenomenon in the context of many other diseases. Together, these observations suggest that loss of tolerance to VGKC-complexed proteins has a low threshold and may account for high observed rates of VGKC-antibodies in control subjects. Finally, their biochemical detection affirms their biological existence in vivo, albeit with non-pathogenic reactivities.

1.3.4 Immunotherapy Response in LGI1- and CASPR2-Autoantibody Syndromes versus those with 'Double-Negative' VGKC-Antibodies

Patients with LGI1-and CASPR2 autoantibodies display striking responses to immunotherapy, boasting improvement rates as high as 96-100% and 86-100%, respectively (Irani et al. 2011; Thompson et al. 2017; van Sonderen, Schreurs, et al. 2016; Gadoth et al. 2017). This is – fundamentally – what makes LGI1 and CASPR2-autoantibody detection so important. By marked contrast, patients with double-negative VGKC-antibodies are poorly responsive, with clinical benefit of immunotherapy demonstrated in only 27-46%, comparable to patients with negative VGKC results (van Sonderen, Schreurs, et al. 2016; Lang et al. 2017). Of interest, the immunotherapy response rates of double-negative VGKC antibody patients (~one third) are also comparable to placebo response rates seen in other neurological diseases (25% in headache, 45% in peripheral neuropathy); (Bittar & Nascimento 2015) , and thus likely largely represent a placebo effect.

1.3.5 Genetic Associations

Perhaps one of the strongest indicators for *lack* of causality of double-negative VGKC-complex antibodies is accrued from HLA data. Recently, a very strong immunogenetic basis was established for LGI1- and CASPR2-autoantibody diseases (Binks, Varley, et al. 2018; Kim et al. 2017; van Sonderen et al. 2017; Mueller et al. 2018). HLA-DRB1*07:01 and HLA-DRB1*11:01 are over-represented in patients with LGI1- (~95% v. 25% in HCs) and CASPR2-autoantibodies (~50% v. 10% in HCs), respectively, with highly significant associations (LGI1 $p=4.1x10^{-26}$; CASPR2 $p=5.7x10^{-6}$). Notably, the HLA-DRB1*07:01 LGI1-autoantibody association appears preserved across populations (Binks, Varley, et al. 2018; Kim et al. 2017; van Sonderen et al. 2017; Mueller et al. 2018). Moreover, 'double-positive' patients with both LGI1- and CASPR2-autoantibodies carried yet another set of distinct HLA alleles (Binks, Varley, et al. 2018). By contrast, patients with intracellular VGKC antibodies lacked distinct HLA associations (Binks, Varley, et al. 2018), denoting their likely clinical irrelevance.

1.3.6 Serological Observations: Intracellular Binding and Non-Pathogenicity of VGKC-Antibodies

Due to the growing evidence that double-negative antibodies showed little clinical relevance, our research group in Oxford investigated their antigenic targets (Lang et al. 2017). Firstly, no double-negative serum IgGs bound surface determinants as expressed on live hippocampal neurons. Contrastingly, the majority of sera from patients with LGI1- or CASPR2-autoantibodies demonstrated neuronal reactivity. Secondly, no double-negative VGKC IgGs bound the extracellular domain of surface-expressed VGKCs. However, around one third bound to intracellular, cytosolic aspects of the Kv1.1/1.2/1.6 subunits (Irani et al. 2010; Lang et al. 2017). In contrast, sera additionally positive for LGI1- or CASPR2-autoantibodies bound *only* extracellular VGKC-complex proteins (LGI1 or CASPR2), and never to intracellular potassium channels. Finally, a small proportion of the double-negative VGKC-antibody sera bound the non-mammalian α-DTX employed in the radioimmunoassays derived from snake venom; some sera were from snake handlers. Thus, these findings established that double-negative VGKC-antibodies often bind targets which are effectively inaccessible or unavailable *in vivo* in humans, thus mitigating their pathogenic potential.

In summary, pathogenic LGI1 or CASPR2-autoantibodies demonstrate robust cell-surface reactivity **(Figures 1.3 and 1.5)**, binding only their specific extracellular VGKC domains on the neuronal surface. Further, strong datasets support their *in vitro* and *in vivo* functionality. LGI1-autoantibodies can disrupt interactions between LGI1 and its receptors ADAM22/23, internalise the LGI1-ADAM complex, and trigger neuronal hyperexcitability and memory deficits *in vivo* passive transfer models (Thompson et al. 2017; Ohkawa et al. 2013; Petit-Pedrol et al. 2018; Ramberger et al. 2020). CASPR2-autoantibody pathogenicity is demonstrated by rodent passive transfer of IgG reproducing human pain manifestations, and evidence that the antibodies can disrupt CASPR2 interactions with contactin-2 (Dawes et al. 2018; Saint-Martin et al. 2018). Thus, autoantibodies to the extracellular VGKC-complex domains are directly pathogenic, mediating their effects through directly binding their targets. Double-negative VGKC-complex antibodies do not recognise the LGI1 or CASPR2 domains of the VGKC on the neuronal surface, but rather, target intracellular epitopes which

are inaccessible *in vivo*, or α-DTX (a non-mammalian, non-existent target in vivo), and show little evidence of pathogenicity.

The features associated with LGI1-, CASPR2- and double-negative VGKC-antibodies are summarised and contrasted in **Figure 1.3 and Table 1.3**.

Table 1.3 Comparisons of autoantibodies against LGI1, CASPR2, and the Double-Negative VGKC-antibodies

Evidence		**Autoantibody**		
		LGI1	**CASPR2**	**Double-Negative VGKC**
Clinical Features	Well-delineated clinical syndromes?	Yes (Irani et al. 2011; Thompson et al. 2017): FBDS Limbic encephalitis (seizures, thermal sensations, encephalopathy, sleep disorder, amnesia, anxiety, emotionality) Dysautonomia Hyponatraemia 65% More rarely, NMT and Morvan's Pain 19% (Ramanathan et al. 2021)	Yes (Irani et al. 2010; Lai et al. 2010; Irani et al. 2012): CNS: limbic encephalitis – seizures, confusion, hallucinations, insomnia PNS: NMT (neuropathic pain, spasms, cramps). Pain in 52% (Ramanathan et al. 2021). Morvan's syndrome: above including insomnia and dysautonomia, often with weight loss	No - heterogeneous, often non-immune, syndromes including varied epilepsies; widespread, non-neuropathic pain; Parkinson's disease; headache; primary psychiatric conditions (van Sonderen, Schreurs, et al. 2016; Olberg et al. 2013; Lang et al. 2017; Binks, Varley, et al. 2018)

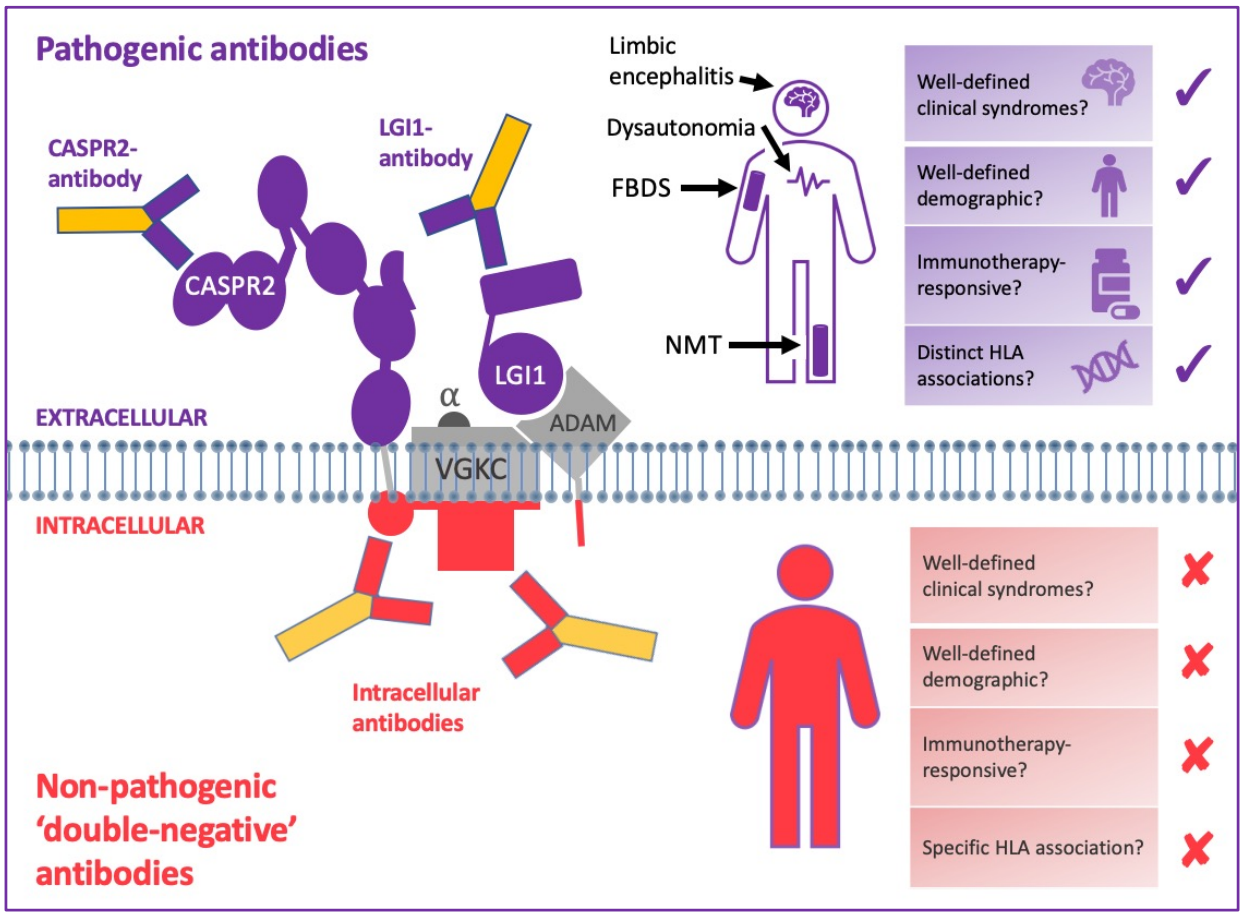
	Demographics: gender ratio and median age of onset	M (2:1) 60-70 years (Thompson et al. 2017)	M (8:1) 50-60 years	No predilection known (M:F 1:1; 18-85 years with no peak onset age); (van Sonderen, Schreurs, et al. 2016; Lang et al. 2017; Binks, Varley, et al. 2018)
	Immunotherapy -Responsive (%)	Highly 100% (van Sonderen, Schreurs, et al. 2016) 97% (Thompson et al. 2017; Gadoth et al. 2017) 96% (Irani et al. 2011) 78% (Lai et al. 2010)	Highly 100% (Lancaster et al. 2011) 86% (van Sonderen, Schreurs, et al. 2016)	Limited, and likely accounted for by referral bias and/or placebo 27% (Lang et al. 2017) 46% (van Sonderen, Schreurs, et al. 2016)
Serological	Epitope	Extracellular (both receptor docking and non-docking domains of this secreted protein); (Irani et al. 2010; Lang et al. 2017; Ramberger et al. 2020)	Extracellular domain (Irani et al. 2010; Lai et al. 2010; Lang et al. 2017)	Intracellular aspects of Kv1 channels, and non-mammalian targets (Lang et al. 2017)
	Rate in healthy controls	<1% (Dahm et al. 2014)	<1% (Dahm et al. 2014)	~5% (Shillito et al. 1995; Vincent et al. 2004; Lang et al. 2017; Meeusen et al. 2012)
	Other associations/ markers	Rarely thymoma <5%	Thymoma 20-50%, especially those with Morvan's and NMT	Possibly malignancy (Paterson et al. 2014; Huda et al. 2014; Jammoul et al. 2016; Tan et al. 2008)

Genetic	HLA associations	HLA-DRB1*07:01 (~95%) (van Sonderen, Schreurs, et al. 2016; Binks, Varley, et al. 2018; Kim et al. 2017; Mueller et al. 2018)	HLA-DRB1*11:01 (~50%) (Binks, Varley, et al. 2018)	Nil (Binks, Varley, et al. 2018)
Evidence of Pathogenicity		Functional blocking of LGI1-ADAM22 interactions (Ohkawa et al. 2013; Ramberger et al. 2020) Internalisation (Thompson et al. 2017; Ramberger et al. 2020) LGI1 IgGs and monoclonal antibodies induce memory deficits *in vivo* (Petit-Pedrol et al. 2018; Ramberger et al. 2020)	Internalisation noted in some studies; pain reproduced in experimental animals (Dawes et al. 2018)	-

Comparison of the associated features of LGI1, CASPR2, and VGKC autoantibodies. Taken and adapted from Michael et al, 2020 (Michael et al. 2020).

PNS, peripheral nervous system; CNS, central nervous system; Kv1, voltage-gated potassium channel – subfamily A member 1; HLA, human leukocyte antigen.

Figure 1.3 LGI1 and CASPR2 – but not Double Negative VGKC – Autoantibodies Predict Characteristic, Immunotherapy-Responsive Syndromes



Pathogenic antibodies (purple) bind to the extracellular domains of LGI1 and CASPR2. Some antibodies against LGI1 bind to the domain which docks with its receptor, a disintegrin and metalloprotease domain (ADAM) molecule. The antibodies which bind extracellular domains are pathogenic, manifesting with characteristic clinical syndromes including pathognomonic FBDS, limbic encephalitis and NMT. Both classically affect older males in their sixth decade and the patients improve markedly with immunotherapies. HLA-DRB1*07:01 is strongly associated to patients with LGI1-autoantibodies, and HLA-DRB1*11:01 in CASPR2-autoantibody syndromes. The non-pathogenic antibodies (red) bind the intracellular domain of VGKCs, and some bind the I^{125}-α-dendrotoxin (α) used in the original VGKC radioimmunoassays. The targets of other double-negative antibodies are unknown. Double-negative VGKC-antibodies are found in a wide array of clinical syndromes, including epilepsy, dementias, primary psychiatric conditions and non-neuropathic pain conditions, which span across a range of age groups, and lack both a clear immunotherapy response and specific HLA association. Taken from Michael et al, 2020 (Michael et al. 2020).

FBDS, faciobrachial dystonic seizures; NMT, neuromyotonia (NMT); VGKCs, voltage-gated potassium channels

1.4 Faciobrachial Dystonic Seizures (FBDS)

1.4.1 Clinical, Radiological, and Electrographic Features of FBDS

Of patients with LGI1-autoantibodies, FBDS occur in approximately 50% (van Sonderen, Thijs, et al. 2016; Navarro et al. 2016; Finke et al. 2017; Flanagan et al. 2015; Gadoth et al. 2017; Aurangzeb et al. 2017). High daily frequency is seen, with up to several hundred events per day, including during sleep (Irani et al. 2011; Flanagan et al. 2015; Navarro et al. 2016; Aurangzeb et al. 2017). FBDS are typically brief, dystonic movements, with duration of only few seconds. Involvement of the ipsilateral arm and face are almost always present (in 100% and 80%, respectively, from early series), whilst involvement of the ipsilateral leg is seen in less than half (40%); (Irani et al. 2013). Occurrence of bilateral FBDS is common (~60%), with some even manifesting bilateral simultaneous or rapidly alternating bilateral independent events (termed by the French, “à bascule”); (Irani et al. 2011; Irani et al. 2013; Navarro et al. 2016).

FBDS are commonly triggered by loud noises or heightened emotion, frequently preceded by sensory aura, and can be associated with altered awareness (Irani et al. 2011; Irani et al. 2013; Navarro et al. 2016). Ictal features including automatisms, vocalisations, speech arrest are observed in around one third (Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015; Navarro et al. 2016; Aurangzeb et al. 2017). Patients frequently sustain falls during FBDS, some with serious injury (Irani et al. 2013; Navarro et al. 2016). Around half exhibit speech arrest or fear immediately following the motor event (Irani et al. 2013). FBDS can occasionally be followed, or less commonly preceded by, focal seizures (Navarro et al. 2016; Aurangzeb et al. 2017).

Ictal EEG changes are infrequent, observed in around one quarter of patients. Radiological features include medial temporal lobe and hippocampal T2-weighted hyperintensities on MRI, with functional studies showing altered metabolism (typically hypermetabolism) in the temporal lobes and basal ganglia. Unilateral T1 signal hyperintensities of the basal ganglia contralateral to the side of the FBDS are also described (Flanagan et al. 2015). The characteristic features of FBDS are summarised in **Figure 1.4 and Table 1.4**.

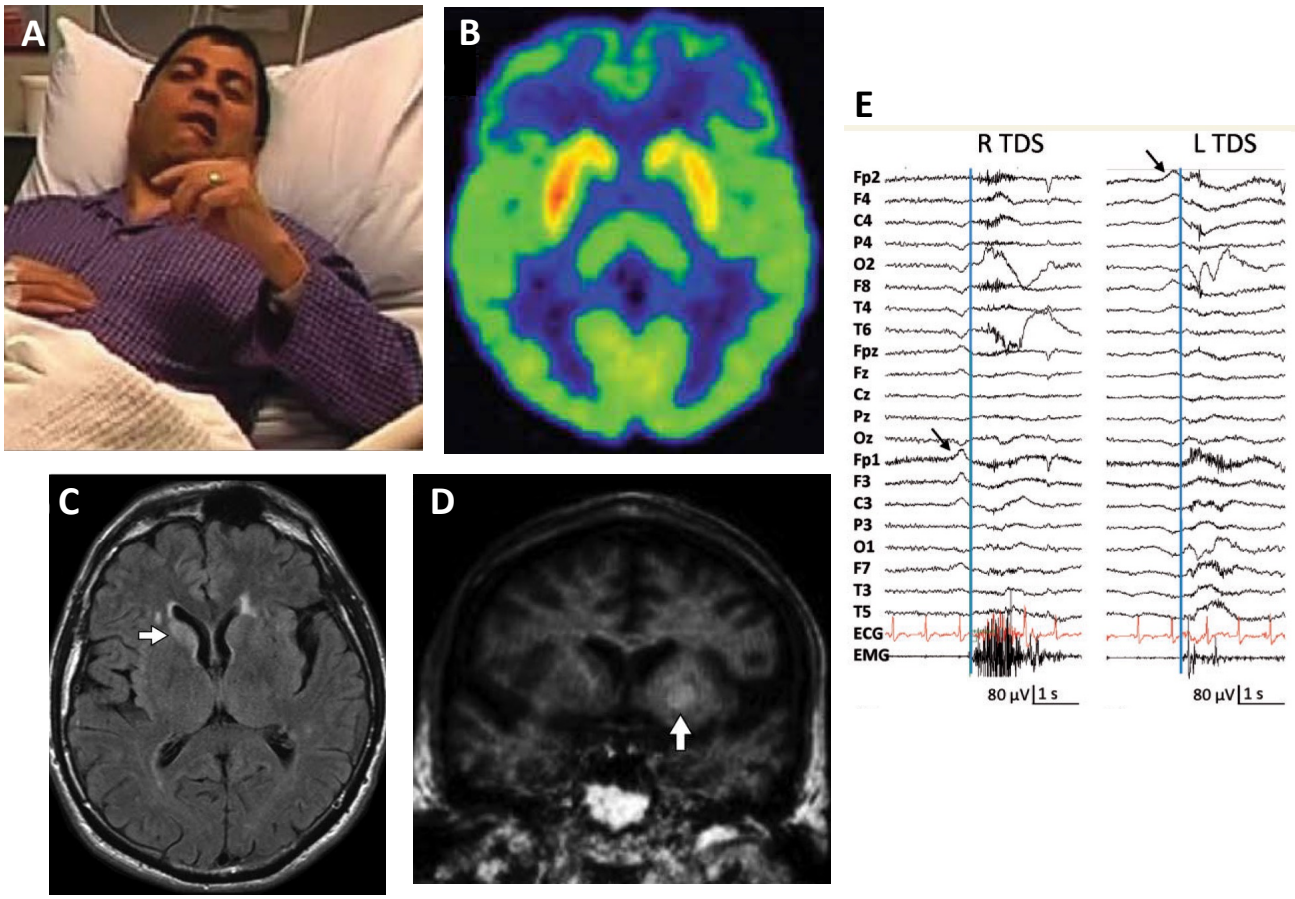
Notably, lack of awareness of the condition has led to misdiagnoses in a substantial proportion of patients, even by Neurologists, with alternative diagnoses including Creutzfeldt-Jakob disease, and 20-38% of patients with FBDS initially receiving diagnoses of psychogenic attacks (Irani et al. 2013; Flanagan et al. 2015). This underscores the need for increased awareness of FBDS, and highlights the potential risks of delayed diagnosis and treatments in this group of patients.

1.4.2 FBDS: Seizure or Movement Disorder?

Since their description, there has been controversy regarding whether FBDS represent a seizure or movement disorder (Morano et al. 2020). Some argue that FBDS show several features supportive of movement disorder. These include their brief duration of typically less than 10 seconds (Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015), dystonic posturing (Irani et al. 2011), radiological basal ganglia changes (Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015; Gadoth et al. 2017), limited response to anti-seizure medications (good or excellent response in only 10-14% of patients); (Irani et al. 2011; Thompson et al. 2017), and EEG correlates in only ~25% (Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015; Aurangzeb et al. 2017).

The original terminology, however, includes 'seizure' within the acronym 'FBDS' itself (Irani et al. 2011), implying an inherent seizure basis, and there is substantial evidence to support an epileptic aetiology. FBDS are highly stereotyped, and are associated with aura, loss of awareness, characteristic ictal features including automatisms and speech arrest, and manifestations immediately after the attacks compatible with postictal features, including confusion and speech arrest **(Table 1.4)**. Further, a proportion of FBDS demonstrate associated epileptic discharges, and their absence in the remainder of attacks does not exclude epileptic aetiology, since scalp surface EEG electrodes may not detect deep epileptic foci. Finally, FBDS occasionally precede focal seizures, further supporting an epileptic basis (Navarro et al. 2016; Aurangzeb et al. 2017).

Figure 1.4 Characteristic Features of FBDS



(A) Ictal still of a patient during an FBDS attack, demonstrating the pathognomonic dystonic posturing of the ipsilateral face and arm. **(B)** Bilateral basal ganglia hypermetabolism seen on FDG-PET during periods of high frequency FBDS. **(C)** MRI brain (FLAIR sequence) with axial views showing T2-weighted hyperintensity of the right caudate nucleus. **(D)** T1-weighted coronal MRI brain demonstrating T1 hyperintensity within the left globus pallidus and putamen pre gadolinium administration. **(E)** Focal cortical slow waves contralateral to FBDS. Focal slow waves detected from left frontal electrodes (Fp1, F3, C3, Fpz, Fz) immediately preceding the onset of a right FBDS (left panel; arrow), and from right frontal electrodes (Fp2, F4, F8, C4, Fpz) immediately preceding a left FBDS (right panel; arrow).

(A+B) taken from Irani et al 2011 (Irani et al. 2011). **(C+D)** taken from Flanagan et al 2015 (Flanagan et al. 2015). **(E)** taken from Navarro et al 2016 (Navarro et al. 2016).

1.4.3 Localisation of FBDS

The localisation of FBDS is also debated, primarily whether they are of cortical or subcortical origin (Morano et al. 2020). Cortical origin is evidenced by ictal EEG electrographic discharges arising from frontotemporal, frontal, frontocentral, and temporal foci contralateral to the side of clinical FBDS (Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015; Navarro et al. 2016; Aurangzeb et al. 2017). Frontal lobe origin is suggested by the startle component and trunk turning associated with some FBDS (Irani et al. 2011), whilst temporal lobe involvement is strongly implicated by the ictal vocalisations, speech arrest, fear and automatisms observed in FBDS. Despite these highly congruent features, Irani et al (Irani et al. 2011) highlight that classical temporal lobe seizures are typically of longer duration, show sustained (>10 seconds) rather than brief motor involvement of the contralateral arm, and do not occur at the high frequencies of FBDS, highlighting the unique nature of FBDS. Navarro et al (Navarro et al. 2016) propose a cortical origin of FBDS since hypermetabolism was observed in the primary motor cortex contralateral to the side of FBDS on 18-Fluorodeoxyglucose (FDG)-positron emission tomography (PET) imaging, which resolved upon resolution of FBDS, and was not seen in controls. Further, a slow, unilateral, frontal electroencephalographic wave was observed immediately prior to a contralateral FBDS in several patients, and polymyography revealed myoclonic patterns and a premyoclonic potential in the cortex.

Alternatively, FBDS may be of subcortical origin, which would not be detected by scalp EEG electrodes. This may explain why only ~25% FBDS demonstrate ictal EEG changes (Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015; Aurangzeb et al. 2017). Regardless of whether the ictal onset zone is cortical or subcortical, a subcortical involvement in FBDS is supported by ictal dystonia, which is highly suggestive of basal ganglia involvement. Indeed, basal ganglia T1 hyperintensities are observed exclusively in LGI1-autoantibody patients with FBDS, and not in those without (Flanagan et al. 2015). Further, basal ganglia T1 hyperintensities are seen bilaterally in those with bilateral FBDS, focal hyper- or hypo-metabolism within the basal ganglia is observed on brain FDG-PET in 43% (Flanagan et al. 2015), and bilateral putamen hypermetabolism is seen in FBDS but not LGI1-autoantibody patients without FBDS, or healthy controls (Zhao et al. 2021). Thus, whilst the precise ictal onset zone has not been determined, the above evidence suggests implication of an extensive cortico-subcortical

network involving the basal ganglia, and frontal and temporal cortices in the generation of FBDS (Morano et al. 2020), highlighting the complexity of the phenomenon of FBDS. Indeed, in patients with LGI1-autoantibody encephalitis, brain FDG-PET glucose metabolic patterns reveal subcortical hypermetabolism of the amygdala and basal ganglia associated with cortical hypometabolism (Zhao et al. 2021). Interestingly, the same study found hippocampal hypermetabolism which appeared to show predilection for the left hippocampus, a finding which remains unexplained at present (Zhao et al. 2021).

Table 1.4 Characteristic Features of Faciobrachial Dystonic Seizures

Feature	**N (%)**	**Reference**
Frequency in patients with LGI1-autoantibodies	34-88%: 60/179 (34) 18/38 (47) 16/30 (53) 26/48 (54) 22/32 (68) 14/16 (88)	 (Gadoth et al. 2017) (van Sonderen, Thijs, et al. 2016) (Finke et al. 2017) (Flanagan et al. 2015) (Navarro et al. 2016) (Aurangzeb et al. 2017)
Age (years)	Median onset 63-68 years: 37-78 (median 63) 36-83 (median 64) 53-92 (median 67) 28-92 (median 68)	 (Flanagan et al. 2015) (Irani et al. 2011) (Aurangzeb et al. 2017) (Irani et al. 2013)
Gender M: F	M: F ratio range 1-3.7: 1 19:10 (1.9: 1) 5:5 (1: 1) 11:3 (3.7: 1) 17:9 (1.9: 1)	 (Irani et al. 2011) (Irani et al. 2013) (Aurangzeb et al. 2017) (Flanagan et al. 2015)
Daily frequency	Median 10-66 FBDS/day: 10-960 (median 66) 6-360 (median 50) median 35 8-200 (median 30) 0.1-9.8/hr (median 0.4/hr = 10/day)	 (Flanagan et al. 2015) (Irani et al. 2011) (Navarro et al. 2016) (Irani et al. 2013) (Aurangzeb et al. 2017)
Duration of motor event	~1 second <3 seconds 3 seconds	(Navarro et al. 2016) (Irani et al. 2011) (Flanagan et al. 2015)

	~5 seconds <10 seconds in 60%	(Aurangzeb et al. 2017) (Irani et al. 2013)
Bilateral **Unilateral**	42-69% 31-58%	(Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015; Navarro et al. 2016)
Arm > face > leg involvement	100% > 76% > 34% 100% > 80% > 40% 100% > 85% > 46%	(Irani et al. 2011) (Irani et al. 2013) (Flanagan et al. 2015)
Ipsilateral face and arm only **Ipsilateral face, arm and leg involvement** **Arm in isolation** **Leg in isolation** **Face in isolation** **Trunk**	67-76% 14-24% 6/86 (7%) 6/86 (7%) 4/86 (5%) 28%	(Irani et al. 2011; Aurangzeb et al. 2017)
Triggers	28-80%: 8/29 (28%): auditory stimuli n=6; heightened emotion n=2 8/10 (80%): heightened emotion n=5, auditory/ loud noise n=2, kinesogenic n=2, concentration n=1 6/13 (46%): movement, startle including auditory, heightened emotion or stress	 (Irani et al. 2011) (Irani et al. 2013) (Navarro et al. 2016)
Aura	8/10 (80%): sensory phenomena (migratory paraesthesiae/ thermal sensations) n=7; auditory hallucinations n=1 3/9 (33%): hemisensory	(Irani et al. 2013) (Navarro et al. 2016)
Ictal Features	Vocalisations 24–39% Automatisms 30% Speech arrest 17-27% 26% had any of: vocalisations, fear, dysphasia, loss of awareness, oral automatisms	(Irani et al. 2011; Navarro et al. 2016) (Irani et al. 2013) (Navarro et al. 2016; Flanagan et al. 2015) (Aurangzeb et al. 2017)

	Loss of awareness present in 66–80% patients but only during few of their FBDS Falls 60-70% with significant injuries in 30-35% including bone fractures and subdural haematomas	(Irani et al. 2011; Irani et al. 2013) (Irani et al. 2013; Navarro et al. 2016)
Postictal features	50%: n=4 speech arrest, n=2 agitation, n=1 fear Postictal confusion 12%	(Irani et al. 2013) (Flanagan et al. 2015; Aurangzeb et al. 2017)
Ictal EEG changes present	13-89%: 3/23 (13%) 3/14 (21%) patients in 5/86 (6%) of events 7/29 (24%) 3/10 (30%) 8/9 (89%)	 (Flanagan et al. 2015) (Aurangzeb et al. 2017) (Irani et al. 2011) (Irani et al. 2013) (Navarro et al. 2016)
EEG abnormality focus	Frontotemporal 3/5 (60%) and 2/7 (29%) Frontal 7/7 (100%), 2/5 (40%) and 2/7 (29%) – contralateral to side of FBDS Temporal 3/7 (43%) and 3/3 (100%)	(Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015; Navarro et al. 2016; Aurangzeb et al. 2017)
MRI changes/ volumetrics	MRI brain normal when FBDS alone present 9/9 (100%) Medial temporal lobe T2-weighted hyperintensities in 25-50% (bilateral > unilateral) Hippocampal T2 high signal in up to 73% Marked bilateral hippocampal atrophy in 25/26 (96%) in CA2/3, CA4/dentate gyrus Unilateral T2 hyperintensities of contralateral putamen and caudate in 1/26 (4%) – 1/6 (17%) Basal ganglia T1 and T2 signal hyperintensities in 31-42%, typically unilateral and contralateral to the FBDS	(Irani et al. 2011) (Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015) (Irani et al. 2011; Navarro et al. 2016; Finke et al. 2017) (Finke et al. 2017) (Irani et al. 2011; Irani et al. 2013) (Flanagan et al. 2015; Gadoth et al. 2017) (Irani et al. 2011)

	Functional imaging: PET temporal hyper-/ hypo-metabolism in 6/8 (75%) and basal ganglia involvement in 5/8 (63%) Volumetric brain MRI: Significantly smaller hippocampal/ total intracranial volumes than controls Smaller pallidal volumes correlating with longer duration of FBDS Diffusion tensor imaging (DTI): no grey matter volume loss or abnormality in white matter structure	 (Irani et al. 2013) (Finke et al. 2017) (Finke et al. 2017)

Summary of the clinical, electrographic, and radiological characteristics of FBDS.

CA, cornu ammonis; PET, positron emission tomography.

1.5 Investigations and Treatments for LGI1-Autoantibody Encephalitis

1.5.1 Diagnostic Criteria for Autoimmune Encephalitis

The aim of diagnostic criteria for autoimmune encephalitis is to provide a logical approach for clinicians for this group of rare disorders, and to thus facilitate prompt diagnosis and early initiation of immunotherapies in this set of potentially reversible syndromes. Importantly, the turnaround time for specialist neuronal autoantibody test results can take several weeks. In the interim, an untreated patient with suspected autoimmune encephalitis may have significant risk of potentially irreversible deterioration, including life-threatening sequelae such as central apnoea, dysautonomia or status epilepticus **(Table 1.1)**. A key emphasis of the updated diagnostic guidelines by Graus et al in 2016 was that a diagnosis of suspected autoimmune encephalitis can be made *without* known autoantibody status, and thus treatment should be initiated early, pending autoantibody results (Graus et al. 2016). Correspondingly, the same principles apply to seronegative syndromes, where, despite a characteristic clinical syndrome, no *known* serum or CSF autoantibody is detected by standard testing, either due to false negative result(s), or due to presence of an underlying novel

autoantibody; this would not refute the possibility of an immune-mediated, immunotherapy-responsive syndrome. In this scenario, live neuronal testing can be requested – any neuronal reactivity demonstrated by the serum/CSF would strongly suggest presence of a novel neuronal surface antibody and allow identification of novel antigenic targets. Similarly, positive autoantibody results are not always relevant to the clinical presentation and may represent detection of an autoantibody analogous to the background healthy control rate. Thus, the clinical assessment and initial investigations such as MRI brain, EEG, and CSF analysis, are paramount in making the diagnosis; subsequently available positive serum and/or CSF autoantibodies should serve as confirmatory.

Graus et al (Graus et al. 2016) devised three levels of evidence for autoimmune encephalitis: possible, probable, and definite. Typically, diagnosis requires: 1. subacute onset (rapid progression within 3 months) of memory deficits, seizures, or altered mental status, *plus* 2. MRI abnormalities, *plus* 3. CSF pleiocytosis and/or EEG abnormalities, and 4. reasonable exclusion of other causes **(Figure 1.5A)**. Positive autoantibody status is required only for 'definite' autoimmune encephalitis, or in the scenario that one of the first three criteria are not met. Important alternative causes to be excluded should include infective viral encephalitis (HSV1, HHV-6), drug/ intoxication, primary psychiatric disease, glioma, other steroid-responsive conditions such as acute disseminated encephalomyelitis (ADEM) and neuromyelitis optica spectrum disorders (NMOSD), prion disease, neurosyphilis, HIV infection, and CNS vasculitis (Graus et al. 2016). Other diagnostic guidelines have been published, which overall, follow similar principles (Rössling & Prüss 2020).

Whilst no formal diagnostic criteria exist which are specific for LGI1-autoantibody encephalitis, once a clinician has suspected autoimmune limbic encephalitis **(Figure 1.5A)**, distinct clinical features such as the demographics of the patient (typically elderly male), and characteristic frequent focal seizures, including FBDS, should provide diagnostic clues to the particular underlying pathogenic autoantibody **(Tables 1.1, 1.3, and 1.4; Figure 1.4)**. Clinician vigilance for autoimmune encephalitis is key. Indeed, Herken et al (Herken & Prüss 2017) recently highlighted that from their cohort (n=100), psychiatric symptoms constituted the initial clinical presentation in 60%, and one third of patients were initially erroneously admitted to a psychiatric ward. They devised 'yellow flags' and 'red flags' of common

symptoms of autoimmune encephalitis for clinicians to be aware, including FBDS, dyskinesias, altered conscious level, aphasia, hyponatraemia, autonomic dysfunction, and catatonia, and abnormal MRI, EEG or CSF.

1.5.2 Investigation Findings

MRI brain, CSF analysis, EEG, and paired serum and CSF autoimmune encephalitis autoantibody panels are standard workup for the diagnosis of autoimmune encephalitis (Graus et al. 2016). Patients with LGI1 autoantibodies commonly show unilateral or bilateral medial temporal lobe T2 hyperintensities on MRI **(Figure 1.5B)**, typically detected in around 70% (Irani et al. 2011; Irani et al. 2013; van Sonderen, Thijs, et al. 2016; Finke et al. 2017). EEG is often abnormal (in ~60%), with epileptiform features, diffuse slowing, or non-specific abnormalities **(Table 1.1)**. In contrast, only ~30% of patients have abnormal CSF **(Table 1.1)**. Hyponatraemia is very common and reported in over 70% of patients during acute illness (Irani et al. 2011; Ariño et al. 2016). Thus, routine, ancillary investigations may provide useful adjunctive paraclinical information to further support the diagnosis whilst autoantibody results are awaited.

1.5.3 The Live Cell-Based Assay

Antibody detection techniques include cell-based assay (CBA) (Irani, Gelfand, Al-Diwani, et al. 2014), live neuronal testing, tissue immunohistochemistry (Graus et al. 2016), and fluorescence immunoprecipitation assay (FIPA) (Waters et al. 2008). The gold-standard for diagnostic testing of neuronal autoantibodies is the CBA (Graus et al. 2016; Rössling & Prüss 2020; Ramanathan et al. 2019; Michael et al. 2020). It is recommended that both serum and CSF are sent for neuronal autoantibody testing in suspected cases of autoimmune encephalitis (Graus et al. 2016). This is primarily due to the fact that a small proportion of patients may harbour autoantibodies only within the CSF compartment and not in the serum. NMDAR-autoantibody encephalitis is one such example, where a large analysis of 250 patients revealed that ~15% lacked serum NMDA-R autoantibodies, but 100% showed CSF autoantibodies (Gresa-Arribas et al. 2014). For LGI1-autoantibody encephalitis, this is less

relevant, since CSF autoantibodies are commonly not detected in 22%-33% of patients (Lehmann-Horn et al. 2020; Muñiz-Castrillo 2021). Further, false positive results almost never occur with CSF analysis and thus a positive CSF autoantibody result alongside a positive serum result can be helpful in the case of diagnostic uncertainty (Graus et al. 2016).

There are many caveats to the interpretation of autoantibody results. Firstly, it is important that serum and CSF samples are sent for autoantibody testing only where there is a high clinical suspicion for an autoimmune encephalitis. Secondly, if an autoantibody is found which does not correlate with the clinical syndrome, one should consider a false-positive result, or that the result is irrelevant, indicating presence of the particular autoantibody at background, healthy control rates. One important study by Dahm et al tested sera from over 4000 healthy controls (HCs) and disease controls (DCs) for presence of various neuronal surface autoantibodies (Dahm et al. 2014). Amongst controls, the seroprevalence of antibodies to the NR1 subunit of the NMDAR was 10% (1% IgG), compared to typically <1% for other autoantibodies, and <0.1% for LGI1 **(Table 1.3)**. This highlights a major challenge in the interpretation of many such results – particularly for NMDAR antibodies – arising from reflexive testing, where the clinical syndrome is vague, yet the serum in isolation is sent for autoantibody testing "just in case". Another similar scenario may arise from individual laboratories' thresholds for positivity; variation in assay cut-offs between laboratories may mean that some results reported as positive at low-titre are likely false-positive and irrelevant to the clinical syndrome. Examples exist of many individuals erroneously diagnosed by these means with receipt of unnecessary, costly immunotherapy with potentially deleterious toxic effects (Bien 2018). Therefore, not only should autoantibody results be sent and interpreted in the appropriate clinical context, but there is also a need for standardization of CBA detection thresholds and diagnostic testing methods between laboratories.

Finally, our understanding of test systems used to detect pathogenic antibodies has advanced. Only antibodies that can 'see' their targets in vivo have clear pathogenic potential. Therefore, "live" CBAs which present only extracellular domains of antibody targets are preferred. These avoid the detection of non-pathogenic proteins and the use of fixative, which can reduce assay accuracy by altering target epitopes and permeabilising cells (Tea et al. 2019). Many diagnostic centres use commercial kits consisting fixed CBAs, largely through

convenience. However, live testing remains biologically intuitive and shows higher sensitivity and specificity and the ability to resolve samples indeterminate by routine commercial kit testing (Tea et al. 2019; McCracken et al. 2017; Waters et al. 2019; Waters et al. 2016). Target-specific CBAs, where individual proteins are over-expressed on the surface of live cells, without detergent or fixative, are used across the range of neuronal surface-directed autoantibodies, including LGI1 and CASPR2 (Irani et al. 2010); **(Figure 1.5D)**. Additionally, using live CBAs, LGI1- or CASPR2-autoantibodies have been detected in patients with immunotherapy-responsive illnesses but without VGKC-complex antibodies (Irani et al. 2013; Becker et al. 2012), highlighting the inadequate sensitivity of VGKC-antibody testing. Hence, direct LGI1- and CASPR2-autoantibody testing should be performed first-line, and not as a second-line, reflexive test following a positive VGKC-antibody result.

Figure 1.5 Autoimmune Encephalitis Diagnostic Criteria, Investigations, Live Cell-Based Assay, and Treatment Response

A

Panel 1

Diagnostic criteria for possible autoimmune encephalitis

Diagnosis can be made when all three of the following criteria have been met:

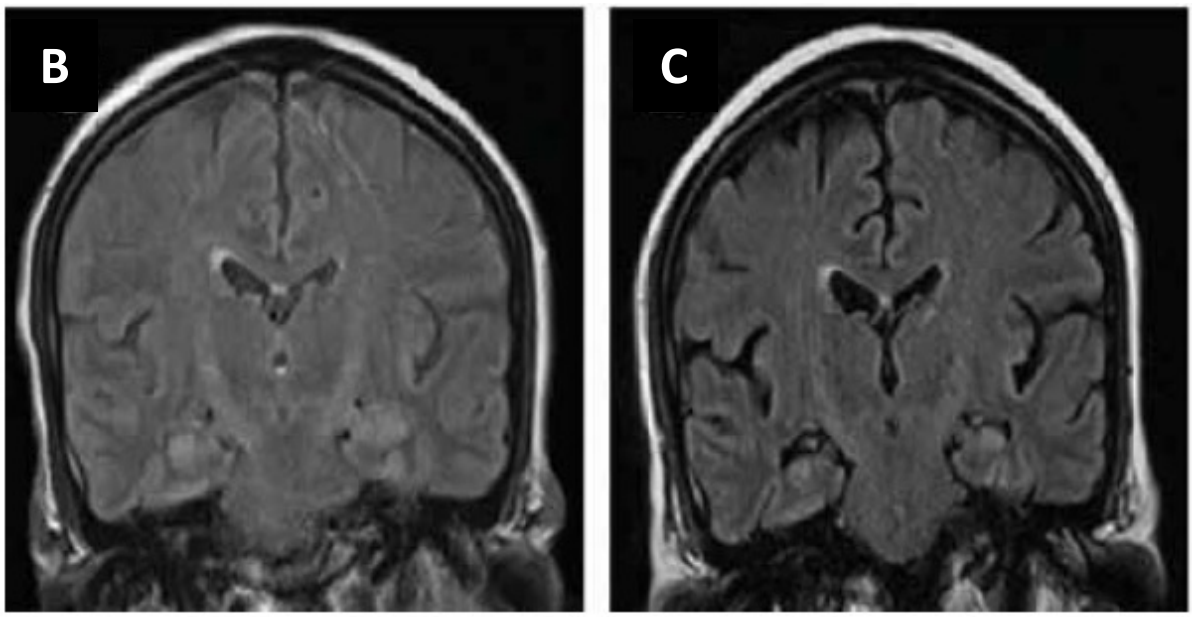
1. Subacute onset (rapid progression of less than 3 months) of working memory deficits (short-term memory loss), altered mental status[*], or psychiatric symptoms
2. At least one of the following:
    - New focal CNS findings
    - Seizures not explained by a previously known seizure disorder
    - CSF pleocytosis (white blood cell count of more than five cells per mm^3)
    - MRI features suggestive of encephalitis[†]
3. Reasonable exclusion of alternative causes (appendix)

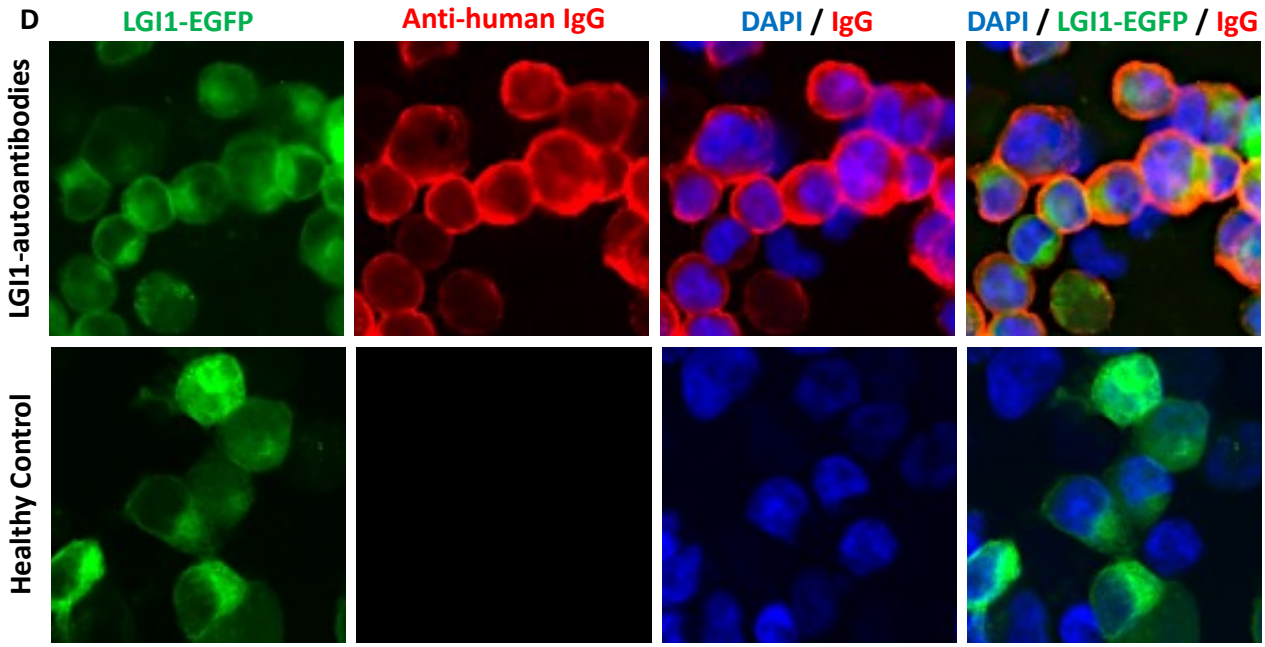
Panel 2

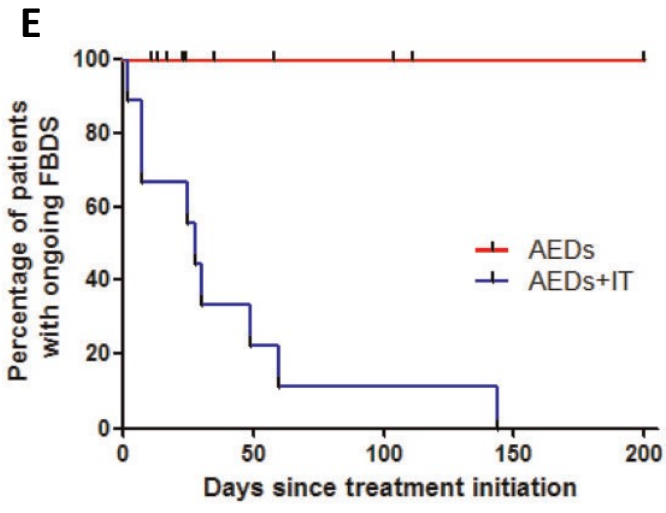
Diagnostic criteria for definite autoimmune limbic encephalitis

Diagnosis can be made when all four[*] of the following criteria have been met:

1. Subacute onset (rapid progression of less than 3 months) of working memory deficits, seizures, or psychiatric symptoms suggesting involvement of the limbic system
2. Bilateral brain abnormalities on T2-weighted fluid-attenuated inversion recovery MRI highly restricted to the medial temporal lobes[†]
3. At least one of the following:
    - CSF pleocytosis (white blood cell count of more than five cells per mm^3)
    - EEG with epileptic or slow-wave activity involving the temporal lobes
4. Reasonable exclusion of alternative causes (appendix)


B
C


D
LGI1-EGFP
Anti-human IgG
DAPI / IgG
DAPI / LGI1-EGFP / IgG
LGI1-autoantibodies
Healthy Control


E
Percentage of patients with ongoing FBDS
100
80
60
40
20
0
0
50
100
150
200
Days since treatment initiation
AEDs
AEDs+IT

(A) Diagnostic criteria for autoimmune encephalitis proposed by Graus et al, 2016. Autoantibody status is not a diagnostic requirement, thus initiation of treatments should not be delayed pending autoantibody results. Taken from Graus et al, 2016 {Graus:2016gq}. Typical MRI changes of autoimmune LGI1-autoantibody limbic encephalitis with bilateral medial temporal lobe T2 hyperintensities and swelling **(B)** and subsequent bilateral hippocampal atrophy at follow-up **(C)**. **B+C** taken from Irani et al, 2011 {Irani:2011kc}. **(D)** LGI1-antibody live cell-based assay. Serum from a patient with LGI1-antibodies binds to the surface of live LGI1-expressing cells (top row) but healthy control sera (bottom row) do not show binding to these cells. LGI1 tagged to EGFP (LGI1-EGFP, green) is expressed on surface of live HEK293T cells; detected with anti-human IgG autoantibodies (red); DAPI nuclear staining (blue). Images taken at x40 magnification. **(E)** No effect on FBDS when treated with AEDs alone (red): proportion of patients with ongoing FBDS 100%, even at 200 days. Dramatic clinical response in FBDS observed when IT added to AEDs (blue), with only ~20% patients with ongoing FBDS after 50 days. Complete cessation of FBDS observed only with IT+AEDs. Taken from Irani et al, 2013 {Irani:2013cj}.

AED, antiepileptic drug – referred to as 'ASM' (anti-seizure medication) throughout thesis.

1.5.4 Therapeutic Algorithms and Treatment Response

Whilst no definitive consensus treatment guidelines exist for autoimmune encephalitis, there are several proposed therapeutic algorithms, for example as recently devised by Ramanathan et al (Ramanathan et al. 2019). General paradigms consist of initiation of fast-acting first-line immunotherapies, to rapidly diminish or 'wash-out' circulating serum autoantibodies, followed by various forms of maintenance immunotherapies, including initiation of 'second-line', long-term steroid-sparing agents, and less frequently, third-line agents. Tumour screening is recommended at disease onset for all cases of autoimmune encephalitis, since tumour removal and treatment often results in favourable response of the neurological syndrome (Dalmau et al. 2008; Ramanathan et al. 2019), suggesting that tumour therapy itself may serve as a form of immunotherapy.

First-line immunotherapies include high-dose intravenous methylprednisolone (IVMP) over 3-5 days, plasma exchange (PLEX; typically 5 cycles in the first instance), and intravenous immunoglobulin (Ramanathan et al. 2019). For those showing partial or lack of response, first line treatments should be repeated, or escalated as appropriate (for example, if initially treated only with IVMP, a cycle of PLEX or additional IVMP should be trialled, etc). IVMP is typically followed by a high-dose oral prednisolone course. In LGI1-autoantibody encephalitis, there is often striking response to steroids, with resolution of seizures within a few days of their initiation (Thompson et al. 2017). For LGI1-autoantibody encephalitis, there is increasing evidence to support a very gradual taper of steroid over several months to one year, since

rapid prednisolone tapering by 20-30mg over 3 months can trigger relapses (Irani et al. 2011; Gadoth et al. 2017). PLEX is primarily effective via removal and thus reduction of circulating levels of pathogenic autoantibodies, although it is thought to have additional mechanisms of action (Sun et al. 2020). These include removal of cytokines, reduction in B cells, increased regulatory T cells, and importantly, stimulating proliferation of autoreactive B cells, rendering them more susceptible to immunosuppressants (Jacob et al. 2021).

Second-line immunotherapies include mycophenolate, azathioprine, cyclophosphamide, and Rituximab. Initiation of second-line agents is typically considered in patients refractory to first-line agents (or with slower response than anticipated), and those with severe presentations (Ramanathan et al. 2019). Whilst Rituximab is now routinely used in some autoantibody-mediated neurological disorders, such as NMOSD (Tahara et al. 2020), limited studies exist for LGI1-autoantibody encephalitis. Small studies of Rituximab use in LGI1-autoantibody encephalitis show tolerability in this predominantly older cohort, and evidence of rapid clinical improvement in some patients (Irani, Gelfand, Bettcher, et al. 2014; Brown et al. 2014), although glucocorticoids appear to be particularly efficacious (Irani, Gelfand, Bettcher, et al. 2014). A recent study analysed Rituximab use in autoimmune encephalitis in Germany (GENERATE Registry), and correlations with long-term clinical outcomes (Thaler et al. 2021). Rituximab appeared to be particularly efficacious in CASPR2- and NMDAR-autoantibody mediated encephalitis, especially when initiated early. In LGI1-autoantibody encephalitis, although both Rituximab and non-Rituximab-treated patients showed substantial clinical improvements (~80% MRS ≤2), the Rituximab-treated group exhibited reduced relapse rates compared to controls. Additionally, Rituximab was found to be safe when used in 149 patients (n=26 with LGI1-autoantibody encephalitis; mean age 61 years).

Finally, in the minority of patients who are refractory to second-line therapies, third-line immunotherapies such as Tocilizumab and Bortezomib may be considered, although these are largely experimental in this group of disorders (Ramanathan et al. 2019). The data on efficacy of use of such plasma-cell targeting agents in autoimmune encephalitides are largely restricted to NMDAR-autoantibody encephalitis (Lee et al. 2016; Behrendt et al. 2016; Scheibe et al. 2017; Shin et al. 2018; Keddie et al. 2018), and overall, suggest both efficacy and safety in severe, refractory cases.

Whilst FBDS are largely resistant to anti-seizure medications, they preferentially respond to immunotherapies **(Figure 1.5E)**; (Irani et al. 2011; Irani et al. 2013; Flanagan et al. 2015; Thompson et al. 2017). Early diagnosis of LGI1-autoantibody encephalitis is crucial, since prompt treatment with immunotherapy can prevent permanent cognitive impairment and is associated with superior clinical outcomes (Thompson et al. 2017). Intriguingly, however, there are examples of a select, small group of patients who exhibit spontaneous resolution without any form of treatment (Flanagan et al. 2015).

ASMs have been consistently shown to cause significant cutaneous reactions in a high proportion (29-50%) of LGI1-autoantibody patients, typically with carbamazepine and phenytoin use – each implicated in around 50% of cases (Irani et al. 2011; Irani et al. 2013; Thompson et al. 2017). Indeed, in patients with ASM-induced cutaneous reactions, severe manifestations including Stevens-Johnson syndrome and intensive care unit admission are observed at a rate of 17% (Irani et al. 2011; Thompson et al. 2017). The high rate of drug rash is likely explicable by the HLA associations of these patients (Binks, Varley, et al. 2018; van Sonderen et al. 2017; Kim et al. 2017). In view of the high rate of such potentially life-threatening side effects, and the preferential response of these patients to immunotherapies, ASMs – particularly carbamazepine and phenytoin – should be used with caution in this group.

1.5.5 Patient Outcomes

At disease onset, nearly 75% of patients with LGI1-autoantibody encephalitis have significant disability, with MRS >2 (Muñiz-Castrillo 2021). Factors identified as poor outcome predictors include CSF LGI1-IgG positivity, increasing age, female sex, status epilepticus, and higher MRS at onset (Muñiz-Castrillo 2021). Outcomes are generally very good, with >80% showing follow-up MRS ≤2 (Binks et al. 2021), and a large cohort of 134 patients were found to have median MRS of 1 at latest follow-up (Muñiz-Castrillo 2021); (median follow-up times 41 and 16.2 months, respectively). Despite these findings, 97% experience severe, persistent verbal and visuospatial memory deficits in association with marked hippocampal atrophy in almost all patients (Finke et al. 2017). Additionally, Binks et al found that only 15% of patients who

were employed at the time of diagnosis returned to work, and that fatigue was the most common long-term deficit across the cohort, present in 52%, and severe in nearly two thirds (Binks et al. 2021). Other prominent long-term deficits identified were anxiety and depression, detected in 33% and 19%, respectively (Binks et al. 2021). Several studies have consistently shown that early initiation of immunotherapy significantly improves cognitive outcomes in patients with LGI1-autoantibodies – both for FBDS cohorts (Thompson et al. 2017) and non-selected cohorts (Finke et al. 2017; Miller et al. 2017). Thus, time to treatment with immunotherapies appears to be the single most important factor which directly influences outcomes in this condition.

Reported relapse rates in LGI1-autoantibody disease are generally between 25-35% (Li et al. 2018; Ariño et al. 2016; Thompson et al. 2017; Sola-Valls et al. 2020; van Sonderen, Thijs, et al. 2016). Rates are variable, however, with some studies reporting rates as low as ~15% (Irani et al. 2011; Shin et al. 2013; Lai et al. 2010), and others as high as 59% (Gadoth et al. 2017). Mortality rates are low, typically 3-6% (Finke et al. 2017; Thompson et al. 2017; Ariño et al. 2016), with deaths largely due to complications of immunotherapies, including sepsis and pulmonary emboli. Only one study reports a two-year case fatality rate of 19% from 38 patients (van Sonderen, Thijs, et al. 2016), which appears to be inconsistent with the aforementioned data from larger cohorts.

1.6 Immunological Concepts: Immunobiological Basis of Autoantibody-Mediated Neurological Disorders

1.6.1 Why and How are Autoantibodies Generated?

With increasing recognition and characterisation of novel autoantibody-mediated neurological disorders, not only is our understanding of the patient clinical phenotypes expanding, but also our knowledge of their immunopathogenesis. Grasp of the underlying autoantibody-mediated cellular neuroimmunology is key, since it will enhance our ability to develop targeted treatments for these patients.

For autoantibody-mediated neurological conditions, one important theme of the literature has been to delineate mechanisms behind autoantibody production and maintenance. These diseases are typically mediated by autoantibodies directed against a single autoantigen located on the neuronal surface. But what triggers production of these pathogenic autoantibodies in the first place? Are these autoantibodies produced at low rates throughout life in individuals with genetic predisposition, remaining quiescent until a certain immune challenge stimulates their overproduction, pathogenicity and clinical manifestations? Or are they produced de novo with close temporal relationship to the individual's disease onset? Where is the primary site of immunisation in these patients, and how do these autoantibodies cause CNS manifestations? The CNS is traditionally considered to be a site of 'immune privilege'. Thus, are the autoantibodies produced peripherally with subsequent entry to the CNS compartment due to compromised immune barriers, or is their primary site of production within the CNS? How are levels of these autoantibodies maintained within the periphery and CNS over the disease course? How, if at all, does the underlying immunobiology differ between patients with underlying tumours, those with short monophasic courses and good responses to immunotherapies, those with relapses, and those who are refractory to immunotherapies? Studies of patients with neurological autoantibody-mediated conditions have helped in part to answer some of these questions, and to provide a robust foundation to our understanding of neuroimmunology.

1.6.2 Immune Tolerance

The adaptive immune system is composed of a variety of T cells, B cells and plasma cells (PCs), which secrete antibodies. In health, these antibodies are produced against foreign antigens, including viral and bacterial antigens. Thus, the adaptive immune system serves to protect us from infection. In autoimmunity, antibodies directed against self-proteins are produced, termed 'autoantibodies'. If these autoantibodies bind to their targets, self-reactivity occurs. For autoantibodies with pathogenic potential, such as neuronal surface-directed autoantibodies, the misdirected autoreactivity is typically deleterious. In health, autoreactivity is limited by a process called 'immune tolerance', consisting of certain regulatory mechanisms within 'immune checkpoints'. Immune checkpoints may be central,

where regulatory mechanisms occur in the bone marrow (BM) during early B cell development, or peripheral, within secondary lymphoid organs (Nemazee 2017).

The developmental programming which occurs during central tolerance to limit self-reactivity consists of three main processes. The first is clonal deletion, or apoptosis of autoreactive B cells. The second is receptor editing, which reprograms the specificity of the B cell through light-chain exchange of the B cell receptor (BCR) so that the BCR switches from autoreactive to innocuous, rescuing the B cell. The third mechanism is via BCR signaling, which regulates the BCR to induce apoptosis, receptor editing, and positive selection of non-autoreactive B cells to facilitate survival, maturation, and migration to the periphery. The efficiency of central tolerance can be demonstrated by the comparative degree of autoreactivity in the immune compartments pre- and post- immune checkpoint control: in normal adults, up to 80% of immature B cells in the BM are autoreactive versus only 10% of naïve B cells in the periphery (Wardemann et al. 2003; Nemazee 2017). Defective or inefficient central tolerance causes defective negative selection and leads to increased numbers of autoreactive B cells. Indeed, defective central tolerance is associated with autoimmune diseases including systemic lupus erythematous, rheumatoid arthritis, type 1 diabetes mellitus, Sjogren's disease, and multiple sclerosis (Nemazee 2017).

In the periphery, B cells are subject to two further peripheral checkpoints which reduce autoreactive B cells via apoptosis and anergy, facilitated by BCR signaling, regulatory T cells and cytokines. Importantly, if a target autoantigen is expressed within a germinal centre (GC), the autoreactive B cells will be eliminated. For autoantibody-mediated neurological conditions, the neuronal surface targets are predominantly expressed within the CNS. Thus, autoreactive B cells with specificity towards neuronal surface antigens may not be eliminated within GCs, highlighting one mechanism by which these autoreactive B cells may evade immune tolerance (Sun et al. 2020). Further, somatic hypermutation occurs within GCs and increases BCR affinity towards their antigen. For autoreactive B cells, somatic hypermutation may increase autoreactivity, highlighting another mechanism by which autoantigen-specific B cells may be accentuated (Sun et al. 2020). In fact, at this peripheral checkpoint, the proportion of autoreactive B cells in healthy subjects is seen to paradoxically increase from 2% in unswitched to 47% in switched memory B cells (Sun et al. 2020).

1.6.3 Evidence for Defective B Cell Immune Tolerance in Neurological Autoantibody-Mediated Syndromes

Both the autoantigen-specific B cells and the autoantigen-specific autoantibodies have been detected in the peripheral circulation of patients with LGI1- and NMDAR-autoantibody syndromes (Lehmann-Horn et al. 2020; Ramberger et al. 2020; Makuch et al. 2018). Since the autoantigen-specific B-cell lineage is responsible for production of pathogenic autoantibodies, examination of B cell subsets from these patients is important. Such studies will allow us to ascertain at which stage of the B cell lineage the autoantibodies appear, and thus to glean insights into which stage immune tolerance malfunctions in these patients. One study cultured B cells from patients with NMOSD and found that aquaporin-4 (AQP4) autoantibodies derived from both antigen-naïve, pre-GC B cell and post-GC memory B cell cultures (Wilson et al. 2018). Presence of AQP4-specific naïve B cells suggests an early, pre-GC loss of central tolerance in NMOSD. Additionally, detection of the AQP4-specific memory B cells suggests their generation from the former, naïve B cells, with further increased affinity for AQP4 via somatic hypermutation in GCs, suggesting that additional disruption of peripheral tolerance checkpoints may be relevant in NMOSD.

1.6.4 Sites of Autoantibody Production and Maintenance: CNS Access

Importantly, in autoantibody-mediated neurological diseases, a consistent pattern of far greater serum autoantibody titres compared to CSF is observed. For example, serum LGI1-autoantibody titres are 127-fold higher than CSF (Lehmann-Horn et al. 2020). However, ratios may even be up to 600-fold greater in serum (Sun et al. 2020). Intuitively, these observations suggest that the primary site of immunisation and autoantibody production is within the periphery, with subsequent entry of autoantibody to the CSF compartment. Indeed, for LGI1-autoantibody encephalitis, Lehmann-Horn et al found shared BCR sequences between CSF and serum LGI1 autoantibodies, evidencing that the autoantigen-specific B cells migrate from the periphery to the CSF compartment (Lehmann-Horn et al. 2020). There are several concepts regarding the methods by which autoantibodies may gain access to the once regarded ‘immune privileged’ CNS space. Sun et al summarise these (Sun et al. 2020).

Inflammatory or infective insults within the CNS may trigger neuronal damage and release soluble neuronal autoantigens into the CSF, which exit the CNS compartment via a CNS lymphatic drainage system into cervical lymph nodes in the periphery. Within lymph nodes, high affinity autoantibodies against these neuronal autoantigens are produced via GC reactions. It is hypothesised that autoreactive B cells then migrate into the CNS compartment via chemotactic factors such as CXCL13. Within the CNS compartment, clonal expansion of the autoantigen-specific B cell may occur, with affinity maturation and secretion of high affinity autoantibodies, which may cause direct neuronal dysfunction if they bind to their autoantigen targets on the neuronal surface. Thus, it is possible that the autoantibodies may enter the CNS via B cell migration from the periphery, IgG transfer across the blood brain barrier, and/ or intrathecal synthesis of the autoantibodies. Crucially, when serum to CSF IgG ratios are less than 400:1, this is considered to be indicative of intrathecal synthesis of the autoantibody, although IgG levels should be normalised to the total IgG levels (Sun et al. 2020). Monoclonal autoantibodies with LGI1- and NRI1- specificities have been cloned from CSF B cells and antibody-secreting cells of patients with LGI1- and NMDAR-autoantibody encephalitis, respectively (Kornau et al. 2020; Kreye et al. 2016). Further, all CSF-derived monoclonal autoantibodies showed evidence of hypermutation and affinity maturation, and the LGI1-specific antibody-secreting cells isolated represented the majority of all CSF antibody secreting cells (Kornau et al. 2020). These observations provide evidence for presence of autoantigen-specific B cells within the CNS compartment, intrathecal clonal expansion and affinity maturation, and intrathecal synthesis of the autoantibodies.

1.6.5 Sites of Autoantibody Production and Maintenance: Proposed Models

For neuronal surface autoantibody-mediated syndromes, two main models of autoantibody production exist. These are centred around GC-predominant mechanisms within the periphery, and long-lived plasma cell (LLPC)-predominant mechanisms within the BM (Sun et al. 2020). The GC-predominant model of autoantibody production assumes ongoing autoantibody production by new GC reactions. Autoreactive B-cells with acquired specificity for their target autoantigen enter GCs and partake in GC reactions, generating short-lived

antibody-secreting cells, which typically secrete IgM autoantibodies. This concept assumes ongoing autoantigen exposure and immunisation, thus generating new autoantigen-specific naïve B cells and recurrent GC reaction cycles with secretion of new autoantigen-specific autoantibodies. The second, LLPC-predominant model of autoantibody production assumes that the autoantibody pool is largely contributed to by BM-resident LLPCs. These autoantigen-specific PCs form following GC reactions, and migrate to the BM where they remain in their niche long-term, secreting autoantibodies for many years.

Studies of patients with neuronal surface autoantibody-mediated syndromes suggest that both GC reactions and LLPCs contribute to the circulating autoantibody pools in these patients.

1.6.6 Germinal Centre Reactions

GC reactions are essential for affinity maturation of antigen-specific B cells and production of high-affinity memory B cells and antibody-secreting PCs (Gatto & Brink 2010; Havenar-Daughton et al. 2016). Thus, whilst the primary function of GC reactions is to ensure sustained immune protection against previously encountered foreign antigens such as infectious pathogens and vaccine antigens, they pose risk of generation and expansion of pathogenic autoreactive B cells, and play a role in the development of autoantibody-mediated diseases and some lymphomas (Gatto & Brink 2010). GCs are specialised structures contained within secondary lymphoid organs such as lymph nodes, spleen, tonsils, and other lymphoid tissues. GCs are organised into dark and light zones. The dark zone is located near T cell areas and contains high density of proliferating B cells with downregulated surface Ig expression termed centroblasts. The light zone contains few B cells, which are small, non-mitotic, express surface Ig, and are termed centrocytes. GC compartmentalisation into light and dark zones depends upon chemokine gradients. CXCL12 is highly expressed within dark zones, and centroblasts express high levels of CXCR4, the receptor for CXCL12, driving their retention within the dark zone. Centrocytes are preferentially localised within light zones due to expression of CXCR5, the receptor for CXCL13, which is highly expressed in GC light zones (Gatto & Brink 2010).

B cell selection and differentiation occurs within the light zone, where specialised follicular dendritic cells (FDCs) and $CD4^+$ T follicular helper (Tfh) cells are localized. FDCs present antigen to B cells, which drives differentiation of selected B cells. Within the dark zone, selected B cells undergo proliferation and affinity maturation. During their proliferation, B cells undergo somatic hypermutation, acquiring high mutation rates in their immunoglobulin variable region genes, resulting in random changes to BCR affinity and specificity. Further, B cell clones with high binding to the immunising antigen undergo preferential selective expansion, allowing affinity maturation, with increasing affinity of the secreted antibodies as GC reactions progress. Overall, GC reactions expand high affinity B cells and are a key source of effector memory B cells or PCs which will ultimately secrete antibodies with high affinity for their antigen (Gatto & Brink 2010; Havenar-Daughton et al. 2016).

Abnormal GC reactions are likely to play a role in the development of autoimmunity. Dysregulated, exaggerated GC reactions have been shown to contribute to autoantibody production in conditions such as SLE and RA. Additionally, ectopic GCs have been shown to form in non-lymphoid organs. One seminal finding for CNS autoimmunity was that of ectopic B and T cell follicles resembling GCs demonstrated in the meninges of patients with MS (Serafini et al. 2004).

1.6.7 Evidence for Germinal Centre Contribution to Autoantibody Production in Neurological Autoantibody-Mediated Syndromes

In NMDAR-autoantibody encephalitis, NR1 subunit autoantigen-specific IgMs were detected in 60% of patients for at least 6 months following disease onset (Makuch et al. 2018). This is far greater than the ~5-day half-life of IgM, indicating ongoing *new* production rather than persistence of NR1-IgMs. Additionally, circulating patient B cells differentiated into antibody-secreting cells *in vitro*, and produced NR1-IgG and -IgM across disease durations (Makuch et al. 2018). These data suggest that ongoing GC reactions triggered by repeated autoimmunization may account for antigen-specific IgM/IgG production in NMDAR-autoantibody encephalitis. Interestingly, B cells isolated from teratomata of NMDAR-autoantibody encephalitis patients have been shown to secrete NR1-specific IgG when

cultured in vitro (Makuch et al. 2018). This indicates that tumour-resident autoantigen-specific B cells are another source of autoantibody production in this condition. Consistently, teratoma removal is associated with decreased autoantibody titres, clinical improvements and better outcomes (Dalmau et al. 2007; Dalmau et al. 2008). Moreover, these ovarian teratomata express the NR1 subunit, and show histological evidence of ectopic GCs, with tertiary lymphoid structures (Tüzün et al. 2009; Makuch et al. 2018). Importantly, these are not identified in teratomata from patients without NMDAR-autoantibody encephalitis. These findings highlight that the tumours themselves act as additional peripheral lymphoid organs, since they contain active GCs which secrete autoantibodies. Finally, C–X–C motif chemokine ligand 13 (CXCL13), a chemokine which serves as a biomarker of GC-reactions (Havenar-Daughton et al. 2016), is elevated in patients with NMDAR-autoantibody encephalitis (Leypoldt et al. 2015; Liba et al. 2016), and LGI1-autoantibody encephalitis (Lin et al. 2019).

1.6.8 Evidence for Long-Lived Plasma Cell Contribution to Autoantibody Production in Neurological Autoantibody-Mediated Syndromes

Only two studies exist which employ direct sampling of the BM compartment in neurological autoantibody-mediated syndromes – one in myasthenia gravis (MG) (Fujii et al. 1986), and one in glutamic acid decarboxylase (GAD)-autoantibody associated disease (Thaler et al. 2019). Neither study employed direct isolation of LLPCs, and their limitations are discussed in Chapters 5 and 6. Rather, the majority of evidence which implicate BM-derived LLPCs in these diseases derives from therapeutic observations. For example, LLPCs do not express CD20, and therefore evade depletion by Rituximab, a monoclonal antibody targeting CD20. However, LLPCs are susceptible to BM ablation used prior to BM stem cell transplantation (Sun et al. 2020). Indeed, in NMOSD, AQP4 autoantibodies persist despite Rituximab therapy, albeit at lower levels (Jarius et al. 2008). However, remission and AQP4-autoantibody seronegativity are achieved in the majority of NMOSD patients who undergo haematopoietic stem cell transplantation (Burt et al. 2019). Finally, the benefits of PC-depletion using Tocilizumab (Lee et al. 2016)and Bortezomib (Behrendt et al. 2016; Scheibe et al. 2017; Shin et al. 2018; Keddie

et al. 2018) in refractory cases of autoimmune encephalitis have already been discussed **(Section 1.5.4)**, implicating LLPCs as strong mediators of the condition.

PCs shall be discussed in detail in the next sections, as a key understanding is important since they form the basis of the experiments conducted in **Chapter 5**.

1.7 Plasma Cell Overview

1.7.1 Plasma Cell Classification and Morphology

Plasma cells are terminally differentiated, non-dividing B cells, which continuously secrete antibodies, thus providing protective immunity (Minges Wols 2005). They are end-stage effectors of the humoral immune response, in both healthy and disease states. Some examples of plasma cells implicated in disease include antibody-mediated autoimmune conditions, such as antibody-mediated encephalitis, malignancy such as multiple myeloma, and rarely, plasmacytoma. It is important to clarify the terminology to classify plasma cells. Circulating antibody-secreting cells found in the bloodstream are known as *plasmablasts* (PBs), and are proliferating, precursor cells of short- and long-lived plasma cells. Antithetically, tissue-resident antibody-secreting cells are termed *plasma cells* – thus terminally differentiated – and are found in the spleen, lymph nodes, and bone marrow (BM); they remain in their respective tissue, or 'niche', for the entirety of their lifecycle (Minges Wols 2005). Plasma cells (PCs) are morphologically distinct, boasting a characteristically abundant cytoplasm, prominent rough endoplasmic reticulum (RER), large Golgi, and a small, condensed nucleus. In fact, their nuclei are often referred to as 'clock-face', 'cartwheel', or 'spokes of a wheel' nuclei due to their chromatin arrangement **(Figure 1.6)** (Hammarlund et al. 2017; Medina et al. 2002). The increased cytoplasmic-to-nuclear ratio and prominent RER serve to accommodate translation of vast

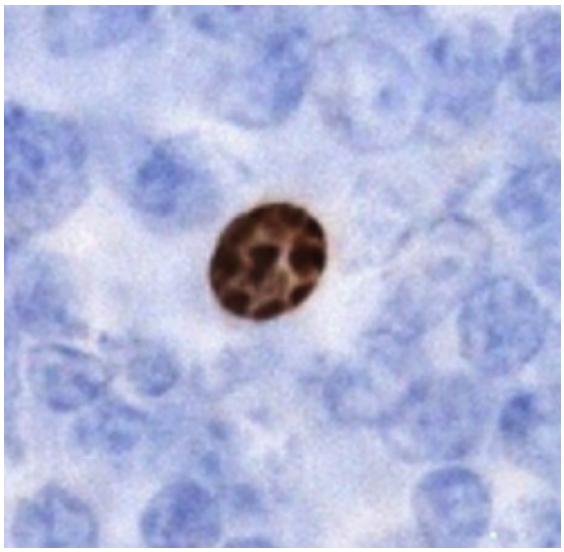


Figure 1.6 Plasma Cell Morphology
Plasma cell with distinctive eccentric 'clock-face' nucleus; Mayer's hematoxylin stain. Taken from Hammarlund et al 2017 (Hammarlund et al. 2017).

amounts of immunoglobulin mRNAs required by the PC to maintain its profound immunoglobulin secretion (Calame et al. 2003). Furthermore, expression of ER-specific markers is 6-fold higher in human BM PCs compared to PCs in other niches, demonstrating their particularly effective and colossal antibody-secreting capacity (Medina et al. 2002). In contrast, B cells have large, granular nuclei and minimal cytoplasm giving high nucleus-to-cytoplasm ratio, and little RER (Minges Wols 2005).

1.7.2 Plasma Cell Life Cycle: 'from the bone marrow, to the blood, to the bone marrow, and back'

Following haematopoiesis and the production of the common lymphoid progenitor cells within the BM, precursor B cells develop into immature B cells through a number of stromal cell-derived factors, including interleukin-7 (IL-7) and C-X-C chemokine ligand-12 (CXCL12). Expression of C-X-C chemokine receptor type 4 (CXCR4; the receptor for CXCL12) on the surface of developing B cells ensures they are retained within the BM, to prevent premature release into the circulation. As developing B cells mature, they lose expression of CXCR4, and migrate from the peripheral region of the BM (the site of haematopoiesis) towards the central sinus. Here, the immature B cells emigrate from the BM as transitional B cells into the systemic circulation and to secondary lymphoid tissues such as the spleen (Minges Wols 2005).

Following antigen encounter through GC reactions, described previously, antigen-exposed naïve B cells may become either memory B cells, or PCs; it is thought that naïve B cells with high-affinity B cell receptors (BCRs; a transmembrane receptor comprised of an immunoglobulin molecule – essentially, a membrane-bound antibody) favour a PC over memory B cell fate. Other naïve B cells undergo apoptosis or anergy. PBs exit the GC within secondary lymphoid organs. If these migrate back to the BM they can reside as PCs. Since PCs express CXCR4 on their surface, it is thought that secretion of CXCL12 by sinusoidal endothelial cells facilitates retention of PBs back into the BM via the central sinus. Within the marrow, akin to precursor B cells, PCs are dependent also on stromal cell-derived factors for survival, namely interleukin-6 (IL-6). Some studies have shown that IL-3, IL-10, and TNF-α are

important cytokines for PC differentiation into immunoglobulin-secreting cells (Minges Wols 2005; Calame et al. 2003).

1.7.3 Molecular Aspects of Plasma Cell Differentiation, Development and Migration

Several molecular mechanisms permit the transition from B cells, to PBs, to PCs (Minges Wols 2005). Chiefly, B lymphocyte-induced maturation protein-1 (Blimp-1), lies at the forefront of PC differentiation (Calame et al. 2003). In cells with strong BCR signals, BCL-6 is downregulated, relieving Blimp-1 repression, allowing termination of the GC reaction and PC differentiation. Firstly, Blimp-1 represses BCL-6 and PAX5 transcription factors found on GC B cells, and secondly c-myc, thus arresting the cell cycle. Moreover, it facilitates downregulation of MHC Class II on the cell surface, and triggers reduced signalling from the B-cell receptor (BCR), since GC-exited PCs no longer require antigen binding or presentation to T cells. Subsequent activation of X-box-binding-protein-1 (XBP-1) induces marked increases in immunoglobulin heavy and light chain mRNAs and consequently promotes immunoglobulin synthesis – the core function of PCs. IRF4 also appears important for immunoglobulin secretion. More recently, and of relevance to LLPCs, it has been shown that the zinc finger transcription factor Krüppel-like factor 4 (KLF4) is expressed in human BM PCs and is important for their differentiation to long-lived, mature PCs (Schoenhals et al. 2016). In summary, Blimp-1 regulates genes required for cell proliferation, GC function and B cell identity, and immunoglobulin secretion. The combination of these molecular events is reflected in the expression profile of PCs at their various stages of differentiation and is cardinal to commitment to a PC phenotype. For example, downregulation of BCR signalling means that the BCR is consequently expressed at low levels on the surface of PBs, but not expressed on mature PCs. Additionally, as indicated by forward scatter using flow cytometry, PCs increase in size as they mature, indicative of their enlarging cytoplasm for more robust antibody production. (Minges Wols 2005; Calame et al. 2003)

Several cell surface markers specifically govern migration and differentiation of B cells to PBs and, ultimately, PCs. Downregulation of the CXCR5 and CCR7 chemokine receptors on PCs

promotes shift from the typical CXCL13- and CCL19- responsiveness of B cells in GCs of the spleen and lymph nodes. This, in conjunction with upregulation of CXCR4 expression on the PC surface drives homing to CXCL12-secreting niches such as the BM. Additionally, PC surface adhesion molecules (e.g. CD44 and very late antigen-4; VLA-4, an integrin) are believed to facilitate stromal cell contact and thus PC retention within the BM – its final destination. (Minges Wols 2005; Calame et al. 2003)

1.7.4 Long-Lived Plasma Cells and Evidence for Contribution to Long-Term Antibody Responses

PCs originating from antigen activation of both B-1 cells (found in the gut and pleura) and marginal zone B cells of the spleen provide an immediate and rapid T cell-independent response to antigen, and thus typically secrete IgM antibodies. These cells peak 7-10 days following primary immunization, have a lifespan of a few days, and are known as short-lived PCs. Since they are non-GC-derived, short-lived PCs secrete low-affinity antibody, acting as a primary defence mechanism whilst B cells with higher affinity antibodies are made. Short-lived PCs typically reside in the spleen and lymph nodes. In contrast, PCs originating from T cell-dependent antigen responses following GC reactions secrete high-affinity antibodies, since they have undergone affinity maturation. These PCs are known as long-lived plasma cells (LLPCs), provide long-term immunological memory, and are found primarily in the BM (Minges Wols 2005; Calame et al. 2003). LLPCs are the central cell of interest in **Chapter 5**.

It is well established that some antigen-specific antibodies are detected in the peripheral blood for many years – in some cases decades – following the initial antigen exposure (Minges Wols 2005). For example, population studies demonstrate that individuals vaccinated against tetanus toxoid can maintain adequate protective antibody levels for 21 years in the absence of boosters (GOTTLIEB et al. 1964), and up to 80% of the population maintain protective antibody levels for up to 25 years post diphtheria vaccination (Kjeldsen et al. 1985). More convincingly, circulating smallpox-specific antibodies have been found in vaccinated individuals over 30 years after the global eradication of smallpox itself, validating that no possible exposure to the variola virus antigen could account for the persistence of these

antibodies (Brynjolfsson et al. 2017). Similarly, another example of prolonged antibody production following infection is the detection of antibodies to particular strains of poliomyelitis virus in Alaskan Eskimos up to 40 years following the epidemic, in the absence of re-infection (PAUL et al. 1951). These studies support a role both for the existence, and ongoing function of, LLPCs.

Two key murine studies changed our understanding of long-term antibody responses, which were traditionally thought to be maintained by continuous differentiation of memory B cells into new populations of antibody-secreting PCs. Firstly, Manz et al (Manz et al. 1997) showed that ovalbumin antigen-specific BM-derived PCs were capable of surviving more than 90 days without any cell division. Moreover, Slifka et al (Slifka et al. 1998) found that lymphocytic choriomeningitis virus (LCMV)-specific BM-derived PCs persisted for more than one year following infection, even despite memory B cell depletion through irradiation. Notably, and of relevance to humans, this PC longevity and antibody secretion constituted more than half of the animal's life. Recently, the persistence of BM PCs without cell division for 10 years was demonstrated in primates also (Hammarlund et al. 2017). Finally, this important study found that tetanus-specific antibody-secreting LLPCs (amongst multiple other virus- and vaccine-antigen specific LLPCs) persist in the primate BM for 10 years, despite memory B-cell depletion with Rituximab. Together these studies verified that BM-derived PCs are indeed long-lived and *contribute to long-term antibody responses independently of both persisting antigen or continuous generation by proliferating B cells.* These data implicate the BM-derived LLPC as the 'ultimate PC', surviving autonomously for extended periods – potentially a lifetime – and capable of secretion of high-affinity antibody. Whilst in the natural, protective state of humoral immunity this is beneficial for the host in providing protection against pathogens, in disease states such as autoantibody-mediated encephalitis this could prove to be deleterious. Such durability displayed by LLPCs could indicate a potential longevity of LGI1- or CASPR2-specific LLPCs of up to a lifetime, which, combined with their ability to reside hidden within their difficult-to-target niche in the BM but also non-dividing and hence insensitive to radiation, could prove to be particularly challenging from a therapeutic perspective.

One important study demonstrated that after clearance of acute LCMV infection in mice, whilst initially 100% of LCMV-specific PCs are located in the spleen at day 8, these splenic PCs

rapidly decline, with 10-fold reductions by day 30, and over 40-fold reductions by day 145. Instead, the BM appears to take over, with around one third of LCMV-specific PCs found in the BM by day 15, and up to 90% at 1 year. The numbers of LCMV-specific PCs within the BM correlated with peripheral levels of LCMV-specific serum antibody, and their persistence was not attributed to ongoing infection within the BM, which was found clear of LCMV. (Slifka et al. 1995) If this paradigm can be translated to sensitisation to self-antigens, it could implicate BM-derived LLPCs as a source of autoantibody production in LGI1- and CASPR2-autoantibody diseases from as early as day 15 post sensitisation – which may even precede the onset of any clinical manifestations by a considerable period of time. Interestingly, recent studies have shown that LLPCs can also survive for at least 22 years in the human gut (Landsverk et al. 2017), although we shall not discuss this in further detail since this population of LLPCs is thought to be responsible for lifelong humoral immunity against enteric pathogens, and is unlikely to contribute to long-term peripheral antibody production to the same extent as BM-derived LLPCs.

1.7.5 Plasma Cell Surface Marker Expression Profiles and BM LLPC Phenotype

Here, we summarise PC surface marker expression profiles, with particular focus on distinguishing features of BM-resident LLPCs, and discuss approaches to the best choice of cell surface markers to isolate this population by flow cytometry, of relevance to **Chapter 5**.

Human PCs express high levels of CD27, CD38, and CD138 on their surface – perhaps as their most defining markers (Cocco et al. 2012; Halliley et al. 2015). CD138 (cluster of differentiation, Syndecan-1), is probably the singular most distinctive PC surface marker and thus widely accepted as a definitive marker for their isolation. This adhesion molecule is a proteoglycan which can bind fibronectin, collagen, and fibroblast growth factor (Medina et al. 2002). In NMO patients, only 15% of $CD19^{+}CD27^{++}CD38^{++}$ antibody-secreting cells expressed CD138 (Wilson et al. 2018), perhaps indicating that its expression is generally associated with a more mature PC phenotype (Cocco et al. 2012). Indeed, CD138 is thought to be a marker of tissue-migrating PCs, upregulated in recently activated GC-derived PBs.

During NMO relapses, for example, IgG-secreting $CD138^{+}HLA\text{-}DR^{+}$ PBs increase in the periphery, and PCs with shared clonal sequences become enriched within the CSF compartment (Chihara et al. 2013). The expression of CD138 on human BM PCs however is 8-fold higher than the level expressed on PCs in other niches, corroborating its high specificity for BM-resident PCs (Medina et al. 2002).

Studies of human antibody-secreting PCs indicate a gradient of successive PC maturation from the tonsil, to the blood, to the BM (Minges Wols 2005; Medina et al. 2002). Notably, BM LLPCs gain CD138, CXCR4, CD44, VLA-4, and importantly, they lose a more classical B cell phenotype (CD19, CD20, HLA-DR, CD22, CD45, CXCR5, CCR7, MHC Class II, PAX-5, and BCR). Moreover, they express Blimp-1, and survival factor Bcl-2 at much higher levels than non-BM PCs, indicative of their resistant, non-apoptotic phenotype. (Minges Wols 2005; Calame et al. 2003; Medina et al. 2002; Cocco et al. 2012)

CD20 is a pan B cell marker, and characteristically downregulated as PCs mature (Cocco et al. 2012). This stands particularly true in BM-derived LLPCs, which almost never (~2-3%) express CD20 (Halliley et al. 2015; Medina et al. 2002). In contrast, in NMO, an average of 60% of $CD19^{+}CD27^{++}CD38^{++}$ antibody-secreting cells expressed CD20, corroborating that CD20 expression correlates with a more immature PC developmental stage (Wilson et al. 2018). Selection for CD20 negativity is a sensible marker to use for cell isolation. CD19 however, is not so straightforward.

CD19 is a co-receptor of the BCR expressed on B cells, and is regulated by PAX5 (Mei et al. 2015). In the periphery, PBs have been defined as $CD27^{hi}CD38^{hi}CD180^{-}$, and either $CD19^{+}$ or $CD19intermediate^{(int)}$ (Chihara et al. 2011). Moreover, they are morphologically comparable to PCs except that they possess a larger nucleus, and only 40% of this population express CD138 – again, both factors compatible with a more immature state. Thus, loss of CD19 typically equates to a more differentiated, mature PC phenotype with longer lifespan. When contrasted within blood, spleen, tonsil and BM, human $CD19^{-}$ PCs are enriched within the BM compartment, but overall $CD19^{-}$ PCs are 2 times less frequent than $CD19^{+}$ PCs (33% versus 67%; (Mei et al. 2015). Classically, human BM-derived LLPCs have been defined as $CD19^{-}CD38^{hi}CD138^{+}$ (Halliley et al. 2015). In this case, high-throughput next generation sequencing

identified that genes of this PC subset exclusively encoded viral antigens such as measles and mumps with last exposure at vaccination 4 decades previously. Although unlikely, it should be considered that BM-derived LLPC phenotypes may differ if they are directed to viral antigens versus self-antigens. Additionally, it is important to note that $CD38^{hi}CD138^{hi}$ antigen-specific LLPCs in the human BM have been shown to be both CD19+ and CD19-, although the majority (~80%) are CD19+ (Brynjolfsson et al. 2017). Indeed, when considering their function, IgG secretion by $CD19^{-}CD38^{hi}CD138^{+}$ LLPCs is greater than that of $CD19^{+}CD38^{hi}CD138^{+}$ LLPCs; moreover, the $CD19^{-}$ subset appear morphologically more consistent with unique features of PCs (Halliley et al. 2015; Mei et al. 2015). Together, these data suggest that the $CD19^{-}CD38^{hi}CD138^{+}$ BM-derived PC subset is the truest, 'bona fide' BM-derived antibody-secreting LLPC.

For our cell sorting strategy, whilst isolation of $CD19^{-}$ PCs may yield the purest population, the disadvantage of this approach would be further limitation of cell numbers whilst attempting to isolate an already extremely rare cell population. Thus, whilst it may not be reasonable to include *both* $CD19^{+}$ and $CD19^{-}$ PC populations, a more balanced approach would be to isolate both '$CD19^{-}$' and 'CD19 low' $CD38^{hi}CD138^{+}$ BM PCs. In the event of low yield of 138^{+} cells from the BM however, it would be reasonable to proceed to isolate *all* $CD19^{+}$ and $CD19^{-}$ $CD138^{+}$ cells, since they have both been shown to be functional antibody-secreting BM-derived LLPC populations, and the $CD19^{+}$ cells would merely represent the 'youngest' cells of the population, yet to lose their CD19 expression. Indeed, one study found that both $CD19^{+}$ and $CD19^{-}$ PCs isolated from the BM of a patient with systemic lupus erythematous (SLE) secreted double-stranded DNA-specific IgG autoantibodies (Mei et al. 2015).

Expression of other cell surface markers may be useful in more accurately further defining the BM LLPCs. As outlined earlier, as B cells differentiate into PCs, they downregulate CXCR5 and thus exhibit reduced responsiveness to CXCL13 (a GC marker), and PCs upregulate CXCR4 and display increased CXCL12 sensitivity. CXCR4 (also known as cluster of differentiation 184; CD184) therefore plays a key role in the homing and retention of PCs in the BM, and as such is found on approximately 70% of human BM LLPCs (Halliley et al. 2015), compared to only ~12% expression on peripheral PBs (Chihara et al. 2013), thus may be a useful additional selection marker. In mice, the number of antigen-specific IgG-secreting PCs within the BM is

3-fold lower in $CXCR4^{-/-}$ versus CXCR4-expressing wild-type controls (Hargreaves et al. 2001). Additionally, a study contrasting expression levels on human tonsil, blood, and BM PCs showed increasing gradient of expression from tonsil, to blood, to BM (Medina et al. 2002) – consideration of such knowledge during our cell selection strategy may facilitate selection of true *bone marrow* LLPCs in the event of peripheral blood contamination of the BM sample.

Human leukocyte antigen (HLA)-DR is expressed by antigen-presenting cells such as B cells. HLA-DR is a marker of GC activity and hence highest in cells newly emerging from GCs such as PBs. Its expression is not restricted to B cells, however, and can be expressed on T lymphocytes also. Whilst most PBs (~80%) express HLA-DR, BM LLPCs almost never express HLA-DR (~1-8% of 138^{+} PCs), and loss of HLA-DR appears to be consistent with more mature LLPCs, and can allow distinction between peripheral PCs and BM LLPCs (Halliley et al. 2015; Mei et al. 2015; Cocco et al. 2012; Medina et al. 2002; Martinez-Murillo et al. 2016). Thus, the presence of CXCR4, and absence of HLA-DR and CD20 are likely reliable cell surface selection markers for identification of BM LLPCs.

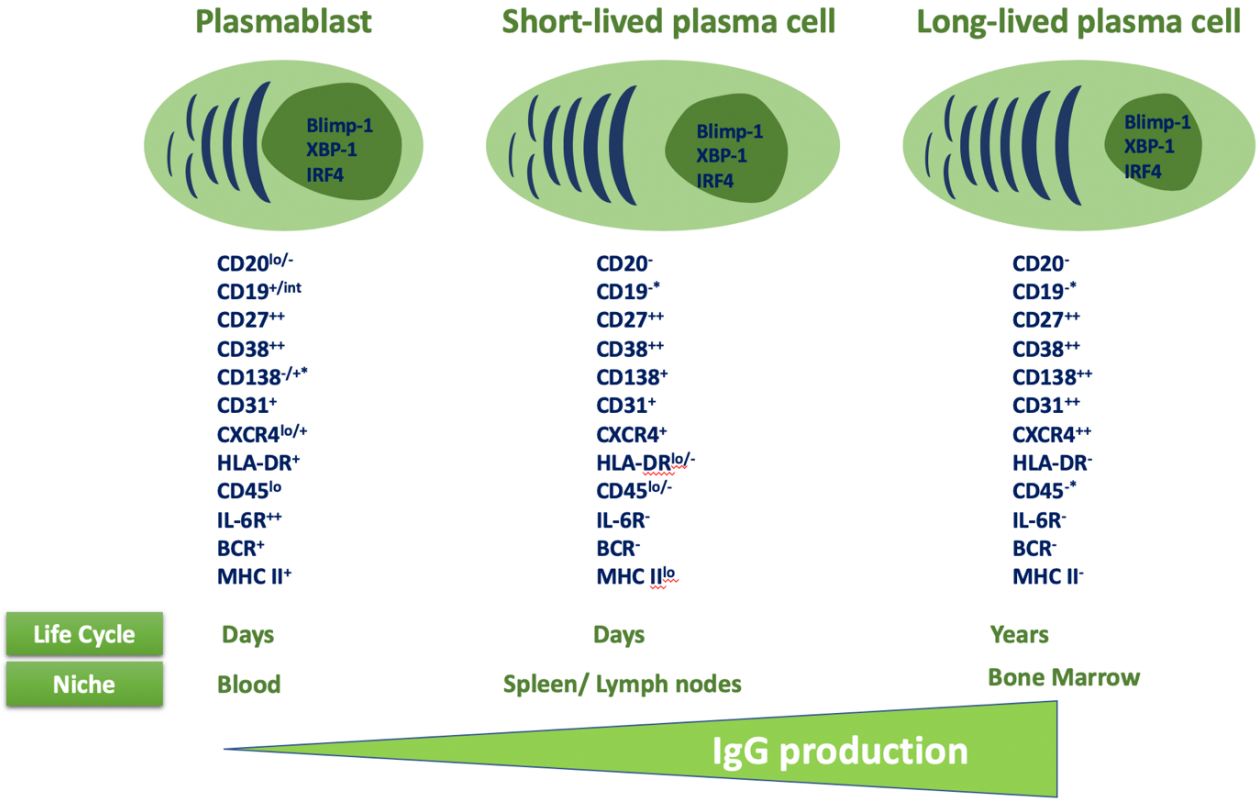
CD31, otherwise known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is expressed on most lymphocytes, monocytes, endothelial cells and platelets. CD31 is found at very high expression on PCs from human tonsil, blood, and BM, although BM PCs show 4-fold higher expression than non-BM PCs (Medina et al. 2002). Some propose that staining for both CD138 and CD31 effectively isolates BM LLPCs, distinguishing from short-lived peripheral PBs (Martinez-Murillo et al. 2016). In both humans and macaques, *double-positive* $CD138^{+}CD31^{+}$ ($CD20^{-}$) cells were defined as the *functional* antibody secreting PCs of the BM, from which most secreted antibodies were IgG (60%), followed by IgA (33%), and IgM (7%) (Martinez-Murillo et al. 2016). Interestingly, CD38, an important adhesion molecule expressed on PCs, binds the ligand CD31, which is likely to signify PC-endothelial cell interactions, rather than PC-PC interactions. (Calame et al. 2003)

The IL-6 receptor (IL-6R) is almost never (~3%) expressed on $CD138^{+}$ BM LLPCs, but highly expressed on peripheral blood PBs (Halliley et al. 2015; Chihara et al. 2011). B cell maturation antigen (BCMA), another receptor expressed on PCs, is thought to be important for PC homing and survival, although it is unclear whether this is more specific to LLPCs (Halliley et al. 2015).

Other PC surface markers include the adhesion molecules CD44 and VLA-4 (an α4β1 integrin dimer comprised of CD49d and CD29) which are believed to facilitate stromal cell contact within the BM (Minges Wols 2005; Calame et al. 2003). Whilst these molecules are likely to be more specific to BM-resident PCs, we cannot use them to accurately define LLPCs. In fact, one study showed similar expression of CD44 across human tonsillar, blood, and BM PCs, and of CD49d between blood and BM PCs (Medina et al. 2002); moreover, another found that CD49d stains the same BM LLPC population as CD31, thus this marker would be superfluous in a panel which already includes CD31 (Martinez-Murillo et al. 2016). These data indicate that neither of the adhesion molecules CD44 nor VLA-4 would be useful selection markers for our panel. Additionally, a constellation of other markers which could be used as negative selection tools for BM-derived LLPCs exist. These include CD24, a marker of early BM-emigrant B cells (Duperray et al. 1990) which would thus be an obvious negative marker for BM-derived LLPCs. However, with an already extensive flow cytometry panel, including powerful *positive* selection markers, use of such additional negative markers would appear unnecessary.

Finally, CD45 (or, leukocyte common antigen; or B220), is expressed on almost all nucleated haematopoietic cells except for erythrocytes (Nakano et al. 1990) and – importantly – many PCs. CD45 has been used in our group to exclude red blood cells (RBCs) from lymph node aspirates, which can be heavily contaminated with blood. This could be considered for our PC panel. However, CD45 expression gradually decreases with increasing PC maturity, with a clear reduction in BM PCs (Medina et al. 2002; Calame et al. 2003; Minges Wols 2005). Yet, despite this trend, the expression of CD45 on BM PCs can be heterogenous (Pellat-Deceunynck 2004; Medina et al. 2002). Whether removal of $CD45^-$ cells from BM aspirates would reliably exclude only RBCs, therefore, is unclear, as it is likely that some BM LLPCs may in fact be $CD45^-$. **Figure 1.7** summarises the above review of PC surface markers and how they vary according to PC maturity.

Figure 1.7 Plasma Cell Surface Marker Expression According to Developmental Stage



Illustrative depiction of PC morphological features and expression of cell surface markers and transcription factors according to developmental stage. As PCs mature, they gain an abundant cytoplasm, prominent rough endoplasmic reticulum, and their nuclei become small and condensed. During their transition from PBs to short-, and, ultimately, long-lived- PCs, PCs lose CD20, CD19, HLA-DR, CD45, MHC Class II, and the IL-6R and BCR. All PCs highly express CD27, CD38, CD138, CXCR4, and CD31, which become maximally upregulated in BM-resident LLPCs. Additionally, all PCs express BCMA, CD44, and VLA-4 (not shown).

The transcription factor PAX-5 is downregulated in PCs (not shown), whilst transcription factors Blimp-1, IRF4, and XBP-1 are upregulated, with increasing gradient of expression from PBs to BM LLPCs.

*Whilst this figure summarises *general* trends in cell surface markers, as outlined within the main text, some BM-LLPCs can be $CD19^{+}$, or $CD45^{+}$, and some PBs do not express CD138.

PC, plasma cell; PB, plasmablast; BM, bone marrow; LLPCs, long-lived plasma cell; BCMA, B cell maturation antigen; VLA-4, very late antigen-4 (an α4β1 integrin dimer comprised of CD49d and CD29); Blimp-1, B lymphocyte-induced maturation protein-1; XBP-1, X-box-binding-protein-1.

1.7.6 Plasma Cell Maintenance: The Importance of Cytokines

Here, we summarise the factors which govern PC survival, including cytokines, and neighbouring cells which contribute to their important microenvironment. We focus on maintenance of BM LLPCs, of relevance to **Chapter 5**, where the aim was to maintain human BM-derived LLPCs in ex-vivo cultures.

Interleukin-6 (IL-6)

Perhaps the best-known cytokine important for PC survival is IL-6, a 26kDa protein produced by antigen-presenting cells, including B cells (Dienz et al. 2009). IL-6 has been found to be markedly elevated in the serum and cerebrospinal fluid of patients with NMO (Chihara et al. 2011), and B cell culture supernatants from SLE patients compared to healthy controls (HCs) (Klashman et al. 1991; Kitani et al. 1992). Since the IL-6R is preferentially expressed on SLE B cells (Kitani et al. 1992), binding of IL-6 promotes their terminal differentiation to antibody-secreting PCs; moreover, IL-6 regulates RAG gene transcription in mature B cells, and some have proposed that its overexpression in autoimmune diseases may promote high-affinity autoantibody production (Hillion et al. 2007). Not only does IL-6 promote antibody production, but consequential binding of the γ-chain of immunoglobulin G receptors (FcγR) to macrophages and dendritic cells induces further synthesis of IL-6, triggering an inflammatory positive feedback loop (Maeda et al. 2010).

IL-6 promotes differentiation of B cells to PCs, likely through induction of IL-21 production by $CD4^+$ T cells, which subsequently acts on B cells (Kitani et al. 1992; Dienz et al. 2009). In vitro, IL-6 has been shown to enhance survival of human PBs and PCs (Jego et al. 2001; Cassese et al. 2003; Chihara et al. 2011; Jourdan et al. 2014). Additionally, and with relevance to our project aims, IL-6 induces antibody secretion by PBs and PCs (Rodriguez-Bayona et al. 2013; Minges Wols et al. 2007; Klashman et al. 1991; Kitani et al. 1992; Dienz et al. 2009; Ellyard et al. 2005; Levy et al. 1990), including specific AQP4-autoantibodies in NMO (Chihara et al. 2011; Wilson et al. 2018). Crucially, its effects appear to be markedly augmented (almost 7-fold) by the addition of other cytokines such as BAFF or APRIL (Jourdan et al. 2014). In mice, IL-6 double knockout resulted in inadequate immune response to pathogens, indicating its

crucial role in PC-driven antibody responses (Kopf et al. 1994). However, whilst responses were defective, PC numbers were adequate compared to control mice, indicating that IL-6 may not be essential for PC formation and survival, or that compensatory mechanisms exist (Kopf et al. 1994).

A Proliferation-Inducing Ligand (APRIL) and B Cell-Activating Factor (BAFF)

A proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF) are tumour necrosis factor (TNF)-ligand family cytokines thought to play key roles in PC survival. They act via three receptors: BAFF receptor (BAFF-R), B-cell maturation antigen (BMCA), and transmembrane activator and cyclophilin ligand interactor (TACI). Evaluation of the expression of these receptors across the various PC compartments reveals downregulation of BAFF-Rs in human PBs and PCs (including BM PCs), with the exception of tonsillar PCs, on which it is highly expressed (Ellyard et al. 2005; Rodriguez-Bayona et al. 2013; Chihara et al. 2011). BCMA, important for PC homing and survival, appears to be almost exclusively expressed by PCs, found in BM PCs, splenic PCs, tonsillar PCs and PBs, but not in B cells (O'Connor et al. 2004; Rodriguez-Bayona et al. 2013; Chihara et al. 2011; Benson et al. 2008). Compared to B cells, BM PCs express heightened levels of BCMA but low expression of TACI and BAFF-R (O'Connor et al. 2004). This highlights the critical role for BCMA in BM PC survival, further corroborated by observations of dramatic reductions (>80%) in BM LLPC survival in BCMA-/- mice compared to wild-type controls (O'Connor et al. 2004). Critically, APRIL has 1000-fold greater affinity for TACI than BAFF (Winter et al. 2012), and binds to BCMA with much higher affinity (nanomolar versus micromolar) than BAFF, which binds to the BAFF-R with 100-fold increased selectivity than BCMA (Cho et al. 2018). These observations suggest that APRIL may play a more central role than BAFF for LLPC survival, predominantly through binding BCMA and activation of signalling cascades such as NFκB, promoting LLPC survival and antibody production (Cho et al. 2018).

In disease states, patients with NMO have high levels of BAFF in the serum and CSF (Chihara et al. 2011), children with opsoclonus-myoclonus syndrome (OMS) have elevated CSF BAFF levels which correlate with disease severity, number of CSF oligoclonal bands, higher serum

chemokines such as CXCL13, and reduce by more than 60% with ACTH treatment (Pranzatelli et al. 2013), and MM patients have 5-fold higher serum levels of both BAFF and APRIL compared to HCs (Winter et al. 2012). These observations either directly or indirectly implicate BAFF and APRIL in PC-mediated diseases. Indeed, whilst some authors found that neither BAFF nor APRIL promote PB survival (Chihara et al. 2011), others report that BAFF increases survival of human PBs in culture, and augments IgG secretion by human BM PCs (Avery et al. 2003). Further, APRIL can preferentially maintain human ASCs in long-term cultures for up to 56 days, enhanced by hypoxic conditions, which interestingly mimic the BM microenvironment (Nguyen et al. 2019). Additionally, APRIL significantly increases survival of human PCs 6.5-fold when used in combination with IL-6 versus alone (Jourdan et al. 2014). BAFF similarly enhances IL-6-induced PC survival effects (Jourdan et al. 2014). Importantly, in BM-derived PC cultures, APRIL and IL-6 increase survival (Chu et al. 2011), and combinations of BAFF and IL-6 markedly enhance both survival and numbers of PCs (O'Connor et al. 2004). These effects are inhibited by addition of TACI-Ig (O'Connor et al. 2004; Chu et al. 2011; Benson et al. 2008), which blocks binding of TACI ligands APRIL and BAFF, highlighting the critical role of both BAFF and APRIL in BM PC survival. In fact, a key murine study demonstrated that *either* BAFF or APRIL in isolation can support BM LLPC survival in vivo, since APRIL knockout mice show BM PC numbers comparable to wild-type, and similarly BAFF blockade with BAFF-Ig in wild-type mice has no effect, but BAFF antagonism in APRIL$^{-/-}$ mice causes depletion of BM PCs (Benson et al. 2008). These studies highlight that BAFF and APRIL are key mediators of BM PC survival and longevity, yet they appear to be dispensable, since neutralization of both is required to deplete BM PCs in vivo. Finally, Mcl-1, an anti-apoptotic protein, is selectively upregulated by BAFF in BM PCs, but not B cells, suggesting its involvement in PC survival (O'Connor et al. 2004; Cocco et al. 2012).

1.7.7 The Plasma Cell Survival Niche: The Bone Marrow Microenvironment

Whilst PCs themselves operate their own mechanisms of survival, such as activated autophagy (Halliley et al. 2015), their environment also plays a fundamental role in sustaining their longevity. Within the BM, various cellular components interplay to support the

maintenance of LLPCs, including reticular stromal cells, and haematopoietic cells such as eosinophils and megakaryocytes, which secrete soluble factors that support PCs within a rich microenvironment.

Bone Marrow Reticular Stromal Cells and CXCL12

Reticular stromal cells found within the BM are thought to be critical for both the development and support of PCs (Minges Wols 2005; Calame et al. 2003). Mesenchymal stromal cells – to which reticular stromal cells belong – are a rare BM population (1:10 000 – 1:100 000), even when compared to BM PCs (1:1000) (Nguyen et al. 2019). Indeed, Minges Wols et al (Wols et al. 2002) showed that BM-derived PCs can survive and secrete antibody for 3 weeks in vitro when co-cultured with stromal cells, compared to only 7 days in those without. Moreover, upregulated IL-6 mRNA expression and IL-6 production was observed in PCs from co-cultures. Indeed, traditional schools of thought are that cell-cell contact is crucial for PC survival (Wols et al. 2002). However, others have shown that stromal cell support of human PCs is contact-independent (Cocco et al. 2012). Moreover, co-culture with stromal cells from IL-6 null mice resulted in diminished antibody levels, and addition of IL-6 to PCs cultured in the absence of stromal cells saw increased PC survival and recovery of antibody secretion (Wols et al. 2002), indicating that the stromal cell-secreted factors rather than cell contact may be more crucial for PC survival. Correspondingly, addition of merely human splenic stromal cell supernatants to cultures enhances antibody secretion by splenic PCs 3-fold due to IL-6 secreted by stromal cells (Ellyard et al. 2005). Others have found that PC cultures with stromal cell supernatants yield superior survival and antibody secretion by PCs compared to co-culture with stromal cells (Nguyen et al. 2019), and Jourdan et al (Jourdan et al. 2014) demonstrate a remarkable cell culture platform of human LLPCs capable of survival for several months in vitro without any contact cell support, functioning with high immunoglobulin secretion rates with merely cytokine supplementation. Together, these data demonstrate that cell-cell contact is not necessary for maintenance of BM-LLPCs ex-vivo. Interestingly, an in vitro differentiation system of human B cells to PCs saw comparable numbers of $CD38^{+}CD138^{+}$ PCs generated with or without direct mesenchymal stromal cell contact, however CD138 expression was much higher in the cells generated with direct contact (Bonnaure et al. 2016).

CXCL12 (also known as stromal cell-derived factor 1α; SDF-1α) is a stromal cell-derived factor key for both the retention and survival of PCs within the BM (Cassese et al. 2003). CXCL12-expressing cells are present uniformly throughout the BM, and are fibroblast-like reticular cells, since almost all express vascular cell adhesion molecule-1 (VCAM)-1. Thus, reticular stromal cells are $CXCL12^{+}(VCAM)\text{-}1^{+}$. Furthermore, almost all $CD138^{+}$ PCs within the BM are in contact with CXCL12-expressing cells, and numbers of antibody-secreting PCs drop dramatically in the BM of $CXCR4^{-}$ mice, indicating that PCs are maintained within the BM niche by CXCL12. (Tokoyoda et al. 2004) CXCR4 drives PC migration and sustenance within the BM, and any subtle changes in its expression can have dramatic effects. The human immunodeficiency warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome is associated with a gain-of-function mutation in CXCR4. A knock-in CXCR4 mouse model mimicking WHIM syndrome found that following immunisation, antigen-specific PCs were not detected in the BM, and specific antibody titres were not maintained (Biajoux et al. 2016). Additionally, CD28, expressed by most BM LLPCs, interacts with CD80 expressed on $CXCL12^{+}$ BM reticular cells, which promotes a CD28-driven LLPC survival signal. Indeed, loss of either CD28 or CD80 leads to dramatic and *selective* loss of the BM LLPC population (Rozanski et al. 2011). Through CD28-CD80/CD86 interactions, BM PCs induce IL-6 production by BM dendritic cells. In turn, IL-6 promotes antibody production (Rozanski et al. 2011). Finally, extracellular vesicles from mesenchymal stromal cells augment ASC survival and IgG secretion in vitro, and may also play a role in PC survival (Nguyen et al. 2018).

Bone Marrow Haematopoietic Cells

Megakaryocytes and eosinophils appear also to be important components of the BM niche, as in mice, they co-localize with LLPCs, and produce the important PC survival factors, APRIL and IL-6 (Winter et al. 2010; Chu et al. 2011); some have even proposed eosinophils as an attractive, novel therapeutic target in PC-mediated autoimmune diseases (Chu et al. 2011). Intriguingly, recent work has shown that T regulatory cells co-localize with PCs in the BM, both preventing PC death during immune stress such as systemic infection, but contrastingly, also regulating the number of PCs and limiting the PC pool through their expression of CTLA-4

(Zaretsky et al. 2017). This may serve as a novel and highly effective mechanism for future therapeutic targets.

Other Factors: Bone Marrow Fat Content and Hypoxia

Other factors, such as oxygen levels, also influence PC survival. Whilst hypoxia does not affect generation of PBs from B cells in culture (Bonnaure et al. 2016), hypoxia is pivotal to the long term maintenance of PCs (Nguyen et al. 2019), and is a prominent feature of the BM microenvironment. Additionally, high glucose uptake by LLPCs through their cell surface Glut1 is thought to enhance their survival, and is also required to glycosylate antibodies (Corcoran & Nutt 2016). In humans, red marrow comprises leucocytes, red cells, and platelets, and is haematopoietically active, whilst yellow marrow is comprised of fat. Over life, the red marrow is gradually replaced by yellow marrow, and even from early adulthood, more than half of the marrow is yellow, with haematopoiesis occurring largely in the ribs, humerus and femur (Brynjolfsson et al. 2018). Analogous to humans, adult rhesus macaques show enrichment of adipocytes in long bones such as the femur, and PCs appear to be surrounded by adipocytes (Hammarlund et al. 2017). In healthy humans, increased BM adipose tissue is associated with lower bone density at the same skeletal sites (Shen et al. 2012), presumed due to osteoclastic activity induced by adiponectin, produced by BM adipocytes (Richards et al. 2007). Additionally, osteoclasts support human PC survival via direct contact (Geffroy-Luseau et al. 2008). Together, these findings suggest that BM fat content may influence PC enrichment at specific sites and, or longevity through induction of osteoclastic activity.

Thus, the BM microenvironment, surrounding cells and secreted factors interplay to regulate the frequency and survival of PCs. The anti-apoptotic protein Mcl-1 (Peperzak et al. 2013), and transcription factors including ZBTB20, IRF4 and Blimp-1 are particularly important (Nutt et al. 2015). Finally, immunoglobulin itself plays a crucial role in BM LLPC survival through expression of the inhibitory FcγRIIb on LLPCs (Xiang et al. 2007).

1.8 Conclusions

This chapter has summarised key concepts regarding both clinical and immunological aspects of these novel autoantibody-mediated autoimmune encephalitic syndromes. These include the highly distinctive clinical syndromes associated with specific autoantibodies, and their preferential and striking responses to immunotherapy. Immunobiological concepts have been explored, with an outline of two key models of autoantibody production: ongoing short-lived, new GC-reactions, generating new autoantibodies, versus LLPC contribution, with continuous, long-term autoantibody secretion from the same LLPC pool, residing within the BM. The data suggest that both GC reactions and secretion by LLPCs occur synergistically within individual patients, contributing to the production and ongoing perpetuation of autoantibody production over the course of disease.

This thesis shall focus on study of both clinical and immunobiological aspects of these diseases, with particular focus on patients with LGI1- and CASPR2-autoantibodies. The overarching goals of this thesis are to expand the clinical phenotypes of these patients, and to examine the concepts of GC-reactions and LLPCs using biological samples. We shall employ use of serum, CSF, and BM, to explore potential contribution of GC-reactions versus BM-derived LLPCs to autoantibody production. By delineating mechanisms of autoantibody production by direct study of LLPCs, this may inform use of more targeted immunotherapies and improved clinical outcomes in these patients and other autoantibody-mediated conditions with LLPC reliance. We anticipate that the findings from this thesis will provide contemporary insights into clinical phenotypes and the underlying immunobiology, with ultimate aim of improving patient care and outcomes through enhanced clinician recognition of these conditions, earlier diagnosis, and more individualised, tailored immunotherapies.

CHAPTER 2. METHODS

METHODS 1. LIVE CELL-BASED ASSAYS

Table(s) 1. Reagents

HEK-293T CELL CULTURE MEDIUM (DMEM FBS 10%, 1% AA)	*Compound*	*Concentration*	*500 ml*
Sterile	DMEM (high glucose)		500 ml
	FBS	10%	50 ml
	Anti-Anti (100X)	1%	5 ml

CBA WASH BUFFER (DMEM HEPES)	*Compound*	*Concentration*	*500 ml*
	DMEM (high glucose)		500 ml
	HEPES		2.3 g

CBA SAMPLE BUFFER (DMEM HEPES, 1% BSA)	*Compound*	*Concentration*	*500 ml*
	DMEM (high glucose)		500 ml
	HEPES		2.3 g
	BSA	1%	5 g

CBA FIXATIVE SOLUTION (PBS, 4% FA)	*Compound*	*Concentration*	*100 ml*
Protected from light	PBS		88.9 ml
	Formaldehyde (36% w/v)	4%	11.1 ml

DAPI SOLUTION 1:1000	*Compound*	*Concentration*	*10 ml*
Protected from light	PBS		10 ml
	DAPI (1 mg/ml stock)	1 μg/ml	10 μl

Dulbecco's Modified Eagle's Medium (DMEM) – high glucose (4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate); Sigma-Aldrich, D6429
Fetal Bovine Serum (FBS), non-USA origin; Sigma-Aldrich, F7524
Anti-Anti (100X) Antibiotic-Antimycotic (Penicillin 10,000 U/ml, Streptomycin 10mg/ml, 25μg/ml Gibco Amphotericin B); Thermo Fisher Scientific, 15420-062
HEPES; Sigma-Aldrich, H3375
Bovine Serum Albumin (BSA) lyophilized powder ≥98%; Sigma-Aldrich, A3983
Dulbecco's Phosphate Buffered Saline (PBS); Life Technologies, 14190-144
Formaldehyde EM 36% w/v Solution; TAAB Laboratories, F003

Methods 1.1 Traditional 24-Well Live Cell-Based Assay Protocol

Sera or cerebrospinal fluid (CSF) samples were tested by live cell-based assay (CBA) as previously (Irani et al. 2010) for reactivity to full-length LGI1 or CASPR2 proteins. 13mm diameter glass coverslips were treated with sterile, filtered 0.01% poly-L-lysine (PLL) solution for one hour, using four glass coverslips in 3 ml PLL/ well in 6-well cell culture plates (clear, flat bottom TC-Treated; Corning® Costar®, 3516). HEK-293T cells were plated onto PLL-treated glass coverslips (4/well in 6-well plates) at a density of $0.75x10^6$/ well in 2ml HEK-239T cell culture medium **(Table(s) 1. Reagents)** and cultured at 37°C, 5% CO_2 for 24 hours.

On day 2, HEK-293T cells were transfected with membrane-bound enhanced green fluorescent protein (EGFP)-tagged LGI1 or CASPR2 cDNA, using 3 μg DNA, 50 μl unsupplemented DMEM **(Table(s) 1. Reagents)**, and 1.5 μl PEI per well. After 6 hours' incubation, the supernatant was removed and replenished with 2 ml/ well fresh HEK-239T cell culture medium, and cells were incubated for a further 18 hours (at 37°C, 5% CO_2) to allow expression of the relevant EGFP-tagged protein.

On day 3, coverslips were transferred to individual wells of 24-well plates cell culture plates (clear, flat bottom TC-Treated; Corning® Costar®, 3526), and live, transfected HEK-293T cells were incubated with samples [neat for CSF; sera 1:20 for LGI1 assay, 1:100 for CASPR2, diluted to a total volume of 200 μl/ well in CBA sample buffer **(Table(s) 1. Reagents)**] for 1 hour at room temperature. Next, supernatants were discarded, and plates were washed thrice in 200 μl/ well CBA wash buffer **(Table(s) 1. Reagents)**. Cells were fixed with 200 μl/ well CBA fixative solution **(Table(s) 1. Reagents)** for 10 minutes at room temperature, and washed three times as above. Next, cells were incubated with 200 μl/ well secondary antibody (donkey anti-human Fcγ-fragment specific whole IgG, Alexa Fluor® 594-conjugated; Jackson laboratories; 709-585-098), diluted to 1:750 in CBA sample buffer for 45 minutes at room temperature in the dark. Coverslips were washed twice with CBA wash buffer 200 μl/ well, followed by two washes with PBS 200 μl/ well, and mounted onto glass slides in fluorescent mounting medium (Dako) containing 4',6-diamidino-2-phenylindole (DAPI) 1:1000. Slides were stored at 4°C in the dark.

Where required, positive samples were quantified by serial titration to establish end-point dilution. Serial dilutions were prepared from the same initial serum sample, using thorough mixing, and replacement of pipette tips between each dilution point to avoid contamination by higher concentration sample.

Methods 1.2 Interpretation of Live Cell-Based Assays

Samples were visualised at 40x on a fluorescence microscope (Leica DM 2500), where cell-surface binding of red fluorescent-labelled secondary antibody was sought, with co-localization to the relevant EGFP-tagged LGI1 or CASPR2 construct. Samples were read blindly against known positive and negative controls. Samples were scored on a scale of 0-4 based on the intensity of the fluorescent cell-surface staining using an established scoring system used within our diagnostic testing laboratory for national and international clinical samples (where 0 = negative, 1 = low positive, 2-3 = positive, 4 = strongly positive). Images were captured at 40x with an Olympus camera using Qimaging QCapture Pro software (version 6.0.0.605). Representative images of live CBAs are shown in Chapter 1 **(Section 1.5.3; Figure 1.5D)**. GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses and data presentation.

METHODS 2. PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION

Table(s) 2. Reagents

PBS 1% BSA	***Compound***	***Concentration***	***500 ml***
Sterile	PBS		500 ml
	BSA (7.5% stock)	1% (v/v)	77 ml

FREEZING MEDIUM (FCS, 20% DMSO)	***Compound***	***Concentration***	***10 ml***
Sterile	FCS	80%	8 ml
	DMSO	20%	2 ml

Ficoll-Paque™ Premium (Ficoll) 1.078 g/ml; Cytiva, 17-5442-03

Fresh whole blood was collected into VACUETTE® NH sodium heparin tubes (Greiner Bio-One, 455051), approximately 8 ml/ tube, and mixed gently frequently. Blood was centrifuged (500g; acceleration 9/ deceleration 5; for 10 minutes at room temperature). Plasma was carefully removed without disturbing the underlying cell layer, and stored in aliquots for freezing. Following plasma separation, the remaining mononuclear blood cells were transferred into 50 ml Falcon centrifuge tubes (polypropylene; CentriStar™ Cap; Corning®, 430829) and diluted 1:1 with PBS 1% BSA **(Table(s) 2. Reagents)**, to a total volume of 35 ml per Falcon tube. Thus, ~17 ml of pelleted cells from typically 3 blood tubes with ~17 ml PBS 1% BSA per Falcon tube.

For Ficoll layering, a ratio of roughly 1:2 of Ficoll: diluted mononuclear blood cells was employed. 15 ml Ficoll was transferred to a 50 ml Falcon tube. 35 ml of well mixed, diluted mononuclear blood cells were carefully layered over the Ficoll, ensuring no mixing of the blood and Ficoll. Falcon tubes were centrifuged for 30 minutes at room temperature (400g; acceleration 3/ deceleration 0).

Next, in order to harvest the peripheral blood mononuclear cells (PBMCs), the 'buffy coat', or interphase between the Ficoll and PBS layer visualised post centrifugation, was carefully transferred to a 50 ml Falcon using a Pasteur pipette. Care was taken to avoid transfer of Ficoll. PBMCs were washed with PBS 1% BSA (typically diluting the PBMCs 1:1 to a total volume of 50 ml), centrifuging at 200g for 10 minutes at room temperature (acceleration 7/

deceleration 7). The supernatant was carefully aspirated, and the PBMC pellet was resuspended in 35 ml PBS 1% BSA, before washing as above. Following aspiration of supernatant from the second wash, PBMCs were resuspended in 10 ml PBS 1% BSA. Cells were counted prior to the third wash, as above. The supernatant was aspirated precisely, and PBMCs were resuspended in the desired medium at the desired volume. PMBCs were kept on ice until further use.

For this thesis, the majority of experiments were conducted using fresh PBMCs, thus at this stage, PBMCs were resuspended in B cell medium (BCM) 'R10' **(Tables 5.4.1 Reagents)**. Alternatively, for freezing, a PBMC concentration of 18-20x 10^6 cells/ ml / Cryovial tube was used. The isolated PBMC pellet was resuspended in BCM, and the cell suspension subsequently slowly diluted 1:1 with 20% freezing medium **(Table(s) 2. Reagents)**. 1 ml of cell suspension was transferred to each 1.8 ml Cryovial at the aforementioned concentration. PBMCs were transferred to -80°C in a Corning® CoolCell® freezing container, to allow for graduated freezing. After 24 hours, PBMC vials were transferred to a liquid nitrogen chamber for long-term cryopreservation.

METHODS 3. CLINICAL

Methods 3.1 Patient Recruitment

107 patients with serum LGI1 autoantibodies were recruited for clinical research between 2016 and 2019. Recruitment followed either direct referral to the Oxford Autoimmune Neurology Service, John Radcliffe Hospital, or notification via the national Association of British Neurologists Rare Disease Ascertainment and Recruitment (RaDaR) Surveillance Unit. Additionally, 218 patients with pharmacoresistant epilepsy (defined by ongoing seizures despite use of 2 or more anti-seizure medications), and 55 seizure-free epilepsy patients were recruited between 2017 and 2020. All patients gave written informed consent (Research Ethics Committee approval 16/YH/0013).

Methods 3.2 Clinical Assessments

Detailed clinical phenotyping of n=107 LGI1-autoantibody patients was performed with 2 experienced clinician colleagues. A structured, pre-defined set of clinical domains was completed by review of clinical records and facilitated by patient and spouse interview.

Interviews were conducted face-to-face during clinical review or via telephone, and consisted detailed history, with specific questions regarding prodromal triggers, gut microbiome, family history, new clinical features including hoarding, obsessive behaviours, emotionality, and subjective perceptions of 'percentage back to normal' ratings. Batteries of validated tests were conducted to measure cognition (Addenbrooke's Cognitive Examination – Revised; ACE-R), and mood (Hospital Anxiety and Depression Scale; HADS).

Functional level of disability was calculated by Modified Rankin Scale (MRS), drawn from patient clinical records and supplemented by clinical observations, and patient and spouse/ carer interviews. Clinician-rated 'percentage back to normal' ratings were calculated from information drawn from clinical records, clinical observations, and patient interviews.

Methods 3.3 Statistical Analyses

GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses and data presentation. All datasets were tested for Gaussian distribution using the Anderson-Darling, D'Agostino and Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov normality tests. All datasets were non-normally distributed, and were evaluated using non-parametric tests. Factors between groups were compared by Fisher's exact test (for example, tumour presence in relapsing versus non-relapsing patients, or presence of falls in FBDS versus non-FBDS patients). Mann-Whitney test was used for comparison of median pre- and post- outcome measure scores (for example MRS score at nadir versus latest follow-up). Where the pre- and post- outcome measures were compared for individual patients, Wilcoxon matched-pairs signed rank test was used. Significance threshold was set as $p<0.05$ throughout.

METHODS 4. GERMINAL CENTRES

Methods 4.2 CASPR2 IgM CBA Development and Optimization with Flow Cytometry Comparison

Methods 4.2.1 CASPR2 IgM Live CBA Development and Optimization

134 sera from the following groups were used: 52 sera from known CASPR2-IgG positive patients (n=25; 7 with longitudinal samples), 20 sera from LGI1-antibody positive patients (n=14), 24 sera from aquaporin-4 (AQP4) antibody-positive patients (n=24), and 38 healthy control (HC) sera (ethical approvals REC16/YH/0013 and REC16/SC/0224).

For the final optimized CBA and flow cytometry experiments, 3 of the original HC sera and 1 of the original CASPR2 sera were unavailable. Thus, 130 sera were used (51 from n=25 CASPR2 patients, 20 sera from n=14 LGI1 patients, 24 sera from n=24 AQP4 patients, and 35 HC sera).

For live CBA, methods were conducted as outlined previously **(Methods 1.1)**, with transfection of EGFP-tagged CASPR2 at 3 μg/well. For initial screening for CASPR2 IgM, sera were tested at 1:100 – that established for CASPR2 IgG assay – and by Alexa Fluor 594®-conjugated goat anti-human Fcμ-specific IgM secondary antibody (A21216; Invitrogen, Carlsbad, CA), at 1:500 dilution, as optimized for use with other antigens (Makuch et al. 2018), and positive samples were quantified by end-point dilution. Initial screening and optimization were performed with select sera. For optimization experiments, primary and secondary antibody dilution and incubation times, and temperature conditions were varied. For the initial experiment, LGI1 IgMs were also sought in LGI1-autoantibody patients, with serum diluted to 1:20 as established for the LGI1 IgG CBA. Depletion studies were not conducted, since the secondary antibody used was previously confirmed to have human-IgM specificity (Makuch et al. 2018).

Finally, to seek presence of CASPR2-IgMs, a large cohort of sera (n=130, outlined above) were tested blindly using the live, optimized CBA (with 2 separate assays on different days), and subsequently by flow cytometry. For flow cytometry, sera from 10 HCs determined as

negative for CASPR2-IgM on optimized CBA were also used (non-blinded), to determine cut-off.

Methods 4.2.2 CASPR2 IgM Quantification by Flow Cytometry

For flow cytometry, samples were incubated for 30 minutes (1:100 dilution) in 10^4 live HEK cells (50% untransfected; 50% stably-expressing CASPR2-EGFP cells). After washing, bound IgM was detected with mouse anti-human IgM Alexa Fluor® 647-conjugated secondary antibody (1:200 for 30 minutes, BioLegend 314536). For standardization of fluorescence intensity, beads with varying binding capacities (0-4) were used (Quantum™ Simply Cellular® anti-Mouse IgG, Bangs Laboratories Inc. BLI # 13359/ 815). As for HEK cells, 10^4 beads were labelled with secondary antibody (beads 1-4; blank beads 0 left unstained). For the final wash, samples, but not beads, were washed in 1:25000 DAPI (in FACS buffer).

CASPR2-IgMs were quantified as the median fluorescence intensity (MFI) of EGFP-expressing cells minus the MFI of cells which did not express EGFP (ΔMFI). For beads, the MFI of any bound AF-647 IgM (APC channel) was determined. Next, for each type of bead (0-4), the MFI was used to establish a logarithmic calibration curve based on the known standardized antibody binding capacity (ABC); (Bangs Laboratories, Inc. QuickCal v2.3). The ΔMFI of each sample was plotted against this standard curve, generating ΔABC values. Cut-off ΔABC for CASPR2 IgM positivity was set as the mean+6 standard deviations of 10 HCs.

A BD LSRII flow cytometer and FlowJo (v10.1r5; FlowJo, LLC, Ashland, OR) software was used for analysis. GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses and data presentation. Clinical data were correlated.

Methods 4.3 Serum IgG Depletion and Live CBA for LGI1-Specific IgM and IgA Antibodies

Methods 4.3.1 Serum IgG Depletion

For serum IgG depletion, beads (Protein G Sepharose®, 4 Fast Flow beads; 17-0618-01; GE Healthcare) were washed four times in PBS (800 rpm for 1 minute in a microcentrifuge) to eliminate ethanol preservative. In order to prevent bead damage, pipette tips were cut throughout. Beads were resuspended to their original volume in PBS. For each serum sample, beads were diluted 1:1 with serum and added to PBS in an Eppendorf. For the LGI1 CBA standard serum dilution of 1:20 **(Methods 1.1)**, 10 μl of serum was added to 190 μl PBS, with 10 μl of beads, to allow for a total diluted serum volume of 200 μl/ well post IgG depletion. Sera were rotated at 20 rpm for 18 hours at 4°C on a sample rotator (SB2 Tube Rotator, Stuart®). The following day, Eppendorfs were centrifuged (800 rpm for 2 minutes) in order to pellet the IgG-bead complexes. Supernatants, or IgG-depleted sera, were aspirated and transferred to clean Eppendorf tubes and stored at 4°C.

For each sample, enough serum was depleted for use for 5 CBAs (200 μl/ well), assuming repeat assays for LGI1-IgM and -IgA, and a final assay for either LGI1-IgG or titration of LGI1-IgM or -IgA positive samples.

Methods 4.3.2 Live CBA for LGI1-Specific IgM and IgA Antibodies

For the LGI1-IgM and -IgA antibody CBAs, 120 IgG-depleted sera were used: 70 LGI1-autoantibody sera from 30 patients, 30 HC sera, and 20 NMDA-R autoantibody sera as disease controls. Live CBAs were conducted as outlined previously **(Methods 1.)**, except for specific modifications to the primary and secondary antibody. First, samples were not diluted in CBA sample buffer, rather, 200 μl of IgG-depleted serum in PBS was used as the primary antibody (serum already pre-diluted to 1:20 in PBS).

All samples were tested for LGI1-IgG Fc on both non-depleted and IgG-depleted serum, to ensure depletion of LGI1 IgG in LGI1-autoantibody patients, and to ensure negativity in control samples (donkey anti-human Fcγ-fragment specific whole IgG, Alexa Fluor® 594-

conjugated; Jackson laboratories; 709-585-098; diluted to 1:750 in CBA sample buffer). All samples were tested for LGI1-specific IgM and IgA on both non-depleted and IgG-depleted serum (Alexa Fluor 594®-conjugated goat anti-human Fcμ specific IgM secondary antibody; A21216; Invitrogen, Carlsbad, CA; 1:500 dilution; Alexa Fluor 594®-conjugated goat anti-human α-chain specific IgA secondary antibody; Jackson laboratories; 109-585-011; 1:250 dilution, as optimized for other antigens by colleagues). Positive LGI1-IgM or -IgA samples were repeated and quantified by end-point dilution.

Methods 4.4 LGI1 Antibody Epitope Specificity Assessment by Live CBA

102 sera from 38 LGI1-antibody positive patients (6/38 with known additional CASPR2-antibodies; characterised by our centre as LGI1-antibody encephalitis; ethical approval REC16/YH/0013) were tested by live CBA as previously **(Methods 1.1)** for reactivity to full-length LGI1 protein, and its two deletion constructs: leucine-rich repeat (LRR) and epitempin (EPTP) repeat. 13 available paired CSF samples from 12 of the 38 patients were also analysed. End-point titrations were determined for each construct using serial dilutions, starting from 1:20 for sera, and neat for CSF. The same dilution mix was used for testing with each LGI1 construct. Next, sera from 9 HCs and 33 disease controls [DCs; 30 multiple sclerosis (MS), 1 NMDA-R antibody encephalitis, 1 AMPA-R antibody encephalitis, and 1 autoimmune peripheral and autonomic neuropathy secondary to immune checkpoint inhibitor; ethical approval REC16/YH/0013] were tested for reactivities to each of the three LGI1 constructs, and where positive, quantified with end-point titrations. Alexa Fluor 594®-conjugated donkey anti-human [IgG Fc-fragment] Fcγ-fragment specific secondary antibody (Jackson laboratories; 709-585-098) was used [1:750 dilution]. Of the 102 samples, 51 were also tested by postdoctoral researcher Dr Melanie Ramberger.

Samples were visualised as outlined in **Methods 1.2**, where cell-surface binding of red fluorescent-labelled secondary antibody was sought, with co-localization to the *relevant* EGFP-tagged LGI1 construct. Images were captured as previously. GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses and data presentation. Clinical data were collated and correlated.

Methods 4.5 CXCL13 ELISA

Serum and CSF samples were collected, centrifuged, and frozen immediately at -80°C in aliquots (ethical approval REC16/YH/0013). Except for validation experiments, aliquots were thawed once immediately prior to analysis. Healthy control CSF samples were acquired from the Neurology Department, Karolinska Institute, Sweden, under the collaborator cohort ethical clause (REC16/YH/0013).

CXCL13 concentrations were measured using the commercially available human CXCL13/BLC/BCA-1 Quantikine ELISA Kit (R&D Systems, DCX130) as per the manufacturer's instructions. Since CXCL13 is detectable in saliva, mask and gloves were worn throughout the procedure to prevent contamination. In brief, standards were prepared as external standard dilution from a stock solution of 5000 pg/ml recombinant human CXCL13 (892426). An initial 10-fold and subsequent 2-fold serial dilutions were prepared in calibrator diluent (RD6-41) to produce standards S1-S7 (500 pg/ml – 7.8 pg/ml). S8 (blank standard) was calibrator diluent only. Serum samples were diluted 1:2 in calibrator diluent, since this was optimized to fall within the concentration range of the standards; CSF samples were run neat and at 1:2 dilution. 100 μl assay diluent (RD1S) was added to each well of human BLC-BCA-1 microplates (892424), precoated with a monoclonal antibody specific for human CXCL13. 50 μl of sample or standard was added to wells; S1-8 were set up in duplicates. Plates were covered with an adhesive strip and incubated at room temperature for 2 hours. Plates were washed 4 times with 400 μl/well wash buffer (wash buffer concentrate 895003 diluted in deionized water), ensuring that microwells were emptied completely between washes, and that wells were not allowed to dry. Next, 200 μl human CXCL13 conjugate (892425) was added to wells, and plates were covered and incubated at room temperature. After 2 hours, plates were washed 4 times, and 200 μl/ well of Substrate solution (equal mix of Colour Reagents A and B; 895000 and 895001) was added. Plates were developed in the dark at room temperature for 30 minutes. The reaction was stopped by addition of 50 μl stop solution (2N H_2SO_4; 895032) to each well. A blue to yellow colour change was observed. The plate was gently tapped on the underside to ensure uniform distribution of the stop solution.

The optical density of wells was measured immediately on a FLUOstar® Omega ELISA microplate reader (BMG Labtech) at 450 nm, ensuring that the underside of the plate was cleaned prior to analysis. A standard curve was prepared from the mean absorbances of the standards minus the blank values using regression analysis. The CXCL13 concentration of the samples was determined using curve-fitting software in GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA); dilution factors were considered for final concentration values.

For validation studies, select samples were measured in duplicates, and intra- and inter-assay reliability was established. Thereafter, samples were measured in singular, owing to cost, and the considerable number of samples which were tested. Serum and CSF cut-off levels were determined as mean+3SD of the HC values. Samples which generated values higher than the highest standard were serially diluted 1:2, 1:4, 1:8 and measured by repeat assay. To determine whether freeze-thawing altered CXCL13 concentrations, 4 serum samples were tested across 10 serial freeze-thaw (FT) cycles on the same assay. Prospective sampling of n=4 serum samples was undertaken from n=4 LGI1-antibody patients. Each sample was divided into 2 aliquots and cryopreserved immediately at -80°C. 1 aliquot was thawed only immediately prior to use for the assay (1 FT cycle). The second vial was repeatedly freeze-thawed and aliquoted for 9 cycles in the same manner; the vial was thawed and allowed to reach room temperature for 30 minutes, Vortexed, and 30µl aliquoted into a new Eppendorf. Both vials were frozen at -80°C: the new aliquot was not thawed until the assay (2 FT cycles), and the original aliquot was serially thawed as above for 9 FT cycles. The subsequent FT cycle was undertaken ≥6 hours at -80°C. The 10 FT cycles for each serum sample were compared on the same assay.

Of 161 serum samples with matched CXCL13 and LGI1-antibody end-titre, 39 were titrated by clinical fellow Dr Alessandro Dinoto, 50 by postdoctoral researcher Dr Melanie Ramberger, and the remainder by Dr Sophia Michael.

GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses and data presentation. All datasets were tested for Gaussian distribution using the Anderson-Darling, D'Agostino and Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov normality tests.

Non-normally distributed datasets were evaluated using the non-parametric tests Mann-Whitney and Spearman's correlation.

METHODS 5. PLASMA CELLS

Methods 5.1 Substantial Ethics Amendment – 'Immune Factors in Neurological Diseases'

5.1.1 Preparatory Phase

A substantial amendment to our study ethics 'Immune Factors in Neurological Diseases' (IRAS project ID 195152; Ethics Ref 16/YH/0013) was required in order to pursue investigation of the bone marrow (BM) compartment of patients recruited under our study. At this point, in December 2017, a meeting was held with other members of our Oxford Autoimmune Neurology Group, to explore any other suggestions for ethics amendments to be submitted at the same time. Most suggestions were carried forward, except for suggestions for amendments to include the use of Apps, which was rejected.

Next, a face-to-face meeting was arranged in December 2017 with Dr Karen Melham, a Senior Clinical Research Support Manager from the Clinical Trials and Research Governance (CTRG) Department, University of Oxford. The proposed changes, feasibility, and necessary requirements for the study amendment were discussed.

5.1.2 Bone Marrow Aspiration Particulars

The Clinical Haematology Team, Churchill Hospital, Oxford University Hospitals, were approached in January 2018 for advice regarding potential feasibility of our research BM aspirations to be performed within their department. This was agreed by the Clinical Lead for Haematology, Dr Graham Collins, provided that their department would be reimbursed for the research samples, and that NHS patients with haematological conditions would take priority. Logistics and patient information, including contraindications and complications of BM aspiration, were discussed.

A/Prof Sarosh R Irani, Dr Sophia Michael, and Dr Karen Melham agreed that:

- BM aspiration would be offered to participants with autoantibody-mediated neurological conditions only, and only to patients adjudged to have capacity to

consent. Therefore, this procedure would not be offered to participants recruited via the consultee consent process.

- Control participants (healthy volunteers) and children would not be eligible for BM aspiration sampling.
- An experienced Haematologist would perform the BM aspirate, which would be taken from the hip bone, under local anaesthetic.
- Whilst BM aspiration has few major risks, the most notable risk is of major haemorrhage, which is extremely rare, occurring at a rate of <1 in 1000 cases. It was agreed that this would be included in the Protocol, and Participant Information Sheet. Additionally, we would supply our study participants with the patient information leaflet used by the Haematology Department to provide more detailed information to that to be outlined in the updated Participant Information Sheet.
- Patients would be advised to be familiar with their aftercare leaflet, and in the instance of any noted bleeding through their dressing site, to apply pressure and seek medical attention immediately.

5.1.3 Preparation of Study Documents

Between January-May 2018, preparation of documents was conducted. For each proposed change, the necessary alterations were made to the relevant study document(s). New documents were also drafted, to allow the recruitment of control participants ('Non-Autoimmune Participant' Information and Consent sheets). A summary of the proposed changes and their individual requirements are summarised in **Chapter 5, Table 5.1**.

With special consideration to the proposed study amendment pertaining to BM aspiration, the following changes were made to our study documents:

- A new 'Bone Marrow Aspiration' section was created within the Participant Information Sheet, including disadvantages and risks.
- The box 'I agree to potential bone marrow aspiration' was added to the Participant Consent Form under the 'Optional' section.

- The Protocol was updated to include complications under ‘Safety Reporting’ 9., and the procedure was included within ’Analysis of Outcome Measures’ 10.3. An Appendix D was created within the Protocol detailing the additional patient information leaflet to be offered to research participants considering undergoing BM aspiration as a research procedure.

5.1.4 Amendment Submission and Approvals

On 29/05/2018, the Substantial Amendment was formally submitted: all study documents, and the IRAS Notice of Substantial Amendment Form were submitted online via the Integrated Research Approval System (IRAS).

A favourable opinion to the amendment was granted by our Research Ethics Committee (REC), Yorkshire and The Humber/ Leeds East, on 22/06/2018. Health Research Authority (HRA) approval was granted on 28/06/2018.

Thereafter, in August 2018, the Oxford University Hospitals Financial Costings Team, was consulted for a cost estimate for each research BM aspiration. This was deemed to be £200 per sample and agreed with the Haematology Department. Local Oxford University Hospitals approval (including financial approval) was granted for BM aspirations on 16/10/2018.

The aforementioned revised and newly drafted study documents and approval letters can be found within Appendix 5.1-5.8, with sections relating to BM aspiration highlighted in Appendix 5.6 and 5.7. A summary of the changes made, and associated requirements, can be found within **Chapter 5 (Table 5.1)**, along with a flowsheet summarizing the above workflow process **(Figure 5.1)**.

Methods 5.2 Antibody Panel for Bone Marrow-Derived Long-Lived Plasma Cells

5.2.1 Optimization: Antibody Titration

Table 5.2.1 Reagents

FACS BUFFER	*Compound*	*Concentration*	*500 ml*
Sterile	DPBS		500 ml
	FCS	2%	10 ml
	HEPES 1M (sterile)	10mM	5 ml
	Sodium Azide 10% stock	0.05%	2.5 ml

Table 5.2.2 Antibody Details

Fluorochrome	**Anti-Human Antibody**	**Species**	**Clone**	**Company**
AF488	CD31	mouse IgG1-κ	WM59	BioLegend
APC	CXCR4	mouse IgG2a-κ	12G5	BioLegend
AF700	CD20	mouse IgG2b-κ	2H7	BioLegend
APC-Cy7	CD45	mouse IgG1-κ	2D1	BioLegend
PacB	CD14	mouse IgG1-κ	63D3	BioLegend
BV510	CD3 CD56	mouse IgG1-κ mouse IgG1-κ	UCHT1 5.1H11	BioLegend
BV605	CD38	mouse IgG1-κ	HIT2	BioLegend
BV650	HLA-DR	mouse IgG2a-κ	L243	BioLegend
BV711	CD27	mouse IgG1-κ	O323	BioLegend
BV785	CD19	mouse IgG1-κ	SJ25C1	BioLegend
PE	ER-Tracker™	-	-	ThermoFisher
PE-Cy7	CD138	Mouse IgG1-κ	MI15	BioLegend

Details of the 13 antibodies titrated are listed, along with their fluorochrome, species, clone, and company. All antibodies are anti-human. ER-Tracker™ Red stain is comprised of BODIPY™ TR and Glibenclamide.

PBMCs were prepared from 50 ml venous blood from a 32-year-old male HC (as per **Methods 2.)**. $41x10^6$ PBMCs were obtained, pelleted, and resuspended in FACS buffer to a concentration of $5x10^6$ cells/ml. $0.5x10^6$ PBMCs (100µL of cell suspension) were plated in each of 66 wells of a sterile 96-well U bottom plate. Serial dilutions were prepared for the 13 antibodies (listed in **Table 5.2.2**) with FACS buffer in a sterile 96-well U bottom plate. Each antibody was titrated with 5 dilutions each (1:25, 1:50, 1:100, 1:200, 1:400) to a total volume of 50 µL, with exception of the ER tracker, which was diluted to 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, as per ranges within manufacturer recommendations. The cells in the plate were pelleted (centrifuged at 2000rpm; acceleration 9/deceleration 9 for 1 minute at 4°C), and the supernatant aspirated. The antibody mixes were added to the cells (50µL/ well), and 50µL FACS buffer only was added to 1 well (for unstained cells). The cells were stained with the antibodies in the dark on ice at 4°C for 30 mins, then washed with 150µL/ well FACS buffer (centrifuged at 2000rpm; acceleration 9/deceleration 9 for 1 minute at 4°C). The supernatant was aspirated, the cells resuspended in 200µL/ well FACS buffer, transferred to cluster tubes, and maintained in the dark on ice at 4°C. Samples were run on a BD™ LSR II Flow Cytometer Machine (BD FACSDiva™ software) and analysed using FlowJo™ v10.5.3. For each data file, the events were increased to >200000 live events, since we aimed to stain antibody secreting plasma cells, a rare population, with the markers of interest.

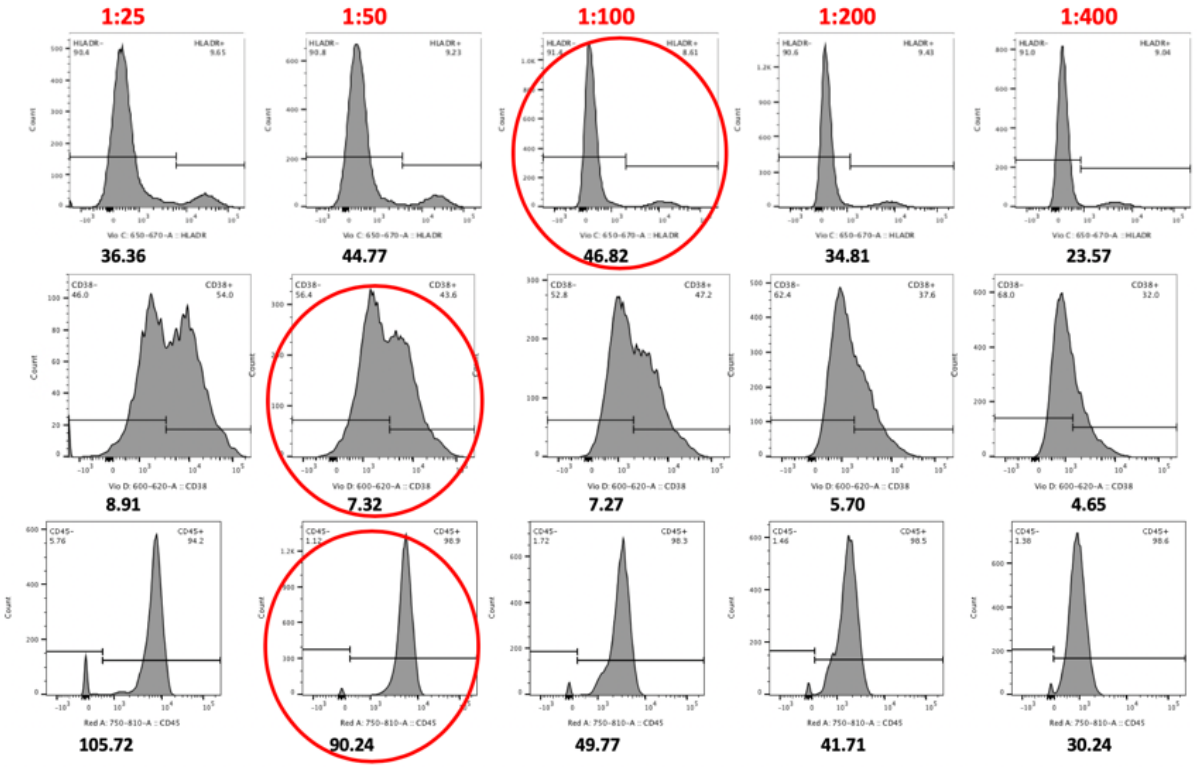
For each antibody, the Separation Index (SI) was calculated for each of the 5 dilutions using the following formula:

Separation Index = [(med Ab+) – (med Ab-)] / SD (Ab-)

For each antibody, the dilution which produced the largest separation index was the optimum dilution, since this gave the maximum separation of the positive and negative populations. This was not always used, however, and there are a few exceptions based on the appearance of the flow cytometry plots (**Figure 5.2.1**). For example, when staining the peripheral blood, only few cells (~9%) stained positively for HLA-DR. This is expected, since the proportion of cells in the peripheral circulation having recently exited GCs and hence acquiring HLA-DR would be memory B cells and/ or plasmablasts – a rare, and short-lived population. The SI with the highest value (46.8) was found for 1:100 dilution, and it is clear that the positive and

negative populations look most distinct and separated at this dilution compared to the others. Hence, 1:100 was selected as the optimum dilution at which the HLA-DR antibody should be used. For CD38, although the highest SI was for 1:25 dilution, separation of both negative and positive populations looked slightly better at 1:50 with a negligible difference in SI, and it was also considered more cost-effective to use slightly less antibody. Moreover, too much antibody (i.e. at 1:25 dilution) may increase non-specific binding, increasing background from the negative population. Thus, use of the CD38 antibody at 1:50 dilution would improve the specificity of the staining also. For CD45, it is clear that most cells (~98%) in the peripheral blood are $CD45^{+}$. As for CD38, similar considerations were made; whilst the highest SI was found for 1:25, the optimum dilution chosen was in fact 1:50, since a slightly better separation of the positive and negative populations was seen at 1:50, with a positive peak still clear and as high as 10^4 on the x-axis.

Figure 5.2.1 Separation Index and Flow Cytometry Plot Examples for Antibody Titrations



Separation Index (SI) and representative flow cytometry plots for antibody titrations. Example plots shown for HLA-DR (top panel), CD38 (middle panel), and CD45 (bottom panel), with increasing dilutions shown from 1:25 to 1:400 in red (left to right). SI for each antibody at respective titration shown in black below each plot. The optimum dilution selected for each of these antibodies is circled in red.

Results for the optimized dilutions of each antibody are shown in **Table 5.2.3**. The ER tracker™ stain failed as no staining was seen on flow cytometry in the expected in Blue/Green A laser (excitation 587nm), despite duplication of the experiment, hence this marker was removed from the panel. Whilst initially the titration experiments were run on a BD™ LSR II Flow Cytometer, for logistical reasons, all experiments thereafter were performed on a BD FACSAria™ Fusion Cell Sorter (Becton Dickinson, San Jose, CA, USA). The respective lasers and filters for both machines are therefore included for the antibody panel in **Table 5.2.3**.

Table 5.2.3 Optimized Antibody Dilutions for Plasma Cell Flow Antibody Panel

LSR II Laser and Filter Parameter	Fusion II Laser and Filter Parameter	Fluorochrome	Antibody	Optimized Dilution
Blue B 488-530/30	B 488-530/30	**AF488**	CD31	1:400
Red C 639-670/30	R 640-670/30	**APC**	CXCR4	1:50
Red B 639-730/45	R 640-730/45	**AF700**	CD20	1:200
Red A 639-780/60	R 640-780/60	**APC-Cy7**	CD45	1:50
Vio H 405-450/50	V 405-450/40	**PacB**	DapiCD14	1:100
Vio G 405-525/50	V 405-525/50	**BV510**	CD3/ CD56	1:50/ 1:200
Vio D 405-610/20	V 405-610/20	**BV605**	CD38	1:50
Vio C 405-670/30	V 405-660/20	**BV650**	HLA-DR	1:100
Vio B 405-710/50	V 405-710/50	**BV711**	CD27	1:50
Vio A 405-780/60	V 405-780/60	**BV785**	CD19	1:100
B-Gr A YG 561-586/15	*N/A*	***PE***	*ER-Tracker™*	*N/A*
Blue YG 561-780/60	YG 560-780/60	**PE-Cy7**	CD138	1:50

Antibody panel developed for isolation of BM-derived LLPCs by flow cytometry. The optimized dilutions for each antibody, based on several titrations, is listed.
The 13 marker/ 11 colour antibody panel is shown (ER-Tracker™ removed as failed), along with the fluorochrome of each antibody and their relative laser and emission filter for the BD™ LSR II and FACSAria™ Fusion flow cytometers.
B, blue; R, red; V/ Vio, violet; B-Gr, Blue-Green; YG, yellow-green. Specified laser excitation wavelengths are in nm.

Methods 5.3 Participants

One healthy control (HC), two disease controls (DCs; one with MG, and one with MS), and two patients with LGI1-antibody encephalitis from the Oxford Autoimmune Neurology Specialist Clinic were selected. Blood was obtained from the HC, thymic tissue from the patient with MG, and BM aspirate from the patients with MS, and LGI1-antibody encephalitis. Full written informed consent was obtained using the updated 'Immune Factors in Neurological Diseases' documents, and the work was performed under Research ethics committee approval 16/YH/0013. Clinical datasets, including patient demographics, serum LGI1-IgG levels, disease onset, and medications, were extracted from case notes. Additional steps required for booking participants for BM aspiration are outlined in **Figure 5.3.1**. A copy of the blue BM referral form can be found within **Appendix 5.9**. Whilst Dr Michael had undertaken BM Aspirate/ Trephine competence training along with Haematology Specialist Registrars at the Churchill Hospital, Oxford University Hospitals (led by Specialist BM Biopsy Nurse Christina Everett, 09/02/2018), it was deemed appropriate for Haematology Specialist Registrars to undertake the research procedures, given their expertise and familiarity with the procedure, and that only a select few participants would be required for this project.

Figure 5.3.1 BM Aspirate Booking Protocol

Autoimmune Encephalitis Research Bone Marrow Aspirate Booking Procedure

Agreements made between Oxford Autoimmune Neurology Group and Haematology Dept., Churchill Hospital after participants consent for BM aspirate under 'Immune Factors in Neurological Diseases':

Booking

- Approximately 10 autoimmune encephalitis patients will be selected for BM aspiration only
- Commence date: summer 2019
- Dr Sophia Michael/ team will select patients, specifically those not on anticoagulation or antiplatelet therapies (with the exception that aspirin is permitted as per British Society for Haematology Guidelines (Keeling et al. 2016)
- Sophia to complete blue BM referral form with patient's details, as done for NHS patients, indicating clearly that this is a research BM aspirate patient, and e-mail a scanned copy to Lynn McNulty, BM Biopsy and Chemotherapy Scheduler, along with a call/ e-mail request, for potential dates **(BM lists are Mon-Thurs - 5 slots 09:15, 10:00, 10:45, 11:30, 12:15)**. NHS patients have priority over research patients.
- Lynn will book an appointment and send an appointment letter via post to the patient, along with a BM information leaflet (the patient will have already seen this during the consent process for BM aspiration under Immune Factors in Neurological Diseases).
- One week prior to the procedure, the participant shall be instructed to attend their local GP practice for blood tests (FBC and coagulation studies). Miss Pip Doling (Deputy Haematology Sister Responsible for BM Lists) or Dr Sophia Michael shall contact the participant's local hospital to ensure that the studies are normal prior to the research appointment.

Procedure

- 1 research patient at a time
- Participants should ensure that they eat breakfast – Sophia will relay this.
- Ethics permits BM aspiration only (not BM biopsy)
- All research participants will undergo same consent process for BM aspiration as NHS patients (procedure information and written consent)
- BM aspirate will be performed by Haematology SpR covering list on that particular day (i.e. no guarantee of consistency of a particular SpR for all research participants)
- Dr Sophia Michael will be present throughout the procedure, and will collect the sample obtained for research
- Should this be required, Entonox and intravenous midazolam use deemed safe by Dr Sophia Michael and Professor Sarosh Irani – no contraindication in autoimmune encephalitis; Entonox required in addition to lidocaine in ~10%
- In case of post procedure bleeding, patients **should not drive** themselves to their BM aspirate appointment (this is particularly relevant for our patients, who may be travelling far) - all patients to be driven to Oxford/ accompanied.
- Emergency cover - all patients undergoing BM aspirate are given post procedure care information regarding checking of dressings, and in the event of bleeding/ other complications, to contact the Oxford Triage service. If deemed necessary, the Triage service would then advise attendance at the local Accident and Emergency Department, or GP Surgery. The same applies to our research participants, even if out-of-area.

Research Costs

The Haematology team shall invoice the Oxford Autoimmune Neurology Group, Nuffield Dept. of Clinical Neurosciences, University of Oxford, on a 6-monthly basis for all BM aspirates undertaken for research purposes (at £200 per sample).

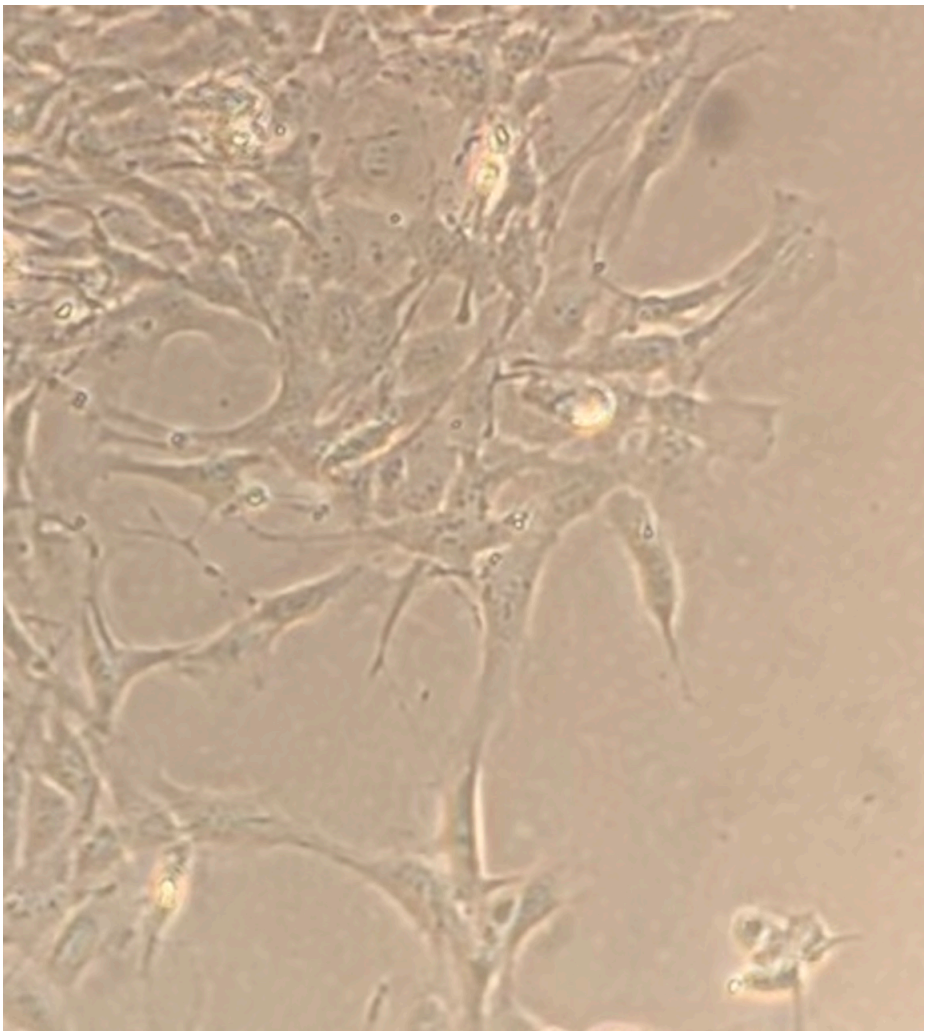
Booking protocol for autoimmune encephalitis participants undergoing BM aspiration. **Figure 5.3.1** summarises the additional procedures required following consent under 'Immune Factors in Neurological Diseases', in order to schedule and prepare a participant for BM aspiration. The protocol was devised by Dr Sophia Michael following a meeting with Miss Pip Doling and Miss Lynn McNulty, and outlines the agreements made between the Oxford Autoimmune Neurology Group, and Haematology Department, Churchill Hospital, for smooth and safe coordination of NHS patient care alongside research patient care.

5.4 Cell Culture Cytokine Mixes

5.4.1 M2-10B4 BM Mesenchymal Stromal Cells

PCs were cultured in the presence or absence of media enriched with BM mesenchymal stromal cell secretome; use of the cells as feeder cells directly within PC cultures was not possible due to the lack of availability of the radiation facility at the time of PC experiments. M2-10B4 mus musculus (mouse) BM mesenchymal stromal cells (gifted from Professor R. Tooze, Leeds) were cultured in IMDM (GlutaMAX™, Phenol Red, 25mM HEPES; Life Technologies Ltd, 31980048) with 10% FCS (Sigma-Aldrich, non-USA origin, F7524) and 1% AA (Penicillin, Streptomycin, Amphotericin B; Life Technologies Ltd, 15240062). Characteristic appearances of M2-10B4 cells are shown in **Figure 5.4.1**. For preparation of enriched media to be used for PC cultures, only M2-10B4 cells at third or fourth passage (P3-P4) were used. M2-10B4 cells were washed, resuspended at $1x10^6$ cells per 12 ml B cell medium (BCM) 'R10' for each plate, and seeded as 2 ml (~$0.2x10^6$ cells)/ well in flat 6-well cell culture plates (Corning® Costar®, CLS3516); constituents of BCM 'R10' are listed in **Table(s) 5.4.1**. On day 3, the enriched BCM (containing M2-10B4 secretome) was collected and frozen at -20°C in sterile Universal containers until use within cytokine mixes for PC cultures.

Figure 5.4.1 M2-10B4 Mesenchymal Stromal Cells



Appearance of M2-10B4 mouse-derived BM mesenchymal stromal cells. Characteristic cell morphology including long fibroblast projections can be seen. Image taken at x100 magnification.

Tables 5.4.1 Reagents

BCM 'R10'	*Compound*	*Concentration*	*500 ml*
Sterile	RPMI (w/o phenol red)		500 ml
	FBS	10%	50 ml
	Penicillin-Streptomycin	1%	5 ml
	GlutaMAX™	1%	5 ml
	HEPES	1%	5 ml
	MEM-NEA	1%	5 ml
	Sodium pyruvate	1%	5 ml
	β-Mercaptoethanol	0.1%	0.5 ml

FACS BUFFER FOR CELL SORTING (PBS 0.1% BSA)	*Compound*	*Concentration*	*500 ml*
Sterile	PBS		493.3 ml
	BSA (7.5% stock)	0.1% (v/v)	6.7 ml

WASH BUFFER (PBS 2% FBS)	*Compound*	*Concentration*	*500 ml*
Sterile	PBS		500 ml
	FBS	2%	10 ml

DAPI SOLUTION 1:1000	*Compound*	*Concentration*	*10 ml*
Sterile; filtered; protected from light	FACS buffer		10 ml
	DAPI (1 mg/ml stock)	1 µg/ml	10 µl

RPMI medium 1640 (1X; - L-glutamine, - phenol red); Thermo Fisher Scientific, 32404-014
Fetal Bovine Serum (FBS); Life Technologies Ltd, 10270-098
Penicillin-Streptomycin (10,000 U/ml/ 10mg/ml); Thermo Fisher Scientific, 15140-122
GlutaMAX™ (100X); Life Technologies Ltd, 35050-061
HEPES (1M); Thermo Fisher Scientific, 15630-080
MEM Non-Essential Amino Acids Solution 100X; Life Technologies Ltd, 11140-035
Sodium Pyruvate (100 mM; 100X); Life Technologies, 11360-070
β-mercaptoethanol (BME); 1:280 dilution in PBS
Dulbecco's Phosphate Buffered Saline (PBS); Life Technologies, 14190-144
Bovine Serum Albumin (BSA); Sigma-Aldrich, A8412

5.4.2 Cytokine Matrix for Cell Culture

Cytokines were reconstituted in sterile FACS buffer **(Table(s) 5.4.1)**. Recombinant human IL-6 (R&D Systems, 206-IL-010) and recombinant human CXCL12/SDF-1α (R&D Systems, 350-NS-050) were reconstituted to 10 µg/ml stock solution; recombinant human APRIL (PeproTech, 310-10C), and recombinant human BAFF (PeproTech, 310-13) were reconstituted to stock concentrations of 100 µg/ml.

For optimization experiments, the cytokine matrix contained 36 different cytokine conditions, divided into 4 main cytokine matrix groups, as shown in **Figure 5.4.2**. Each of the individual 36 cytokine mixes was made up in BCM in sterile 1.5 ml Eppendorf Tubes® as per the following tables within **Table 5.4.2**. For each condition, 100 µl of cytokine mix was transferred from the Eppendorf Tubes to 96-well cell culture plates as per the plate layouts in **Table 5.4.2**

(96-well round bottom cell culture plates with lid; tissue culture treated, non-pyrogenic, polystyrene; Corning Costar®; ref 3799). The eventual optimized cytokine condition for BM LLPC cultures was IL-6 10 ng/ml, CXCL12 10ng/ml, APRIL 200 ng/ml, and BAFF 100 ng/ml (**Table 5.4.2; pink Table 'IL-6 plus CXCL12 plus APRIL plus BAFF'; Eppendorf 5**). For each plate, 6 ml of 1x concentrated cytokine mix was prepared (since PCs were sorted into 60 wells of each plate, with a total volume of 100 µl/well). For plates with M2-10B4 secretome, 3 ml of 2x concentrated cytokine mix was prepared, using 50 µl/well cytokine mix plus 50 µl/well BCM enriched with M2-10B4 secretome.

Figure 5.4.2 Cytokine Matrix Plate Layout for PC Cultures

Plate 1 or 2	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		IL-6 100	IL-6 100 CXCL12 10	IL-6 100 CXCL12 100	IL-6 100 APRIL 200	IL-6 100 CXCL12 10 APRIL 200	IL-6 100 CXCL12 100 APRIL 200	IL-6 100 BAFF 100	IL-6 100 CXCL12 10 BAFF 100	IL-6 100 CXCL12 100 BAFF 100		
C		IL-6 100	IL-6 100 CXCL12 10	IL-6 100 CXCL12 100	IL-6 100 APRIL 200	IL-6 100 CXCL12 10 APRIL 200	IL-6 100 CXCL12 100 APRIL 200	IL-6 100 BAFF 100	IL-6 100 CXCL12 10 BAFF 100	IL-6 100 CXCL12 100 BAFF 100		
D		IL-6 10	IL-6 10 CXCL12 10	IL-6 10 CXCL12 100	IL-6 10 APRIL 200	IL-6 10 CXCL12 10 APRIL 200	IL-6 10 CXCL12 100 APRIL 200	IL-6 10 BAFF 100	IL-6 10 CXCL12 10 BAFF 100	IL-6 10 CXCL12 100 BAFF 100		
E		IL-6 10	IL-6 10 CXCL12 10	IL-6 10 CXCL12 100	IL-6 10 APRIL 200	IL-6 10 CXCL12 10 APRIL 200	IL-6 10 CXCL12 100 APRIL 200	IL-6 10 BAFF 100	IL-6 10 CXCL12 10 BAFF 100	IL-6 10 CXCL12 100 BAFF 100		
F		Blank - BCM	CXCL12 10	CXCL12 100	APRIL 200	CXCL12 10 APRIL 200	CXCL12 100 APRIL 200	BAFF 100	CXCL12 10 BAFF 100	CXCL12 100 BAFF 100		
G		Blank - BCM	CXCL12 10	CXCL12 100	APRIL 200	CXCL12 10 APRIL 200	CXCL12 100 APRIL 200	BAFF 100	CXCL12 10 BAFF 100	CXCL12 100 BAFF 100		
H												

Plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A		No M2-10B4 secretome			Cytokines plus M2-10B4 secretome							
B		IL-6 100 APRIL 200 BAFF 100	IL-6 100 CXCL12 10 APRIL 200 BAFF 100	IL-6 100 CXCL12 100 APRIL 200 BAFF 100	IL-6 100 APRIL 200 BAFF 100	IL-6 100 CXCL12 10 APRIL 200 BAFF 100	IL-6 100 CXCL12 100 APRIL 200 BAFF 100					
C		IL-6 100 APRIL 200 BAFF 100	IL-6 100 CXCL12 10 APRIL 200 BAFF 100	IL-6 100 CXCL12 100 APRIL 200 BAFF 100	IL-6 100 APRIL 200 BAFF 100	IL-6 100 CXCL12 10 APRIL 200 BAFF 100	IL-6 100 CXCL12 100 APRIL 200 BAFF 100					
D		IL-6 10 APRIL 200 BAFF 100	IL-6 10 CXCL12 10 APRIL 200 BAFF 100	IL-6 10 CXCL12 100 APRIL 200 BAFF 100	IL-6 10 APRIL 200 BAFF 100	IL-6 10 CXCL12 10 APRIL 200 BAFF 100	IL-6 10 CXCL12 100 APRIL 200 BAFF 100					
E		IL-6 10 APRIL 200 BAFF 100	IL-6 10 CXCL12 10 APRIL 200 BAFF 100	IL-6 10 CXCL12 100 APRIL 200 BAFF 100	IL-6 10 APRIL 200 BAFF 100	IL-6 10 CXCL12 10 APRIL 200 BAFF 100	IL-6 10 CXCL12 100 APRIL 200 BAFF 100					
F		APRIL 200 BAFF 100	CXCL12 10 APRIL 200 BAFF 100	CXCL12 100 APRIL 200 BAFF 100	APRIL 200 BAFF 100	CXCL12 10 APRIL 200 BAFF 100	CXCL12 100 APRIL 200 BAFF 100					
G		APRIL 200 BAFF 100	CXCL12 10 APRIL 200 BAFF 100	CXCL12 100 APRIL 200 BAFF 100	APRIL 200 BAFF 100	CXCL12 10 APRIL 200 BAFF 100	CXCL12 100 APRIL 200 BAFF 100					
H												

The plate layout for the 36-condition cytokine matrix is shown across 2 plates divided into the 4 main groups: IL-6 and CXCL12 group (yellow), IL-6, CXCL12 and APRIL group (green), IL-6, CXC12 and BAFF group (blue), and IL-6, CXCL12, APRIL and BAFF group (pink). Each group contains 9 cytokine combinations, each repeated in wells directly below. The concentrations of cytokines displayed are in ng/ml. Note the plate layout design which includes a free perimeter of wells (grey) around the wells containing cytokine mixes for PC cultures, in order to avoid any effects of evaporation and thus changes to cytokine concentrations during the cell culture process. No PCs were sorted into these grey wells, which were filled with BCM only. Finally, for experiments where BCM containing M2-10B4 secretome was used in the cultures, plate 1 was set up as shown, and plate 2 was prepared in the same manner but with the addition of the M2-10B4 secretome to a half-volume, twice concentrated cytokine mix. Plate 3 was prepared as shown. For experiments where the M2-10B4 secretome was not used, only Plate 1, and Plate 3 (only the left half of the plate marked 'No M2-10B4 secretome' was prepared; wells B2-G4).

Table 5.4.2 Cytokine Matrix Culture Mixes

IL-6 plus CXCL12	Condition (ng/ml)	Standard 1x Concentrations			2x Concentrated		
		IL-6 (µl)	CXCL12 (µl)	BCM (µl)	IL-6 (µl)	CXCL12 (µl)	BCM (µl)
Epp 1	IL-6 100	1	0	99	2	0	98
Epp 2	IL-6 100 CXCL12 10	1	0.1	98.9	2	0.2	97.8
Epp 3	IL-6 100 CXCL12 100	1	1	98	2	2	96
Epp 4	IL-6 10	0.1	0	99.9	0.2	0	99.8
Epp 5	IL-6 10 CXCL12 10	0.1	0.1	99.8	0.2	0.2	99.6
Epp 6	IL-6 10 CXCL12 100	0.1	1	98.9	0.2	2	97.8
Epp 7	Blank - BCM	0	0	100	0	0	100
Epp 8	CXCL12 10	0	0.1	99.9	0	0.2	99.8
Epp 9	CXCL12 100	0	1	99	0	2	98

IL-6 plus CXCL12 plus APRIL	Condition (ng/ml)	Standard 1x Concentrations				2x Concentrated			
		IL-6 (µl)	CXCL12 (µl)	APRIL (µl)	BCM (µl)	IL-6 (µl)	CXCL12 (µl)	APRIL (µl)	BCM (µl)
Epp 1	IL-6 100 APRIL 200	1	0	0.2	98.8	2	0	0.4	97.6
Epp 2	IL-6 100 CXCL12 10 APRIL 200	1	0.1	0.2	98.7	2	0.2	0.4	97.4
Epp 3	IL-6 100 CXCL12 100 APRIL 200	1	1	0.2	97.8	2	2	0.4	95.6
Epp 4	IL-6 10 APRIL 200	0.1	0	0.2	99.7	0.2	0	0.4	99.4
Epp 5	IL-6 10 CXCL12 10 APRIL 200	0.1	0.1	0.2	99.6	0.2	0.2	0.4	99.2
Epp 6	IL-6 10 CXCL12 100 APRIL 200	0.1	1	0.2	98.7	0.2	2	0.4	97.4
Epp 7	APRIL 200	0	0	0.2	99.8	0	0	0.4	99.6

Epp 8	CXCL12 10 APRIL 200	0	0.1	0.2	99.7	0	0.2	0.4	99.4
Epp 9	CXCL12 100 APRIL 200	0	1	0.2	98.8	0	2	0.4	97.6

IL-6 plus CXCL12 plus BAFF	**Condition (ng/ml)**	**Standard 1x Concentrations**				**2x Concentrated**			
		IL-6 (µl)	**CXCL12 (µl)**	**BAFF (µl)**	**BCM (µl)**	**IL-6 (µl)**	**CXCL12 (µl)**	**BAFF (µl)**	**BCM (µl)**
Epp 1	IL-6 100 BAFF 100	1	0	0.1	98.9	2	0	0.2	97.8
Epp 2	IL-6 100 CXCL12 10 BAFF 100	1	0.1	0.1	98.8	2	0.2	0.2	97.6
Epp 3	IL-6 100 CXCL12 100 BAFF 100	1	1	0.1	97.9	2	2	0.2	95.8
Epp 4	IL-6 10 BAFF 100	0.1	0	0.1	99.8	0.2	0	0.2	99.6
Epp 5	IL-6 10 CXCL12 10 BAFF 100	0.1	0.1	0.1	99.7	0.2	0.2	0.2	99.4
Epp 6	IL-6 10 CXCL12 100 BAFF 100	0.1	1	0.1	98.8	0.2	2	0.2	97.6
Epp 7	BAFF 100	0	0	0.1	99.9	0	0	0.2	99.8
Epp 8	CXCL12 10 BAFF 100	0	0.1	0.1	99.8	0	0.2	0.2	99.6
Epp 9	CXCL12 100 BAFF 100	0	1	0.1	98.9	0	2	0.2	97.8

IL-6 plus CXCL12 plus APRIL plus BAFF	Condition (ng/ml)	Standard 1x Concentrations					2x Concentrated				
		IL-6 (µl)	CXCL12 (µl)	APRIL (µl)	BAFF (µl)	BCM (µl)	IL-6 (µl)	CXCL12 (µl)	APRIL (µl)	BAFF (µl)	BCM (µl)
Epp 1	IL-6 100 APRIL 200 BAFF 100	1	0	0.2	0.1	98.7	2	0	0.4	0.2	97.4
Epp 2	IL-6 100 CXCL12 10 APRIL 200 BAFF 100	1	0.1	0.2	0.1	98.6	2	0.2	0.4	0.2	97.2
Epp 3	IL-6 100 CXCL12 100 APRIL 200 BAFF 100	1	1	0.2	0.1	97.7	2	2	0.4	0.2	95.4
Epp 4	IL-6 10 APRIL 200 BAFF 100	0.1	0	0.2	0.1	99.6	0.2	0	0.4	0.2	99.2
Epp 5	IL-6 10 CXCL12 10 APRIL 200 BAFF 100	0.1	0.1	0.2	0.1	99.5	0.2	0.2	0.4	0.2	99
Epp 6	IL-6 10 CXCL12 100 APRIL 200 BAFF 100	0.1	1	0.2	0.1	98.6	0.2	2	0.4	0.2	97.2
Epp 7	APRIL 200 BAFF 100	0	0	0.2	0.1	99.7	0	0	0.4	0.2	99.4
Epp 8	CXCL12 10 APRIL 200 BAFF 100	0	0.1	0.2	0.1	99.6	0	0.2	0.4	0.2	99.2
Epp 9	CXCL12 100 APRIL 200 BAFF 100	0	1	0.2	0.1	98.7	0	2	0.4	0.2	97.4

Cytokine matrix dilution tables shown for each of the 36 individual cytokine conditions of the cytokine matrix. 4 tables are shown, 1 for each of the 4 main cytokine groups: IL-6 and CXCL12 group (yellow table), IL-6, CXCL12 and APRIL group (green table), IL-6, CXC12 and BAFF group (blue table), and IL-6, CXCL12, APRIL and BAFF group (pink table). The indicated required volumes of each cytokine are based upon the following stock concentrations: IL-6 10 µg/ml, CXCL12 10 µg/ml, APRIL 100 µg/ml, BAFF 100 µg/ml. Calculations for standard 1x and 2x concentrations are shown. 100 µl total volume/well was used for all experiments except for the HC peripheral PB culture (200 µl/well). Typically, cytokine conditions were prepared as 2x concentrated mixes so that they could be diluted by half, to their 1x concentration by addition of equal volumes of either BCM, or BCM enriched with M2-10B4 secretome, depending on the experimental setup. The indicated volumes for each condition were scaled up based upon how many wells were required for the particular experiment, based upon the required total number of duplicate wells and/or plates.

5.5 PC Cultures

5.5.1 FMOs and Single Stained Controls

For Fluorescence Minus One (FMO) controls, fresh PBMCs were resuspended to a concentration of $10x10^6$ cells/ml in FACS buffer **(Table(s) 5.4.1)**. $0.5x10^6$ cells (50 µl) were added to each well of a 96-well plate. Cells were pelleted by centrifugation at 2000rpm, acceleration/ deceleration 9/9, for 1 minute and wells aspirated. 50µl/well of each FMO antibody mix **(Table 5.5.1)** was added to the cells and incubated for 30 minutes at 4°C in the dark. A fully stained sample **(Table 5.5.1;** 'all') was additionally prepared as a positive control. All wells except for 'FMO -CD14' were washed in 200 µl DAPI solution **(Table(s) 5.4.1)**; 'FMO -CD14' cells were washed with 200 µl FACS buffer only. After washing, stained cells were resuspended in 200 µl FACS buffer and transferred to sterile polypropylene FACS tubes (BD Falcon™). Stained cells were maintained on ice at 4°C in the dark.

Table 5.5.1 Fluorescence Minus One (FMO) Controls

Laser and filter	Fluorochrome	Ab	Optimised Dilution	All (µl)	(-) CD31	(-) CXCR4	(-) CD20	(-) CD45	(-) CD14	(-) CD3	(-) CD38	(-) HLA-DR	(-) CD27	(-) CD19	(-) CD138
B 488 530/30	AF488	CD31	1:400	1 (of 1:8)		1 (of 1:8)	1 (of 1:8)	1 (of 1:8)	1 (of 1:8)	1 (of 1:8)	1 (of 1:8)	1 (of 1:8)	1 (of 1:8)	1 (of 1:8)	1 (of 1:8)
R 639 670/30	APC	CXCR4	1:50	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
R 730/45	AF700	CD20	1:50	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
R 780/60	APC-Cy7	CD45	1:50	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0
V 405 450/50	PacB	DapiCD14	1:100	0.5	0.5	0.5	0.5	0.5		0.5	0.5	0.5	0.5	0.5	0.5
V 525/50-505	BV510	CD3	1:50	1.0	1.0	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0
V 610/20	BV605	CD38	1:50	1.0	1.0	1.0	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0
V 670/30	BV650	HLA-DR	1:100	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5		0.5	0.5	0.5
V 710/50	BV711	CD27	1:50	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		1.0	1.0
V 780/60	BV785	CD19	1:25	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0		2.0
YG 780/60	PE-Cy7	CD138	1:50	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
				FACS buffer per sample	FACS buffer per sample	FACS buffer per sample	FACS buffer per sample	FACS buffer per sample	FACS buffer per sample	FACS buffer per sample	FACS buffer per sample	FACS buffer per sample	FACS buffer per sample	FACS buffer per sample	FACS buffer per sample
				39.0	40.0	40.0	40.0	40.0	39.5	40.0	40.0	39.5	40.0	41.0	40.0

Preparation of FMO Controls*. FMOs were prepared in FACS buffer in sterile conditions in the dark. For each FMO, 0.5 million cells were prepared in 50 µl. For each experiment, FMO controls were prepared using fresh PBMCs from HCs, obtained on the same day as the cell culture. In this manner, PBMCs and FMOs were prepared in advance of the thymic or BM sample, which allowed their analysis and experimental setup on the cell sorting machine in parallel to preparation of the sample for culture. This ensured that PBs isolated from blood, and PCs isolated from thymus and BM, were prepared in a prompt manner, to maximize their survival and functionality for cell culture.

*FMO Controls were modified as per the cell sorting strategy (e.g. thymic PCs were isolated based upon a truncated panel, and isolation of HC PBs included CD56).
FMO, Fluorescence Minus One.

For single stained controls, 1 drop of compensation beads (Anti-mouse Ig, κ; 51-90-9001229; Compensation Particles Set; BD™ CompBeads, 552843) was added to each well containing single antibody mixes (1µl antibody in 50µl FACS buffer) and incubated for 30 minutes at 4°C in the dark. After washing (centrifugation at 2000rpm, acceleration/ deceleration 9/9, for 1 minute in a total volume of 200 µl/ well FACS buffer), wells were aspirated, and beads resuspended in 200µl FACS buffer. Single stained beads were transferred to sterile polypropylene FACS tubes (BD Falcon™) and 1 drop of negative compensation beads (Negative Control; 51-90-9001291; Compensation Particles Set; BD™ CompBeads, 552843) added to each tube. Stained beads were maintained on ice at 4°C in the dark.

5.5.2 Sample Preparation, PC Isolation, and Cell Culture

Peripheral Blood Mononuclear Cells

~70 ml peripheral venous blood was taken from a 38-year-old male HC, 6 days after the onset of an upper respiratory tract infection, in order to enrich for peripheral circulating PBs. PBMCs were isolated on a Ficoll density gradient as outlined previously **(Methods 2.)**. 10^8 PBMCs were obtained. Following washing (1300rpm; acceleration/ deceleration 9/9, 5 minutes) in 20ml wash buffer **(Table(s) 5.4.1)**, 40x10^6 PBMCs were stained with the newly developed PC antibody panel (full stain, plus FcR blocking solution 1:20; Human TruStain FcX™; BioLegend, 422301) in a total volume of 500 µl at 4°C in the dark for 30 minutes. Stained cells were washed (as above) in 20 ml FACS buffer containing 200 µl DAPI solution **(Table(s) 5.4.1)** and resuspended to 10x10^6 cells/ml in FACS buffer. The stained cells were strained through a cell strainer into sterile polypropylene FACS tubes (Falcon®, Stemcell™ Technologies; 38030) with a total volume of 4 ml/tube. In parallel, FMOs and single stained controls were prepared as above (**Methods Section 5.5.1**).

For the HC PB culture, PBs (live, $CD45^+CD3^-CD56^-CD14^-CD20^+CD19^+CD27^+CD38^+$ cells) were purified from the fresh PBMC sample with a cell sorter. 5 PBs / well were sorted directly into each well of pre-prepared plates containing the 36 cytokine combinations (**Methods Section 5.4.2**) in a total volume of 200 µL per well. A total of 2 plates were prepared; 72 wells, with each of the 36 cytokine conditions tested in duplicate. Plates were maintained on ice at 4°C

until completion of cell sorting and transferred promptly to an incubator. PBs were incubated in a dedicated oligoculture humidified incubator (PHCbi) at 37°C and 5% CO_2 for 10 days. Supernatants for several timepoints were taken sequentially from the same culture plate. At timepoints day 3, 5, and 7, 100 µL of supernatant was carefully aspirated from each well and frozen at -20°C, and replenished with 100 µL of fresh 2x concentrated respective cytokine mix. The culture was terminated at day 10, with supernatants stored from this timepoint also. Supernatants from each of the 4 timepoints were tested for total IgG and total IgM levels by ELISA.

Thymic Tissue

Fresh thymic tissue was obtained from a 29-year-old female with MG and positive AChR autoantibodies undergoing thymectomy for thymic hyperplasia. Cell preparation and staining were undertaken by Drs Philip Ambrose and Mateusz Makuch, and cell sorting by Drs Mateusz Makuch and Jakob Theorell. The thymic tissue was transported on ice at 4°C in sterile BCM from QMC Hospital, Nottingham, to the John Radcliffe Hospital, Oxford (~2 hours; 100 miles). The sample was manually dispersed before passing through sterile CellTrics® cell filters (50 µm). Total tissue suspension in FACS buffer was centrifuged and counted. The suspension was adjusted to a concentration of $5x10^6$ cells/ml, which were stained with newly developed PC antibody panel on ice at 4°C in the dark for 30 minutes. In parallel, FMOs and single stained controls were prepared (**Methods Section 5.5.1**).

For the MG thymic PC culture, PCs (live, $CD3^-CD14^-CD45^+CD138^+$ cells) were purified from the thymic cell suspension with a cell sorter. 20 PCs were sorted directly into each well of pre-prepared culture and ELISpot plates containing the 36 cytokine combinations **(Methods Section 5.4.2 and Section 5.6)** in a total volume of 100 µL per well. Each condition was tested in duplicate. Plates were maintained on ice at 4°C until completion of cell sorting and transferred promptly to an incubator. PCs were incubated in a dedicated oligoculture humidified incubator (PHCbi) at 37°C and 5% CO_2. For ELISpot, the number of IgG- and IgM-secreting PCs were assessed after 36 hours. At day 3, the remaining culture was terminated and the 100 µL of supernatant from each well was aspirated and frozen at -20°C. Day 3 supernatants were tested for total IgG and total IgM by ELISA.

Bone Marrow Aspirate

Approximately 8 ml of fresh BM aspirate was taken from the right posterior iliac crest of participants using a 21 gauge needle. This was aspirated directly into a VACUETTE® NH sodium heparin tube (Greiner Bio-One, 455051) and mixed gently frequently. Whilst BM aspirates are usually taken into tubes containing EDTA, we elected to use heparin tubes to avoid any potential detrimental effects on PC functionality caused by EDTA. Additionally, slides of smeared BM aspirate were prepared by the Haematology Specialist Registrar, for morphological review. The BM aspirate sample was transported on ice at 4°C as promptly as possible (~15 minutes) from the Churchill Hospital, to the John Radcliffe Hospital site (1.3 miles). Due to high fatty content of the BM aspirate, and high susceptibility to coagulation, the sample required thorough mixing, and aspiration required a large bore pipette. The 8 ml BM aspirate sample was diluted with wash buffer **(Table(s) 5.4.1)** to a total volume of 35 ml. BM mononuclear cells (MNCs) were separated using Ficoll density gradient by careful transfer of the BM sample on to a 15 ml layer of Ficoll-Paque™ Premium (Cytiva, 17-5442-03) in a 50ml CentriStar™ Cap polypropylene centrifuge tube (Corning®, 430829). They were centrifuged at 400g, acceleration/ deceleration 3/0, for 30 minutes at room temperature, and carefully harvested with a Pasteur pipette, ensuring minimal aspiration of Ficoll. BM MNCs were counted, and washed (1300rpm, acceleration/ deceleration 9/9, for 5 minutes) twice in 30 ml wash buffer. Supernatant was aspirated precisely, and pelleted BM MNCs were resuspended thoroughly in 500 µl FACS buffer and labelled with antibodies against CD3 (clone UCHT1, BV510, mouse IgG1-κ, BioLegend), CD14 (clone 63D3, Pacific Blue, mouse IgG1-κ, BioLegend), CD45 (clone 2D1, APC/Cy7, mouse IgG1-κ, BioLegend), CD19 (clone SJ25C1, BV785, mouse IgG1-κ, BioLegend), CD20 (clone 2H7, AF700, mouse IgG2b-κ, BioLegend), CD138 (clone MI15, PE/Cy7, mouse IgG1-κ, BioLegend), CD27 (clone O323, BV711, mouse IgG1-κ, BioLegend), CD38 (clone HIT2, BV605, mouse IgG1-κ, BioLegend), CD31 (clone WM59, AF488, mouse IgG1-κ, BioLegend), CXCR4 (clone 12G5, APC, mouse IgG2a-κ, BioLegend), and HLA-DR (clone L243, BV650, mouse IgG2a-κ, BioLegend) in a total volume of 1000 µl at 4°C in the dark for 30 minutes **(Table 5.5.2)**. BM MNC Fc receptors were blocked with Human TruStain FcX™ (BioLegend, 422301). Stained cells were washed in 200 µl DAPI solution **(Table(s) 5.4.1)** in 20ml FACS buffer and resuspended to a concentration of $20x10^6$ cells/ml in 4 ml FACS buffer.

The stained cells were passed through a cell strainer into a single sterile polypropylene FACS tube (Falcon®, Stemcell™ Technologies; 38030) with the total 4 ml volume contained in a single tube. In parallel, FMOs and single stained controls were prepared (**Methods Section 5.5.1**).

Table 5.5.2 BM MNC Full Staining Panel

Fusion 2 Laser and Filter	Fluorochrome	Ab	Optimized Dilution	Full Stain (µl)/ 4M pellet	Full Stain (µl)/ 80M cells
B 488-530/30	**AF488**	CD31	1:400	0.125	2.5
R 640-670/30	**APC**	CXCR4	1:50	1	20
R 640-730/45	**AF700**	CD20*	1:50	1	20
R 640-780/60	**APC-Cy7**	CD45	1:50	1	20
V 405-450/40	**PacB**	CD14	1:100	0.5	10
V 405-525/50	**BV510**	CD3	1:50	1	20
V 405-610/20	**BV605**	CD38	1:50	1	20
V 405-660/20	**BV650**	HLA-DR	1:100	0.5	10
V 405-710/50	**BV711**	CD27	1:50	1	20
V 405-780/60	**BV785**	CD19*	1:25	2	40
YG 561-780/60	**PE-Cy7**	CD138	1:50	1	20
		FcR block			25
		FACS buffer			500 (+250 µl flush)

Preparation of full PC stain of BM MNCs. For 80 million cells acquired from each BM aspirate, the BM MNC cells were stained in the full antibody panel in a total staining volume of 1000 µl (including antibody volumes, and FACS buffer flush volumes which were required to ensure staining of cell pellet, due to persistent lipid layer coating Falcon tube following washes). BM MNCs were stained with the above antibodies in a 50ml CentriStar™ Cap polypropylene centrifuge tube (Corning®, 430829) in sterile conditions at 4°C in the dark for 30 minutes.

*For staining of BM cells, CD20 and CD19 concentrations were increased to 1:50 and 1:25, respectively, due to their heightened expression on BM MNCs compared to that of PBMCs (upon which the panel was optimized).
BM MNCs, bone marrow mononuclear cells.

A BD FACSAria^{TM} Fusion Cell Sorter (Becton Dickinson, San Jose, CA, USA) was used to determine cell phenotypes, and LLLPC populations were purified from the fresh BM MNCs as live, CD3-CD14-CD20-CD19-/lowCD138+ cells. BM LLPCs were sorted directly into pre-prepared cell culture plates containing the 36 cytokine combinations **(Methods Section 5.4.2)** in a total volume of 100 µL per well both with and without enriched BCM. Plates were maintained on ice at 4°C until completion of cell sorting and transferred promptly to an incubator. PCs were incubated in a dedicated oligoculture humidified incubator (PHCbi) at 37°C and 5% CO_2. Cultures were terminated at respective timepoints and 100 µl of supernatant from each well was aspirated and frozen at -20°C*.

BM LLPC cultures for LGI1-antibody patients JR879 and JR051 consisted of sorting various LLPC cell numbers (ranging from single cell to 20/well) in pre-prepared plates containing the optimized cytokine condition (IL-6 10 ng/ml, CXCL12 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml in a total volume of 100 µl /well) both with and without enriched BCM for culture durations of 3 or 7 days. Experimental details can be found in **Chapter 5, Table 5.5**. Supernatants were then tested for presence of specific LGI1-IgG by live 96-well CBA. For JR051, LLPCs were additionally sorted directly into ELISpot plates containing the 36 cytokine combinations with and without enriched BCM **(Methods Section 5.4.2 and Section 5.6)** using 20 LLPCs in a total volume of 100 µl per well. For ELISpot, IgG-secreting PCs were enumerated at day 1.

**Modifications to BM LLPC Isolation and Culture Protocol:*

For the MS BM LLPC culture, a fresh 4 ml BM aspirate was obtained from the femur of a 74-year-old patient with MS undergoing a total knee replacement for advanced osteoarthritis. The patient had a 35-year history of secondary progressive MS, was wheelchair-bound, and had only ever been treated once with IVMP, approximately 30 years prior. She was not taking any immunosuppressive medications. In this experiment, the sample was acquired intraoperatively directly into a sterile Universal container, and there was delay in transfer of the sample from the operating theatre to the researcher (~30 minutes). There was an approximate 45-minute interval from the time of BM aspirate sampling to time of transfer to

a VACUETTE® NH sodium heparin tube (Greiner Bio-One, 455051), and the sample was visibly highly coagulated. 10 LLPCs/ well (live, $CD3^-CD56^-CD14^-CD20^-CD19^{-/low}CD138^+$ cells) were sorted into pre-prepared cell culture plates containing the 36 cytokine combinations **(Methods Section 5.4.2)** in a total volume of 100 µL per well both with and without enriched BCM. This experiment did not yield sufficient numbers of PCs (as shown in **Chapter 5, Figure 5.8**). LLPCs were cultured for 7 days, and day 7 supernatant total IgG levels were measured by Ultrasensitive ELISA **(Methods Section 5.7.2)**.

All cell sorting was performed on a BD FACSAria™ Fusion Cell Sorter (Becton Dickinson, San Jose, CA, USA), and FlowJo™ (v10.5.3; FlowJo, LLC, Ashland, OR) software was used for analysis. All cell culture plates were covered with AlumaSeal II film (Sigma-Aldrich, Z721530-50EA) to minimise evaporation of supernatants during long-term storage at -20°C.

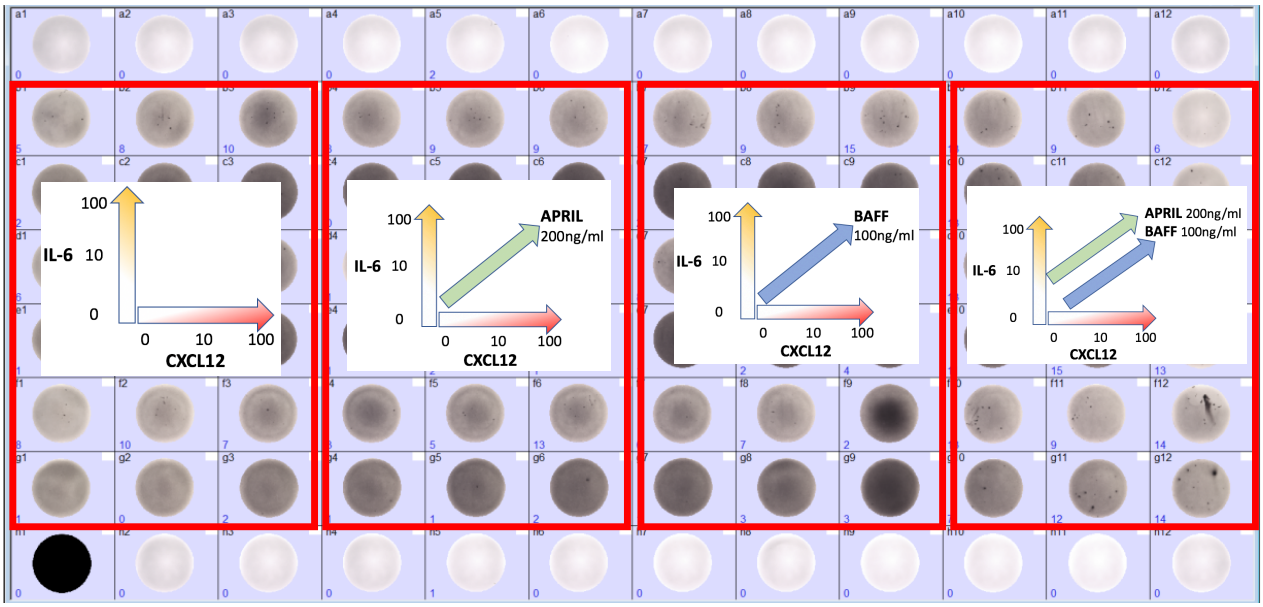
5.6 ELISpot Functional Assays

IgG- and IgM-secreting PCs were detected and enumerated by functional ELISpot assays. ELISpot assays were performed using the Human IgG ELISpot^{BASIC} kit (Mabtech; 3850-2A) and Human IgM ELISpot^{BASIC} kit (Mabtech; 3880-2A).

To ensure maximal antibody binding capacity, PVDF-based membrane plates (type MSIPS4510, Millipore; Mabtech 3654-TP-10) were activated with 15 µl 35% ethanol/well for 1 minute, and washed 5 times with sterile water, 200 µl/well, ensuring that the plate was not allowed to dry. Plates were coated with 100 µl/well of 15 µg/ml anti-Ig monoclonal antibody (IgG MT91/145; IgM MT11/12; Mabtech) overnight at 4°C. The following day, plates were washed 5 times with 200 µl/well sterile, filtered PBS. Plates were blocked with 200 µl/well of BCM 'R10' **(Table(s) 5.4.1 Reagents)**, to ensure use of the same 10% serum as contained in the PC sorting medium. After 30 minutes' incubation at room temperature, the medium was removed, and 100 µl/well of the respective cytokine mix was added to the plates (for plate layouts used in individual experiments, see **Figure 5.6**). PCs were sorted directly into the plates (20/well for both thymic PCs and JR051 LGI1 BM PCs). To ensure high quality, live, functional cells, plates were transported at 4°C with minimal agitation, and promptly transferred to an incubator. PCs were incubated in a dedicated oligoculture humidified

incubator (PHCbi) at 37°C and 5% CO_2 for 24 hours. The plate was not moved during this time. After 24 hours, the plates were emptied and washed 5 times with 200 µl/well PBS. Plates were incubated with 100 µl/well of 1 µg/ml anti-human Ig biotinylated monoclonal antibody (IgG MT78/145-biotin; IgM MT22-biotin; Mabtech, diluted in PBS/0.5% FCS) for 2 hours at room temperature. Plates were washed 5 times with PBS, 200 µl/well, and then incubated with 100 µl/well Streptavidin-ALP (1:1000 in PBS-0.5% FCS) for 1 hour at room temperature. Subsequently, plates were washed 5 times with PBS, 200 µl/well. Next, 100 µl/well of substrate solution (BCIP/NBT-plus substrate; Mabtech; 3650-10) was added and developed for 25-30 minutes until distinct spots emerged. Colour development was stopped by extensive washing in tap water, and plates were left to dry. Spots were detected and counted using an ELISpot plate reader (Autoimmun Diagnostika gmbh ELISpot Reader Classic).

Figure 5.6 ELISpot Plate Layouts



ELISpot plate layouts depicting the 36-condition cytokine matrix, using 100 µl/well of cytokine mix. For each of the 4 cytokine groups, the layout of individual conditions is as shown in **Figure 5.4.2**. As in **Figure 5.4.2**, the cytokine conditions were set up in duplicates, repeated in the wells directly below. For the thymic PC culture, the duplicate conditions were tested for detection of IgG-secreting PCs, and the other for IgM-secreting PCs. For the JR051 BM LLPC culture experiment, the duplicate wells were both used for IgG-secreting PCs (i.e. the plate was not coated with antibody to IgM, and no IgM detection antibody was used).

5.7 ELISA Techniques

5.7.1 ELISA

PC culture supernatants were collected for measurements of total Ig using the Human IgG ELISA Quantitation Set (Bethyl Laboratories, E80-104) and Human IgM ELISA Quantitation Set (Bethyl Laboratories, E80-100) as per the manufacturer's instructions. In brief, 100 µl of diluted coating antibody [affinity purified goat anti-human IgG-Fc coating antibody A80-104A-9 or affinity purified goat anti-human IgM coating antibody A80-100A-11 diluted to 1:100 in coating buffer (0.05 M carbonate-bicarbonate in distilled water; pH 9.6; E107] was added to each well of 96-well ELISA plates (Nunc MaxiSorp C bottom; 445101/E105). Plates were incubated at room temperature for 1 hour and washed 5 times with 200 µl/well wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20 in distilled water; pH 8.0; Sigma T9039/E106), ensuring that microwells were emptied completely between washes, and that wells were not allowed to dry. 200 µl blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA in distilled water; pH 8.0; blocking buffer, Sigma T6789/E104) was added to each well and incubated at room temperature for 30 minutes. Plates were washed 5 times.

Standards and samples were diluted in sample diluent (0.05% Tween 20 in blocking buffer; 10% Tween 20, Sigma P7949/E108). Standards were prepared as external standard dilution. For IgG, a 1000 ng/ml initial dilution was prepared by diluting 5 µl of human reference serum (RS10-110-4) with 22 ml of sample diluent. 2-fold serial dilutions in sample diluent were then prepared, producing standards S1-S7 (500 ng/ml-7.8 ng/ml). For IgM, standard 1 (1000 ng/ml) was prepared by dilution of 10 µl of human reference serum (RS10-110-4) with 4.4 ml of sample diluent. 2-fold serial dilutions were then prepared in sample diluent, producing standards S1-S7 (1000 ng/ml-15.6 ng/ml). 100 µl/well of standards S1 – S7 were pipetted into the standard wells, and 100 µl/well of sample diluent into the blank wells; all 8 set up in duplicates. Samples from PC culture supernatants were diluted in sample diluent to fall within the concentration range of the standards, based upon their expected total Ig concentration. 100 µl/well of sample was added to wells at the same time as the standards, and plates were incubated for 1 hour at room temperature. Plates were washed 5 times and 100 µl of HRP detection antibody (HRP conjugated goat anti-human IgG-Fc detection antibody A80-104P-87

diluted to 1:150,000 in sample diluent or HRP conjugated goat anti-human IgM detection antibody A80-100P-36 diluted 1:100,000 in sample diluent; using 2-step dilutions) was added to each well. Plates were incubated at room temperature for 1 hour and washed 5 times. 100 μl of TMB substrate solution (E102) was added to each well and plates were developed in the dark at room temperature for 15 minutes. Colour development was monitored carefully, and the reaction was stopped by rapid addition of 100 μl of stop solution (0.18 M H2SO4 diluted in deionized water) to each well, in the same order as the TMB substrate. The plate was gently tapped on the underside to ensure uniform distribution of the stop solution throughout the microwells in order to completely terminate the reaction. Absorbance was measured immediately on a FLUOstar® Omega ELISA microplate reader (BMG Labtech) at 450 nm, ensuring that the underside of the plate was cleaned prior to analysis. A standard curve was prepared from the mean absorbances of the standards minus the blank values using regression analysis. Then, the total IgG or IgM concentration of the samples was determined using curve-fitting software in GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA); dilution factors were considered for final concentration values.

For the HC PB culture, supernatants from each of the 4 timepoints (days 3, 5, 7, and 10) were tested for total IgG and total IgM levels by ELISA. They were initially tested for IgG at 1:50 dilution, as performed for memory B cell culture supernatants within our research group, however despite good standard curves, no IgGs were detected for any well from the day 3, 5, 7, or 10 plates. Considering that only 5 PBs were cultured in each well, and that PCs are terminally differentiated cells, the expected supernatant Ig levels were deemed to be much lower than those found in memory B cell cultures. Thus, the ELISAs were repeated at 1:3 dilution for total IgG and IgM levels, with positive results (**Chapter 5, Figure 5.5**). For the MG thymic PC culture, day 3 supernatants were tested at 1:2 dilution for total IgG and IgM levels **(Chapter 5, Figure 5.6)**.

5.7.2 Ultrasensitive ELISA IgG

For select experiments, supernatants were measured for total IgG using a high sensitivity Human total IgG Platinum ELISA kit (eBioscience; Invitrogen BMS2091) according to the manufacturer's instructions. The microwell plate (precoated with monoclonal antibody to

human total IgG) was washed twice with 400 µl/ well wash buffer (PBS with 1% Tween 20), ensuring that microwell contents were emptied completely between washes, and that wells were not allowed to dry. Standard dilutions were prepared as external standard dilution (human total IgG standard reconstituted in distilled water, and subsequently 2-fold serial dilutions in assay buffer). 100 µl/well of standard dilutions (S1 – S7; 0.1 µg/ml – 0.002 µg/ml) were pipetted into the standard wells, and 100 µl/well of assay buffer (PBS with 1% Tween 20, 10% BSA) into the blank wells; all 8 set up in duplicates. 100 µl/well of each prediluted PC culture supernatant sample was added to the sample wells[+]. 50 µl/well of HRP-conjugated antibody solution (1:100 in assay buffer) was added to all wells and incubated at room temperature for 1 hour on a microplate shaker set at 400 rpm. After washing plates 4 times, 100 µl/well TMB substrate solution was added and incubated at room temperature for 30 minutes in the dark. Colour development was monitored carefully. After 30-35 minutes, when the highest standard developed a dark blue colour, and was deemed to have reached an OD of 0.9 by an ELISA reader at 620 nm (reference wavelength), the substrate reaction was terminated by quickly adding 100 µl/well of stop solution (1M phosphoric acid). The plate was gently tapped on the underside to ensure uniform distribution of the stop solution throughout the microwells in order to completely stop the reaction. The absorbance of each microwell was read immediately on a FLUOstar® Omega ELISA microplate reader (BMG Labtech) at 450 nm, ensuring that the underside of the plate was cleaned prior to analysis. A standard curve was prepared from the mean absorbances of the standards minus the blank values using regression analysis, and the total IgG concentration of the samples was determined using curve-fitting software in GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA); dilution factors were considered for final concentration values.

[+]At this point, standards and samples of PC supernatants from the MS BM culture were left for 18 hours overnight at 4°C in order to maximise binding of any low levels of IgG contained within the samples. Following incubation, plates were washed four times prior to the addition of HRP-conjugated antibody. From this point, the assay protocol was continued as above. This modification was conducted following detailed discussion with Thermo Fisher Scientific.

Sample Dilution: MS BM Culture

Whilst the manufacturer's instructions recommend predilution of serum samples to 1:500,000, we elected to test our PC culture supernatants at a much lower dilution, owing to extremely low anticipated levels of IgG compared to those contained in the serum. Due to limited sample (100 µl supernatant from each well of the MS BM culture), it was not possible to assay each sample in duplicate. However, the manufacturer's performance characteristics indicate good reproducibility with an overall intra-assay coefficient of variation of 5.3%. Thus, the ultrasensitive ELISA was initially conducted using a 1:2 dilution of 50 µl PC supernatant with 50 µl assay buffer (without duplicates). Since this did not yield any positive results for detectable IgG in any well, technical advice was sought from Thermo Fisher Scientific. The recommendation was to increase sensitivity by overnight incubation of samples (as outlined above). The assay was repeated with the overnight incubation, however in this case since only 50 µl supernatant was remaining, all PC supernatant samples were run neat using a total volume of 50 µl. The volume of the standards was also reduced to 50 µl/well.

Sample Dilution: LGI1 and CASPR2 Monoclonal Antibodies

Dilution details of the patient-derived LGI1 (n=2) and CASPR2 (n=2) monoclonal antibodies, are shown in **Table 5.7.2**. Each antibody was diluted from its respective stock concentration to the defined target concentrations required for the experiment.

Table 5.7.2 Monoclonal Antibody Dilutions for Ultrasensitive ELISA and Live 96-Well CBA

mAb	mAb stock concentration (µg/ml)	Dilution for target 1µg/ml concentration	Dilution for 100 ng/ml	Dilution for 50 ng/ml	Dilution for 10 ng/ml	Dilution for 5 ng/ml	Dilution for 1 ng/ml
LGI1 LRR5678	1000	1µl stock+1000µl diluent*	50µl of 1µg/ml+450µl diluent	100µl of 100ng/ml+100µl diluent	20µl of 100ng/ml+180µl diluent	20µl of 50ng/ml+180µl diluent	20µl of 10ng/ml+180µl diluent
LGI1 EPTP5676	500	2µl stock+1000µl diluent	50µl of 1µg/ml+450µl diluent	100µl of 100ng/ml+100µl diluent	20µl of 100ng/ml+180µl diluent	20µl of 50ng/ml+180µl diluent	20µl of 10ng/ml+180µl diluent
CASPR2 E8 memory	520	1µl stock+520µl diluent	50µl of 1µg/ml+450µl diluent	100µl of 100ng/ml+100µl diluent	20µl of 100ng/ml+180µl diluent	20µl of 50ng/ml+180µl diluent	20µl of 10ng/ml+180µl diluent

CASPR 2 E9 memory	250	1µl stock+250µl diluent	50µl of 1µg/ml+450µl diluent	100µl of 100ng/ml+100µl diluent	20µl of 100ng/ml+180µl diluent	20µl of 50ng/ml+180µl diluent	20µl of 10ng/ml+180µl diluent

Dilution details of patient-derived monoclonal antibodies from stock concentrations to each of 5 target concentrations selected for validation experiments: 100 ng/ml, 50 ng/ml, 10 ng/ml, 5 ng/ml, 1 ng/ml.
*Diluent used for dilution of mAbs: ultrasensitive ELISA - assay buffer provided in kit; live 96-well CBA - PBS.
mAb, monoclonal antibody.

5.8 Live 96-Well Cell-Based Assays

96-well cell culture plates (clear, flat bottom TC-Treated; Corning® Costar®, 3595) were treated with 100 µl/ well sterile, filtered 0.01% PLL solution. HEK-293T cells ($0.23x10^6$/ml) were plated into the 96-well plates with 23,000 cells/ well in 100 µl volume HEK-239T cell culture medium **(Table(s) 1. Reagents)** and cultured at 37°C and 5% CO_2 for 24 hours. On day 2, HEK-293T cells were transfected with EGFP-tagged LGI1 or CASPR2 cDNA. For each plate, 25.2 µg DNA was added to 420 µl unsupplemented DMEM **(Table(s) 1. Reagents)** containing 12.6 µl PEI for 10 minutes prior to transfection and added to a final volume of 10 ml HEK-239T cell culture medium. The 100 µl cell supernatant was carefully removed from each well of the culture plates, taking care not to disturb cells. 100 µl of transfection mix was quickly added to each well, ensuring that wells were not allowed to dry. After 6 hours' incubation, the supernatant was removed and replaced with 100 µl/ well fresh HEK-239T cell culture medium and cells were incubated for a further 18 hours.

On day 3, supernatants were aspirated completely, and live transfected HEK-293T cells were incubated with samples for 1 hour at room temperature. For PC culture supernatant assays, 40 µl/ well of neat PC culture supernatant was used. For monoclonal antibody (mAb) validation assays, 40 µl/ well of mAb diluted in PBS was added **(Table 5.7.2)**. After 1 hour, supernatants were discarded, and plates were washed twice in 150 µl/ well CBA Wash Buffer **(Table(s) 1. Reagents)**. Addition of all solutions to wells was done carefully at 45° pipetting angles to ensure non-disruption to cells on the well surface. Cells were fixed with 100 µl/ well CBA fixative solution **(Table(s) 1. Reagents)** for 10 minutes at room temperature. Plates were

washed twice in 150 μl/ well CBA wash buffer. Cells were incubated with 100 μl/ well secondary antibody (goat anti-human IgG Fc cross-adsorbed unconjugated secondary antibody; Invitrogen, 31125) diluted to 1:750 in CBA sample buffer **(Table(s) 1. Reagents)** for 45 minutes at room temperature in the dark. Cells were washed twice in 150 μl/ well CBA wash buffer and incubated with 100 μl/ well tertiary antibody (donkey anti-goat IgG (H+L), Cross-Adsorbed antibody, Alexa Fluor® 568; Life Technologies, A-11057) diluted to 1:750 in CBA sample buffer for 45 minutes at room temperature in the dark. Cells were washed once in 150 μl/ well CBA wash buffer, and once in 150 μl/ well PBS. Finally, cells were stained with 40 μl/ well DAPI solution 1:1000 **(Table(s) 1. Reagents)** for 15 minutes in the dark at room temperature. Wells were washed in 150 μl/ well PBS, and stored in 150 μl/ well PBS at 4°C in the dark.

For the mAb experiments, patient-derived mAbs were diluted to various concentrations as shown in **Table 5.7.2**. LGI1 and CASPR2 mAbs were incubated with HEK-293T cells expressing EGFP-tagged LGI1 and CASPR2, respectively. For the PC culture supernatants, samples were run neat (i.e. akin to CSF testing), and tested for reactivity to membrane tethered EGFP-tagged LGI1 on the live HEK293T cells. In order to increase sensitivity, all PC culture supernatants and mAb validation experiments were conducted with a tertiary antibody, as outlined above. Samples were visualised with a fluorescent microscope (Olympus CKX53 Inverted LED Microscope), where cell-surface binding of red fluorescent-labelled tertiary antibody was sought, with co-localization to the relevant EGFP-tagged LGI1 or CASPR2 construct. mAb experiments were all repeated and scored blindly. Images were captured at 20x with a Hamamatsu Spark CMOS C11440-36U camera using Volocity Acquisition Lite software (version 6.4). GraphPad Prism v8 (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses and data presentation.

CHAPTER 3. CLINICAL FEATURES:

Expanded Clinical Phenotypes and Outcomes of Patients with LGI1-Autoantibodies

3.1 Introduction

Patients with LGI1-autoantibody syndromes have a recently described form of autoimmune encephalitis (Irani et al. 2010) which typically manifests with an immunotherapy-responsive and pathognomonic syndrome of faciobrachial dystonic seizures (FBDS), other complex focal seizures and cognitive impairment, comprising a limbic encephalitis (LE) (Irani et al. 2011; Thompson et al. 2017). Owing to their recent description and rarity, however, the wider clinical phenotypes and detailed clinical outcomes remain less well-defined.

During this DPhil, my clinical assessments of LGI1-autoantibody patients in the inpatient and outpatient settings at the John Radcliffe Hospital, Oxford, revealed several observations.

3.1.1 Initial Clinical Observations:

1. Select LGI1-autoantibody patients had potential prodromal associations, including shingles, vaccinations, and abrupt shifts in diet, in the few weeks prior to the onset of their neurological syndrome.
2. Two patients with LGI1-autoantibody encephalitis displayed highly unusual, complex manual motor behaviours consisting of task-imitation, for example acting out ingestion of tablets or winding string around the fingers using imaginary objects.
3. Relapses were observed in several patients despite prompt and adequate treatments with immunotherapies.
4. Patients and/ or their spouses reported persistent long-term symptoms such as cognitive impairment which impacted their quality of life, including employment. Additionally, certain patients were found to have new-onset, obsessive tendencies including hoarding.

These observations led to several aims which were explored in cohorts during my DPhil.

3.1.2 Aims:

1. To explore whether LGI1-autoantibody syndromes show environmental triggers such as infections including herpes viruses, vaccinations, or abrupt dietary changes.

2. To describe novel clinical features and establish their frequencies in a large cohort of patients with LGI1-autoantibodies.
3. To define the proportion of clinical relapses and relapse patterns in a large cohort of patients.
4. To evaluate long-term patient outcomes and define factors that may associate with more favourable outcomes.

3.1.3 Methodology

Patients were recruited for clinical research following either direct referral to the Oxford Autoimmune Neurology Service, John Radcliffe Hospital, or via notification to the national Association of British Neurologists Rare Disease Ascertainment and Recruitment (RaDaR) Surveillance Unit.

Detailed clinical phenotyping of 107 LGI1-autoantibody patients was performed. A structured, pre-defined database of clinical characteristics was completed by review of clinical records and facilitated by patient and spouse interview (during clinical review or via telephone). Detailed methods are outlined in **Methods (Chapter 2, Section 3. 'Clinical'**).

3.2 Results

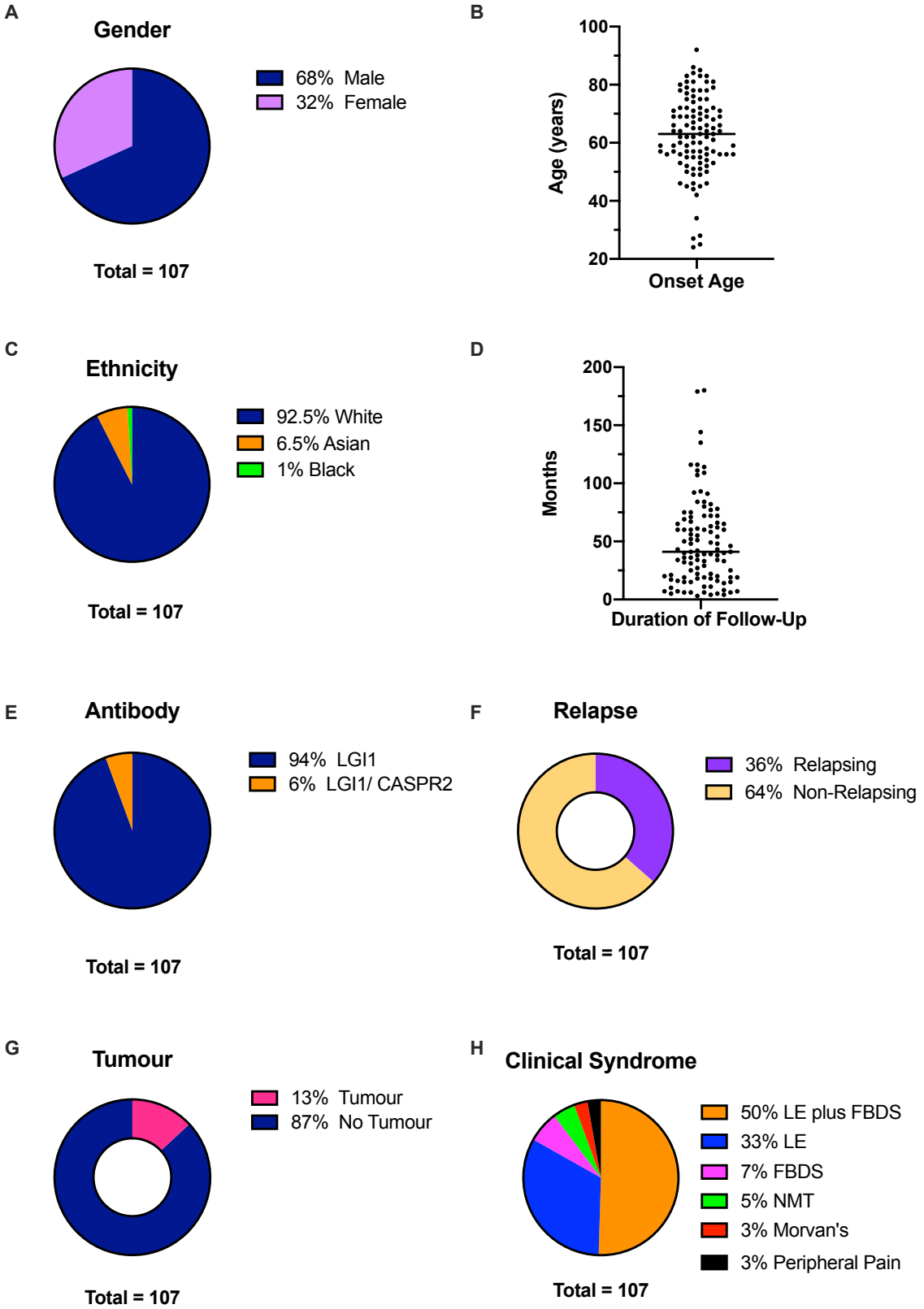
3.2.1 Cohort Demographics and Clinical Phenotypes

68% (73/107) of patients were male, with median age at onset of 63 years (range 24-92 years), and 93% of patients were White British or European **(Figure 3.1A-C)**. The clinical follow-up period was up to 15 years, with median duration of 41 months **(Figure 3.1D)**. 6/107 (6%) had additional CASPR2-autoantibodies **(Figure 3.1E)**. 36% (39/107) of patients experienced relapses **(Figure 3.1F)** with follow-up duration of 5-179 months (median 52 months), and the remainder had a monophasic course (follow-up period 3-180 months; median 34 months). 13% (14/107) had a tumour at any stage during their lifetime **(Figure 3.1G)**.

The most common clinical syndrome was limbic encephalitis (LE) plus FBDS (54/107, 50%), followed by LE alone (35/107, 33%) and FBDS alone (7/107, 7%); **(Figure 3.1H)**. Of the three patients with Morvan's syndrome, one lacked CASPR2 autoantibodies, but had detectable AChR autoantibodies with an associated thymoma. The two other Morvan's patients both had LGI1- and CASPR2-autoantibodies and one had a thymoma. Isolated peripheral nervous system syndromes were rare, occurring in 3% (3/107). Detailed breakdowns are summarised in **Table 3.1**.

Six patients had atypical presentations **(Table 3.1)**. Three presented with simultaneous, new-onset additional autoimmune diseases (two with IgG4-related disease, and one with giant cell arteritis affecting one eye). Another patient had profound neuropsychiatric manifestations, with a temporal relationship matching the onset of LGI1-autoantibody encephalitis, leading to criminal offences. One patient developed LGI1-autoantibody encephalitis with FBDS five years following the onset of likely frontotemporal dementia, with prosopagnosia and behavioural changes. Finally, we observed a patient with an unusual sleep disorder at onset of encephalitis. Of interest, another patient had concurrent anterior horn cell disease, which was diagnosed one year following the onset of LGI1-autoantibody encephalitis symptoms.

Figure 3.1 LGI1-Autoantibody Patient Cohort Demographics



Gender distribution **(A)**, age at disease onset **(B)**, ethnicity **(C)** and duration of clinical follow-up **(D)** shown for n=107 LGI1-autoantibody patients. Antibody status shown indicating proportion of patients LGI1-only, versus those with both LGI1- and CASPR2-autoantibodies **(E)**. Proportion of patients with relapses **(F)** and tumour at any stage during lifespan **(G)** shown. Clinical syndromes of patients with LGI1-autoantibodies represented in pie chart, with most common presentation as limbic encephalitis plus FBDS **(H)**.

Table 3.1 LGI1-Autoantibody Cohort Demographics

Clinical Characteristic	n	%
Gender	M 73/107 F 34/107	68 32
Age at onset	24-92 (median 63)	-
Ethnicity	White 99/107 - White British 97/99 - White European 2/99 Asian 7/107 Black 1/107	92.52 6.54 0.93
Follow-up duration (months)	3-180 (median 41)	-
Additional CASPR2 autoantibodies	6/107	6
Relapsing	39/107	36
Tumour (at any stage)	14/107	13
Clinical Syndrome	LE plus FBDS* 54 LE only[$] 35 FBDS only 7 NMT[ƒ] 5 Morvan's syndrome 3 PN 3 *LE + FBDS including n=3 atypical presentations (LE + FBDS + PN + neuropsychiatric, LE + FBDS + sleep disorder, LE + FBDS + dementia) and n=1 patient with concurrent motor neuron disease [$]LE only including n=2 atypical presentations (LE + GCA, LE + IgG4-RD) [ƒ]NMT including n=1 atypical presentation (NMT + IgG4-RD double-positive)	50.47 32.71 6.54 4.67 2.80 2.80

Breakdown of LGI1-autoantibody cohort demographic data and clinical syndromes for n=107 patients.

LE, limbic encephalitis; FBDS, faciobrachial dystonic seizures; NMT, neuromyotonia; PN, peripheral nerve; GCA, giant cell arteritis; IgG4-RD, IgG4-related disease.

3.2.2 Associated Autoimmune Diseases

32% of patients had a history of other autoimmune diseases in addition to their LGI1-autoantibody syndrome **(Figure 3.2A)**, consistent with previously reported rates (Binks et al. 2018). 24% of this group had more than one additional autoimmune disease. The most frequent underlying autoimmune conditions were dermatological and thyroid disease **(Figure 3.2B)**. Of interest, 5 of the 34 (15%) patients with other autoimmune conditions had initial onset of their autoimmune condition near to the onset of LGI1-autoantibody encephalitis. Remarkably, three patients, with spontaneous idiopathic urticaria, giant cell arteritis and Graves' disease, showed *simultaneous* onset with LGI1-autoantibody encephalitis. The two patients with IgG4-related disease developed the condition 1 year pre- and 7 months post- their respective LGI1-autoantibody syndrome. One patient with refractory, relapsing encephalitis had relapses which correlated clearly with exacerbations of allergic eosinophilic sinusitis. A polypectomy resulted in dramatic improvements in both short-term memory and personality. Detailed breakdowns are summarised in **Table 3.2**.

Additionally, 4 patients had additional positive serum autoantibodies (2 GAD-, and 2 AChR-autoantibodies – both in patients with thymoma), although these findings were not classified as additional autoimmunity since they were not accompanied by related clinical manifestations.

Of those with available data, 19/97 (20%) had a positive family history of either underlying autoimmunity or neurological disease **(Figure 3.2C and Table 3.2)**. A family history of neurological disorders was present in 11/19 (58%) of these patients. Of interest, one patient had a first-degree relative with MG, and another had a relative with encephalitis lethargica.

Table 3.2 Associated Autoimmune Diseases in LGI1-Autoantibody Patients

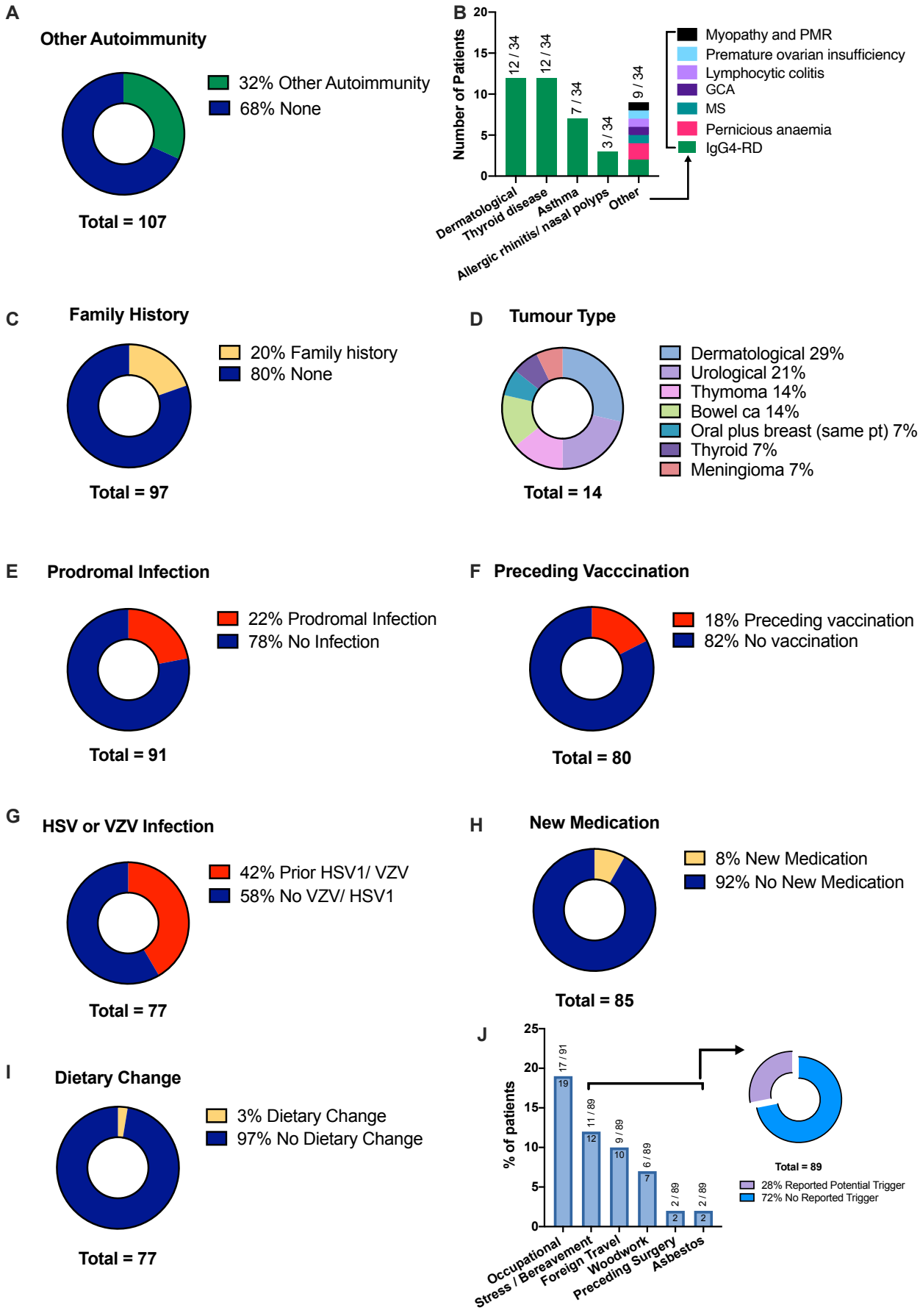
Clinical Characteristic	n	%
Other Autoimmunity	34/107 - **Dermatological 12/34** (6 psoriasis +/- psoriatic arthritis, 3 atopic eczema, 1 spontaneous idiopathic urticaria, 1 vitiligo, 1 lichen planus and bullous pemphigoid) - **Thyroid disease 12/34** (9 hypothyroidism, 2 Graves' disease, 1 post-partum thyroiditis) - **Asthma 7/34** - **Allergic rhinitis and nasal polyps 3/34** - **Other 9/34** (2 IgG4-RD, 2 pernicious anaemia, 1 MS, 1 GCA, 1 lymphocytic colitis, 1 premature ovarian insufficiency, 1 myopathy and polymyalgia rheumatica) - **>1 co-existent autoimmune condition 8/34**	32 24
Relevant Family History (autoimmunity, or neurological)	19/97* - **13 Neurological disorders in 11/19** (4 epilepsy, 3 PD, 1 encephalitis lethargica, 1 MG with thymoma, 1 cerebellar ataxia in two first-degree relatives, 1 MS, 1 poliomyelitis, 1 headache) - **5 Autoimmune disorders in 4/19** (3 thyroid disorders, 1 coeliac disease, 1 T1DM) - **Dementia 5/19** (2 AD, 1 Lewy body dementia, 2 vascular dementia) - **Psychiatric 4/19** (2 schizophrenia, 1 bipolar disorder, 1 unknown psychiatric disorder requiring institutionalisation)	20

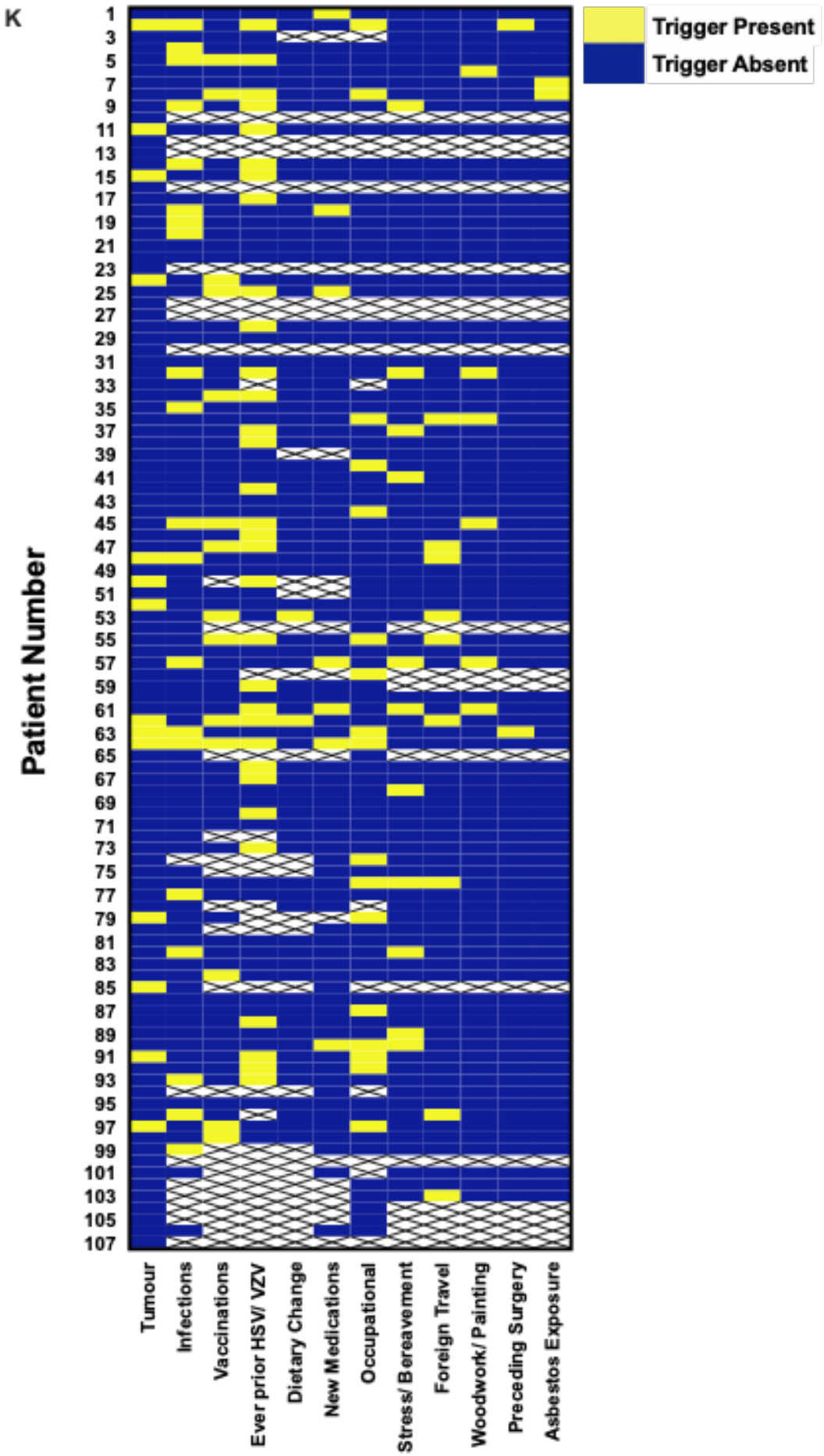
Details of autoimmune diseases found in LGI1-autoantibody patients and their relatives, including family history of neurological disease.

*family history was unknown in 10/107

IgG4-RD, IgG4-related disease; MS, multiple sclerosis; GCA, giant cell arteritis; PD, Parkinson's disease; MG, myasthenia gravis; T1DM, type 1 diabetes mellitus; AD, Alzheimer's disease.

Figure 3.2 Potential Triggers of and Associations with LGI1-Autoantibody Disease




K
Patient Number
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85
87
89
91
93
95
97
99
101
103
105
107
Tumour
Infections
Vaccinations
Ever prior HSV/ VZV
Dietary Change
New Medications
Occupational
Stress/ Bereavement
Foreign Travel
Woodwork/ Painting
Preceding Surgery
Asbestos Exposure
Trigger Present
Trigger Absent

(A) Proportion of patients with additional autoimmune diseases, with breakdown of particular autoimmune diseases shown in **(B)**. Proportion of patients with a family history of autoimmune or neurological disease **(C)**. Representation of tumour types found in n=14 LGI1-autoantibody patients **(D)** at any stage (both active tumours and past history of tumours included). Proportion of patients with preceding infection **(E)** or vaccination **(F)** within 4 and 2 months, respectively, prior to disease onset. Percentage of patients with history of prior HSV1 or VZV infection **(G)**. Frequency of patients with new medications **(H)** or dietary changes **(I)** shown, which could potentially influence gut microbiome. 28% of patients had additional potential triggers, including occupational exposures, stress, and foreign travel **(J)**. Heatmap for all identified triggers across all n=107 individual patients **(K)**.

PMR, polymyalgia rheumatica; GCA, giant cell arteritis; MS, multiple sclerosis; IgG4-RD, IgG4-related disease; ca, cancer

3.2.3 Triggers: Associations with Infections, Vaccinations and Tumours

Whilst clear HLA-associations of LGI1-autoantibody disease have been identified (Binks et al. 2018; Kim et al. 2017; van Sonderen et al. 2017), indicating a strong immunogenetic basis for the disease, evidence for any complementary/ corresponding environmental triggers is lacking. In contrast, two well-known triggers exist in NMDAR-autoantibody encephalitis: presence of an underlying ovarian teratoma (Dalmau et al. 2007; Dalmau et al. 2008) and preceding herpes simplex virus-1 encephalitis (Leypoldt et al. 2013).

3.2.3.1 Tumours

Tumours are found in up to one third (32%) of patients with CASPR2 autoantibodies (n=19), most commonly thymoma in 26% (Irani et al. 2010). The same series found no tumours in 55 patients with LGI1 autoantibodies.

In our cohort, 13% had tumours **(Figure 3.1G)**; two cancers occurred in the same patient. The most common tumours were dermatological, although the majority (basal cell carcinomas) likely represent the frequency of age-matched healthy controls **(Figure 3.2D and Table 3.3)**. Tumour detection and onset of LGI1-autoantibody syndrome coincided in those with thymomas, thyroid follicular cancer, bladder cancer and meningioma. Thymomas, which represented 14% of tumours, and thyroid cancer, are the tumour types typically associated with paraneoplastic neurological disease. From our cohort, both patients with thymomas had Morvan's syndrome (one with additional CASPR2 autoantibodies), and the thyroid cancer occurred in a neuromyotonia patient (also with CASPR2 autoantibodies). Thus, 2 of 6 (33%)

patients with additional CASPR2 autoantibodies had an associated tumour, and 1 of 6 (17%) had a thymoma.

The only tumours which recurred were the 2 malignant thymomas. Both patients with thymoma had maternal family history of bowel cancer. Finally, Bowen's disease was diagnosed in a patient who had been treated with 3 years' immunotherapy. In this case, it is possible that the Bowen's disease may have occurred due to the long-term immunosuppressive treatment, a known risk factor (Akhdari et al. 2006).

3.2.3.2 Prodromal Infections and Vaccinations

Next, we sought evidence for environmental triggers in LGI1-autoantibody syndromes. Specifically, we asked about prodromal infections or vaccinations occurring in the 3 months prior to onset. 20/91 (22%) patients with available data had prodromal infections **(Figure 3.2E)**, occurring at a median time of 4 weeks (range 0-16 weeks) prior to manifestation of LGI1-autoantibody disease symptoms. The prodromal infections were most frequently of the upper and lower respiratory tract, found in 9 of the 20 (45%); **(Table 3.3)**.

14/80 (18%) patients received vaccinations 1-8 weeks (median 3.5 weeks) prior to onset **(Figure 3.2F)**, most commonly influenza, in 11/14 (79%); **(Table 3.3)**.

32/77 (42%) had a history of prior herpes simplex virus (HSV) or varicella zoster virus (VZV) infection in the form of cold sores or shingles, respectively **(Figure 3.2G and Table 3.3)**. 2 of the 13 patients with shingles developed the infection after their encephalitis diagnosis, whilst receiving immunotherapies (one whilst taking prednisolone and mycophenolate, and the other during monthly Omalizumab therapy for spontaneous idiopathic urticaria). One patient who had no manifestations of oral cold sores for 10 years developed an eruption at simultaneous onset of LGI1-autoantibody encephalitis and Graves' disease.

3.2.3.3 Gut Microbiome

Another aetiological factor implicated across several autoimmune conditions is the gut microbiome. For example, Clostridium perfringens is overrepresented in the gut microbiome

of NMO patients and has been proposed to play a role in NMO pathogenesis (Cree et al. 2016). Specific gut microbiota patterns have yet to be explored in LGI1-autoantibody syndromes. Here, we asked about any factors which could potentially alter the gut microbiome, such as new medications, including antibiotics, or sudden dietary changes, in the 12 weeks leading up to symptom onset. 7/85 (8%) had commenced new medications prior to onset **(Figure 3.2H and Table 3.3)**. Only 2/77 (3%) patients had sudden dietary changes prior to onset of encephalitis **(Figure 3.2I and Table 3.3)**. Intriguingly, one of these patients had LGI1-specific IgA autoantibodies, which were found in only 3 patients (see **Chapter 4, Section 4.4**). While the close temporal relationship of disease onset with the dietary and medication changes found in these nine patients is of interest, it will be prudent to explore the microbiome profiles of those patients with and without preceding evidence of dietary changes, compared to controls, in future work.

3.2.3.4 Occupational Exposures

Finally, potential occupational risks were detected in 17/91 (19%), especially occupations with associated chemical or particle exposures. Some examples include one patient who worked as a farmer with regular pesticide use and dichlorodiphenyltrichloroethane (DDT) exposure 40 years prior, an abattoir worker, three carpenters, and two industrial chemists. Finally, 25/89 (28%) patients with available data reported other potential triggers, most commonly severe stress (12% of cohort) or foreign travel (10% of cohort) within the 12 weeks leading up to disease **(Figure 3.2J and Table 3.3)**.

Presence or absence of identified potential triggers are summarised across n=107 patients **(Figure 3.2K)**. This reveals some trends, with multiple potential triggers present in some patients (for example, 6 triggers are present in patient 64).

Table 3.3 Potential Triggers of LGI1-Autoantibody Disease

Trigger	Event Type n (%)	Timing in Relation to LGI1 Onset
Tumour	**14/107 (13%)**	
	• Malignant thymoma with pleural metastases (B2 subtype)	Onset
	• Malignant thymoma with pleural metastases – high grade stage 4b (B3 subtype)	Onset
	• Thyroid follicular neoplasm	Onset
	• Bladder cancer (non-invasive papillary urothelial ca)	Onset
	• Meningioma	Onset
	• BCC	5 years post
	• BCC	4 years post
	• BCC	5 years pre
	• Bowen's disease (squamous cell skin ca in situ)	8 years post
	• Bowel ca	4 years post
	• Rectal ca	3 years post
	• Oral squamous cell ca	5 years pre
	• Breast ca (ductal ca in situ)	1 year pre
	• Prostate adenoca	18 months pre
	• Penile ca	8 years pre
Prodromal Infection	**20/91 (22%)**	
	• Epididymo-orchitis	Onset
	• Dental infection plus viral meningoencephalitis	1 week pre
	• Mosquito bites	2 weeks pre
	• LRTI n=2	4 and 12 weeks pre
	• URTI n=7	8 weeks pre n=1, 4 weeks pre n=3, 1 week pre n=3
	• H. pylori gastritis	4 weeks pre
	• V1 shingles	8 weeks pre
	• Cellulitis	4 weeks pre
	• UTI	8 weeks pre
	• Diarrhoeal illness n=2	4 and 12 weeks pre
	• Tick bites n=2	12 and 16 weeks pre

Preceding Vaccination	**14/80 (18%)** • Shingles and influenza • Pneumococcal and influenza • Influenza n=9 • Travel vaccinations n=3* *No further details regarding travel vaccinations	 5 weeks pre 1 week pre 1 week pre (n=2), 2 weeks pre (n=2), 3 weeks pre (n=2), 4 weeks pre (n=2), 6 weeks pre 5, 6 and 8 weeks pre
History of prior HSV or VZV infection	**32/77 (42%)** • **HSV n=21 (66%)** • **VZV n=14 (44%)** • Shingles (n=13) ** • Adult chickenpox (n=1) **n=3 patients had both HSV and shingles infections	 First clinical manifestations of oral cold sore 2-60 years prior to onset 1-45 years prior to onset (n=11) Post onset (n=2) 30 years prior to onset
Gut Microbiome	**New medications 7/85 (8%):** • Statin (n=2) • Antibiotics (n=2; flucloxacillin, trimethoprim and nitrofurantoin) • Metformin (n=1) • Topiramate for migraine prophylaxis (n=1) • Herbal/ over-the-counter (n=2; weight loss patches from China, cranberry juice) **Dietary change 2/77 (3%):** Sudden shift from high meat consumption to entirely vegetarian plus new probiotic use (daily yoghurt drinks) for hypercholesterolaemia (n=1) Sudden shift from predominantly meat- to fish-based diet for 1 month during business trip to Thailand (n=1)	 1-8 weeks pre 3-12 weeks pre

Occupational Exposure	**17/91 (19%)** • Carpenter (n=3) • Car engineer (n=3) • Lamb carcass packager; abattoir worker (n=2) • Industrial/ analytical chemist (n=2) • Train driver (n=2) • Farmer (n=1) • Heating and ventilation engineer (n=1) • Aircraft engineer (n=1) • Printer (n=1) • Baker (n=1)	
Other Potential Triggers	**25/89 (28%)** • Significant stress/ bereavement (n=11) *** • Foreign travel (n=9): Thailand, Panama, Turkey (n=2), Spain, Portugal, France, Mediterranean, unknown • Woodwork/ house renovations/ emulsion painting (n=6) • Preceding surgery (n=2): nasoplasty with post-operative haemorrhage and haemodynamic shock; cataract surgery • Asbestos (n=2): identified on work site; regularly worked with asbestos in laboratory ***4 patients had overlap of two potential triggers (stress due to home renovation n=2, renovation of a holiday home n=1, stress and foreign travel n=1)	0-8 weeks pre 0-12 weeks pre Regularly, and 2-4 weeks pre onset 4-12 weeks pre 1 month pre onset 6 years pre onset

Details of potential triggers found in LGI1-autoantibody patients, including tumours, and environmental factors including infections, vaccinations, dietary changes, and occupational exposures. Both active and historical tumours are listed.

B2 and B3 denotes thymoma subtype; ca, cancer; BCC, basal cell carcinoma; LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection; H. pylori, Helicobacter pylori; V1, ophthalmic division of the trigeminal nerve; UTI, urinary tract infection; HSV, herpes simplex virus; VZV, varicella zoster virus

3.2.4 Seizure Semiologies

98/107 (92%) of patients had seizures. Of the remaining patients, 8 had no seizures owing to peripheral manifestations of neuromyotonia or isolated peripheral nerve syndromes, and 1 had Morvan's syndrome without associated seizures. In patients with seizures, 29/98 (30%), 33/98 (34%), 26/98 (27%), 5/98 (5%), and 5/98 (5%) had 1, 2, 3, 4 or 5 different semiologies, respectively **(Figure 3.3A)**. The median number of seizure semiologies per patient was 2. We compared this to our pharmacoresistant epilepsy cohort (n=218) whose phenotyping I contributed clinical assessment, data collection of seizure frequency and anti-seizure medications, and blood sampling for immune phenotyping. Within this group, 40/218 (18%), 110/218 (50%), 63/218 (29%), 5/218 (2%), and 0/218 (0%) had 1, 2, 3, 4 and 5 different seizure semiologies. The median number of semiologies per patient was 2 (data not shown). When the number of seizure semiologies in LGI1-autoantibody encephalitis was compared to patients within the pharmacoresistant epilepsy cohort, the proportion of patients with ≥ 3 seizure types was greater in the LGI1-autoantibody patient group (10% versus 2%, p=0.0039**; Fisher's exact test**; Figure 3.3C)**.

Seizures were classified into 11 distinct event types, including semiologies we and others have observed frequently in LGI1-autoantibody patients. The most common seizure type was FBDS (62% of patients with seizures), followed by focal with loss of awareness (44%) and generalised tonic-clonic (35%); **(Figure 3.3B)**. 7% of patients had seizures consisting of piloerection, and 2 patients (2%) had ictal tachy-/brady-cardia; one required permanent cardiac pacemaker placement due to focal seizures associated with significant ictal bradycardia.

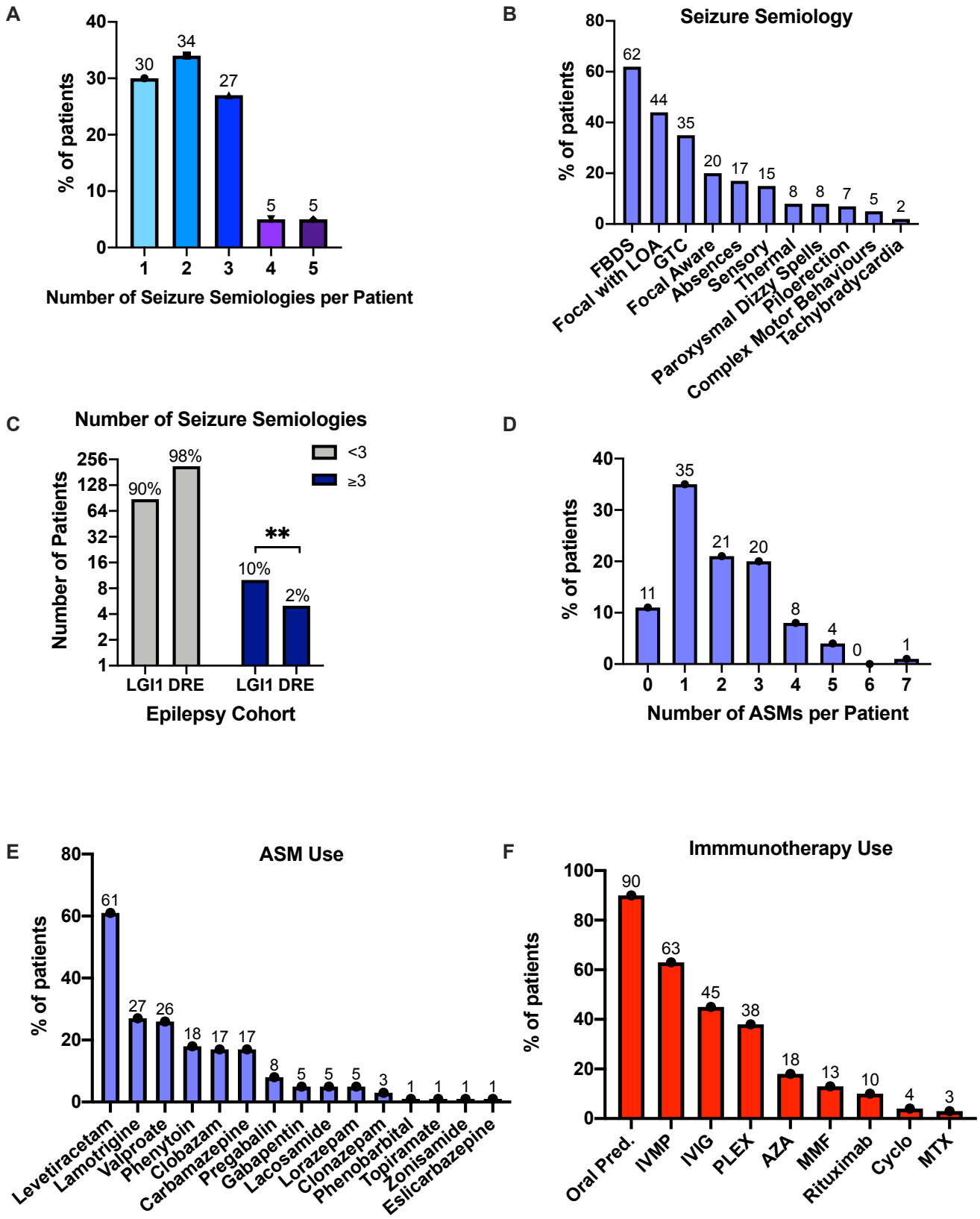
Information regarding anti-seizure medications (ASMs) was unknown in 1 patient. Of 94/106 (89%) patients treated with ASMs, the indications were as follows: 88/94 (94%) for seizures (2 of whom also had additional indication of pain), 5/94 (5%) for neuropathic pain, and 1/94 (1%) as a mood stabilizer. 88 of the 98 (90%) patients with seizures were treated with ASMs. Patients received a median number of 2 ASMs (range 0-7). The maximum number of ASMs trialled by individual patients is shown in **Figure 3.3D**. The most commonly used ASMs were levetiracetam (61%), lamotrigine (27%), and sodium valproate (26%; **Figures 3.3E and 3.3G)**.

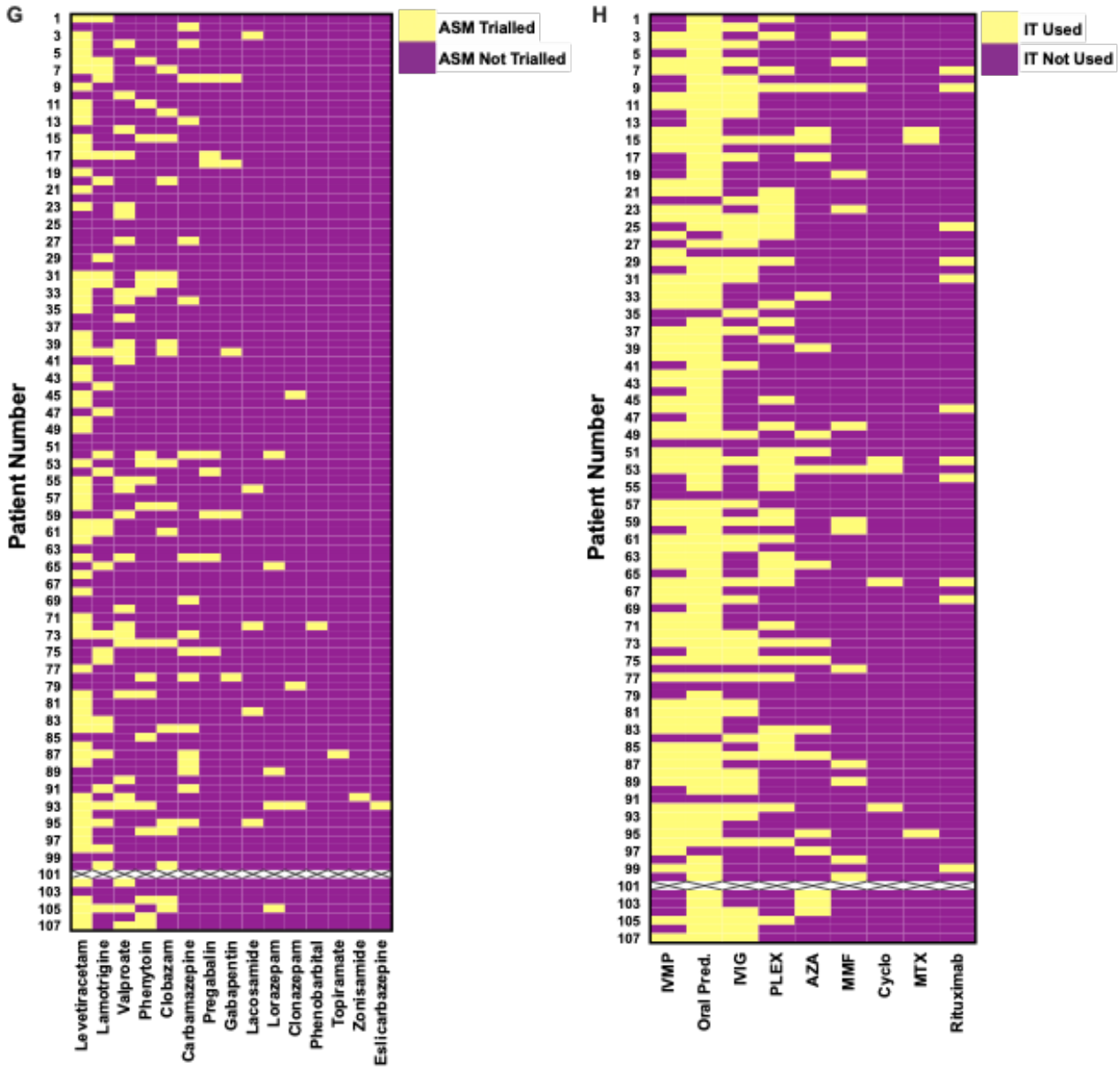
3.2.5 Immunotherapies

Information regarding immunotherapies was unknown in 1 patient. 102/106 (96%) of patients were treated with immunotherapies. 64/106 (60%) received only first-line immunotherapies such as steroids, intravenous immunoglobulin, and/or plasma exchange. The most commonly used immunotherapies were oral prednisolone (90%), intravenous methylprednisolone (IVMP; 63%) and IVIG (45%). 42/106 (40%) required second-line immunotherapies such as azathioprine, mycophenolate, and Rituximab. The median number of second-line agents trialled per patient was 1 (range 1-3). Unexpectedly, 1 of the 42 received a second-line agent without prior first-line treatments. Azathioprine was the most common second-line agent (18%; **Figures 3.3F and 3.3H)**.

Steroid-induced hyperglycaemia or diabetes, weight gain, irritability and insomnia were the most common side effects. Other select examples include cataracts, osteoporosis with fractures (n=2), proximal myopathy, and Achilles tendonitis secondary to steroids. Steroid-induced psychosis occurred in one patient. Another patient, on long-term steroids, developed multiple cerebral abscesses due to Nocardia infection.

Figure 3.3 Seizure Types, and Anti-Seizure Medication and Immunotherapy use in LGI1-Autoantibody Patients





(A) Proportion of patients with given number of seizure semiologies (1-5). Data from n=98 patients with seizures. **(B)** Proportion of patients displaying each of 11 distinct seizure types. **(C)** Comparison of proportion of patients with ≥ 3 seizure semiologies in LGI1-autoantibody versus drug-resistant epilepsy (DRE) cohorts. **(D)** Maximum number of ASMs (0-7) trialled by patients throughout disease. Data from n=106 patients. **(E)** ASM use shown by percentage of patients 'ever trialled' for each drug. Data from n=94 patients treated with ASMs. **(F)** Immunotherapy use by percentage of patients 'ever trialled' for each immunotherapy. Data from n=106 patients with available data. 4/106 (4%) were not treated with any form of immunotherapy. **(G)** ASM and **(H)** immunotherapy use across cohort: each of n=107 patients are shown across rows (alternate patient numbering). 15 different ASMs **(G)** and 9 different immunotherapies **(H)** are denoted in columns, where yellow indicates drug use in that particular patient, and purple indicates that the drug was not trialled. One patient with no available treatment data is demarcated by 'X'.

LOA, loss of awareness; GTC, generalised tonic clonic; DRE, drug-resistant epilepsy; ASM, anti-seizure medication; Pred., prednisolone; IVMP, intravenous methylprednisolone; IVIG, intravenous immunoglobulin; PLEX, plasma exchange; AZA, azathioprine; MMF, mycophenolate; Cyclo, cyclophosphamide; MTX, methotrexate.

3.2.6 Relapses

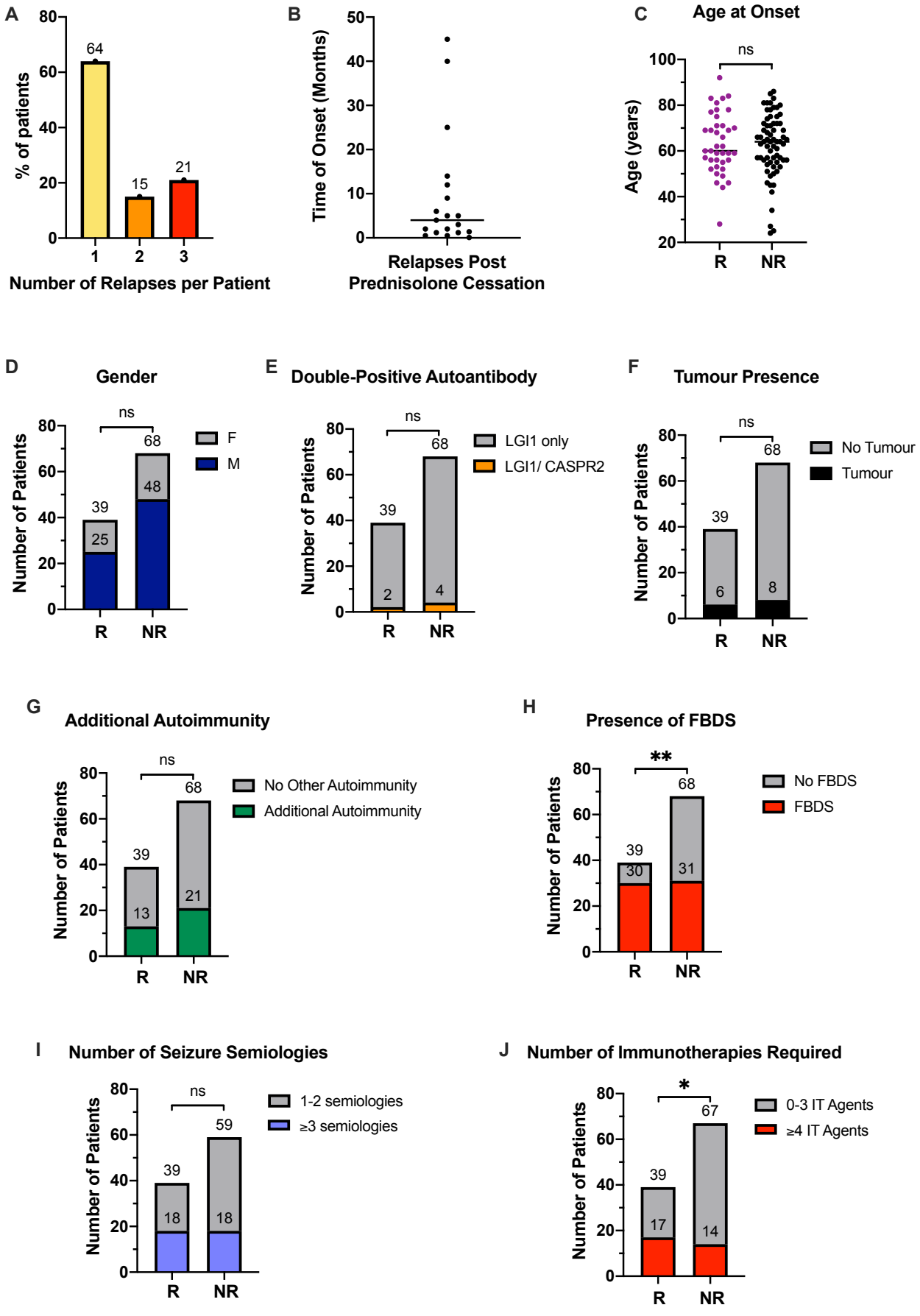
Next, we identified the proportion of patients with relapses, and examined relapse patterns. 36% (39/107) of patients had relapses **(Figure 3.1F)**. A total of 61 relapses occurred in 39 patients, with a median of 1 relapse per patient (range 1-3); **(Figure 3.4A)**. Strikingly, 36% of relapsing patients showed multiple relapses **(Figure 3.4A)**. In the patients with relapses, only 10 (26%) were reinvestigated for tumours at the time of subsequent relapse(s). Details regarding 2 of the 61 relapses were unknown. Of the 59 relapses with available information:

- 28/59 (47%) occurred during prednisolone wean
- 19/59 (32%) occurred 3 days – 45 months after prednisolone cessation
- 4/59 (7%) occurred during weaning or 1-12 weeks after cessation of mycophenolate, methotrexate or azathioprine
- 3/59 (5%) occurred shortly after reduction of ASM dose
- 3/59 (5%) showed no potential trigger
- 2/59 (3%) occurred in immunotherapy-naïve patients

The most common precipitant of disease relapses was found to be weaning of prednisolone dose (47%). Relapses which developed in patients at any time after prednisolone cessation were also explored. Such relapses occurred at a median time of 4 months following discontinuation of prednisolone (range 3 days – 45 months); **(Figure 3.4B)**. Of these, 4 relapses occurred in 3 patients despite concurrent therapy with either mycophenolate or azathioprine. Additionally, one of the patients without an obvious trigger relapsed despite 6 doses of cyclophosphamide, and one of the patients who relapsed during prednisolone weaning did so despite concurrent mycophenolate therapy. These data suggest that this subgroup of patients may be preferentially sensitive to the immunomodulatory effects of steroids. The majority of the cohort (90%), however, received oral prednisolone (**Figure 3.3F)**, yet only 36% of the cohort developed clinical relapses **(Figure 3.1F)**. Moreover, 3% of relapses occurred in immunotherapy-naïve patients (typically first episodes treated with ASMs alone), and, crucially, just over one third of relapsing patients showed multiple relapses **(Figure 3.4A)**. Thus, relapses *per se* are not fully explicable by selective steroid dependency.

We examined several potential risk factors for relapses by comparing their associations in the relapsing (n=39) and non-relapsing (n=68) patient groups. Firstly, age did not appear to associate with relapse (median age at disease onset 60 versus 64 years in relapsing and non-relapsing patients, respectively; p=0.90, Mann-Whitney); **(Figure 3.4C)**. Gender did not influence risk of relapse (64% male versus 71% in relapsing and non-relapsing patients, respectively; p=0.52, Fisher's exact test); **(Figure 3.4D)**. Presence of additional CASPR2 autoantibody was not associated with relapse (5% in relapsing versus 6% in non-relapsing patients; p>0.99, Fisher's exact test); **(Figure 3.4E)**. Similarly, tumours did not significantly correlate with relapses (present in 15% versus 12% of relapsing and non-relapsing patients, respectively; p=0.77, Fisher's exact test); **(Figure 3.4F)**. Presence of additional autoimmunity was no more frequent in the relapsing group (33% versus 31% in non-relapsing group; p=0.83, Fisher's exact test); **(Figure 3.4G)**. FBDS, however, appeared to confer risk of relapse, present in 30/39 (77%) relapsing patients, compared to less than half (31/68; 46%) of non-relapsing patients (p=0.002**, Fisher's exact test; no p value correction for multiple comparisons); **(Figure 3.4H)**. Higher numbers of seizure semiologies did not appear to associate with relapses, and 46% of relapsing patients had 3 or more seizure semiologies compared to 31% of those without relapses (p=0.14, Fisher's exact test); **(Figure 3.4I)**. Finally, number of immunotherapy agents trialled significantly associated with relapse. 17/39 (44%) of relapsing patients required 4 or more immunotherapies during their disease course compared to only 14/67 (21%) of non-relapsing patients (p=0.016*, Fisher's exact test); **(Figure 3.4J)**. Whilst this finding likely reflects increased use of immunomodulatory agents as a consequence of relapse, it may suggest that relapsing patients are more refractory to immunotherapy compared to non-relapsing patients.

Figure 3.4 Relapse Characteristics of LGI1-Autoantibody Patients



(A) Proportion of patients with given number of relapses (1-3) shown. Data from n=39 patients with relapses. **(B)** Distribution of relapse onset time following prednisolone discontinuation in 19 relapses which occurred at any time after prednisolone cessation (median onset time 4 months; range 3 days – 45 months). **(C)** Comparison of age at initial disease onset in relapsing (R; n=39) and non-relapsing (NR; n=68) patients. **(D)** Gender distribution amongst relapsing and non-relapsing patients. Comparison of proportion of patients with additional CASPR2 autoantibody **(E)**, tumour **(F)**, additional autoimmune disease **(G)**, and FBDS **(H)**, within relapsing and non-relapsing groups. Proportion of patients with 3 or greater seizure semiologies **(I)**, and those requiring 4 or more immunotherapy agents throughout disease duration **(J)** in relapsing versus non-relapsing patients.

IT, immunotherapy

3.2.7 Investigations

3.2.7.1 Ancillary Investigation Findings

Serum sodium level was unknown in 35/107 patients. Hyponatraemia was detected at presentation in 51/72 (71%) patients **(Figure 3.5A)**. Median sodium level in those with hyponatraemia was 126 mmol/L (range 109-134 mmol/L); **(Figure 3.5B)**.

67/105 (64%) patients with available data underwent CSF analysis. CSF was abnormal in 35/67 (52%), either due to elevated white cell count and/ or protein, and/ or presence of CSF LGI1 or CASPR2 autoantibodies **(Figure 3.5C)**. Only 2 patients had an elevated protein (≥0.6 g/L) in isolation.

102 patients with available data had MRI brain imaging. MRI was abnormal in 62/102 (61%); **(Figure 3.5D)**. Acutely, radiological findings relevant to disease were considered abnormal. These included hippocampal swelling and increased T2-weighted signal within the hippocampus, amygdala, temporal lobe or basal ganglia, but not other findings such as small vessel disease. Other studies found abnormalities in 73-74% at initial MRI, with hippocampal T2 hyperintensity (van Sonderen, Thijs, et al. 2016; Finke et al. 2017).

79/107 (74%) patients underwent electroencephalography (EEG). EEG was abnormal in 52/79 (66%); **(Figure 3.5E)**. 32/52 (62%) had epileptiform discharges and 35/52 (67%) had features of encephalopathy with generalised slowing.

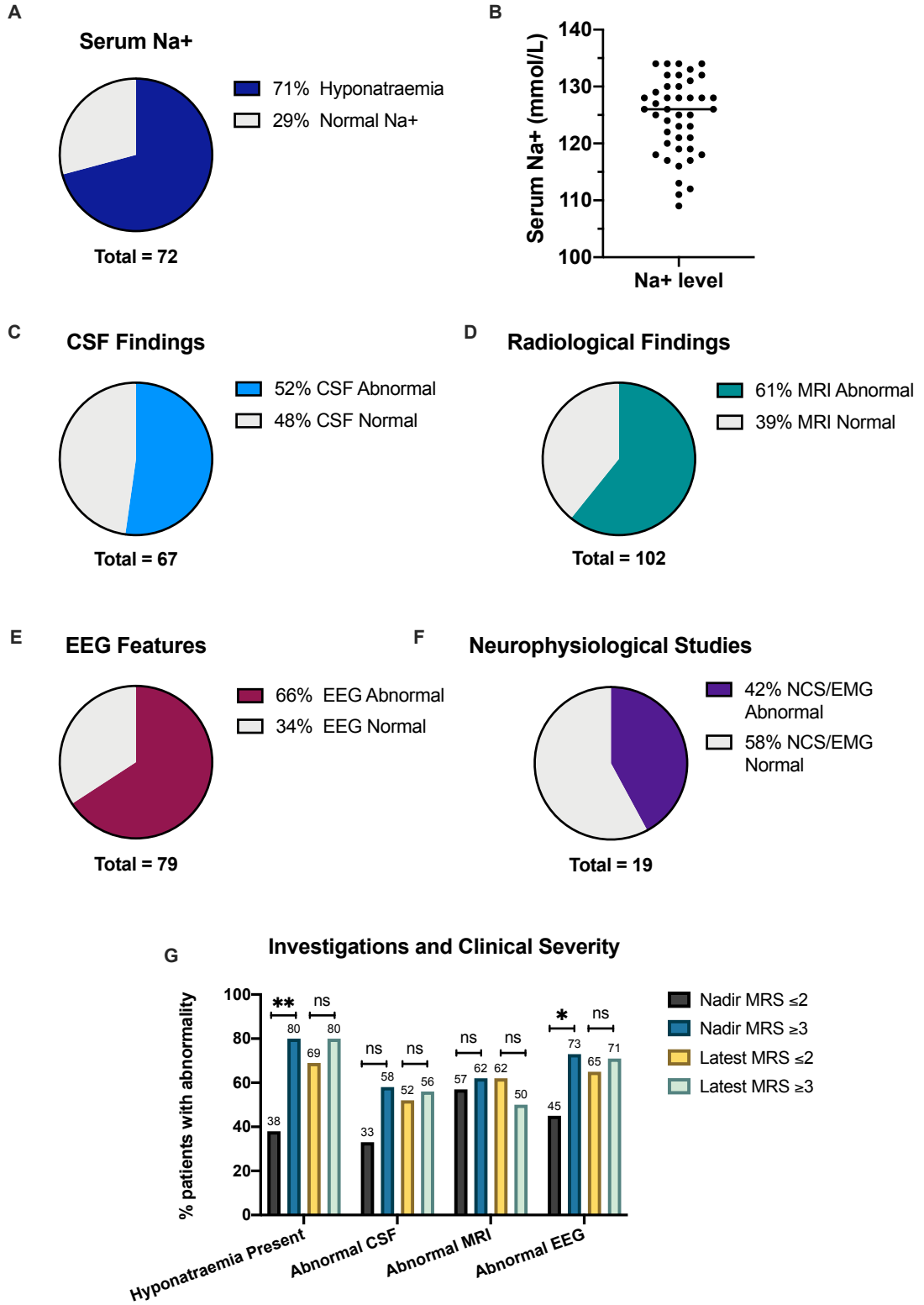
Only 19/107 (18%) patients had nerve conduction studies plus electromyography. These were abnormal in 10/19 (53%), with 8/19 (42%) having findings of peripheral nerve hyperexcitability or peripheral neuropathy **(Figure 3.5F)**. Carpal tunnel syndrome was found in one patient, and another showed active and chronic denervation, consistent with a concurrent diagnosis of anterior horn cell disease.

4 of the 107 patients had no documented evidence of whether paraneoplastic imaging screening was undertaken at onset. Of the remaining 103, 80 (78%) had imaging, typically CT or PET-CT of the thorax, abdomen and pelvis in 79/80 (99%); one patient had a skeletal survey only. Only 5/107 patients (5%) were tested for additional paraneoplastic antibodies or tumour markers at onset. 23/103 (22%) did not undergo any form of paraneoplastic screening (whole body imaging, pelvic ultrasound, or paraneoplastic neuronal antibody panel).

3.2.7.2 Ancillary Investigations and Clinical Outcomes

Next, we studied whether clinical outcomes related to investigation findings. Presence of hyponatraemia and abnormal EEG both significantly associated with clinical severity, occurring in a higher proportion of patients with nadir MRS ≥3 compared to those with nadir MRS ≤2 (hyponatraemia 80% versus 38%, respectively, p=0.002**; abnormal EEG 73% versus 45%, respectively, p=0.031*; Fisher's exact test). The presence of abnormal CSF or MRI showed non-significant trends towards higher clinical severity. None of hyponatraemia, abnormal CSF, MRI, or EEG found at initial investigations correlated with the final ascertained (latest) MRS **(Figure 3.5G)**.

Figure 3.5 Investigation Findings in LGI1-Autoantibody Patients



(A) Proportion of cohort with hyponatraemia at initial investigations. **(B)** Distribution of serum sodium level in patients with hyponatraemia (n=51). Percentage of patients with abnormal CSF **(C)**, MRI head **(D)**, EEG **(E)**, and neurophysiological studies **(F)**, at initial investigation. **(G)** Correlation of abnormal investigation findings with clinical severity at disease nadir (nadir MRS ≤2 versus MRS ≥3) and with clinical outcomes (latest MRS ≤2 versus MRS ≥3).

Na+, sodium; CSF, cerebrospinal fluid; EEG, electroencephalogram; NCS, nerve conduction studies; EMG, electromyography; MRS, Modified Rankin Scale

3.2.8 Clinical Features

3.2.8.1 Common Clinical Features Across Cohort

We examined the frequency of clinical features associated with LGI1-autoantibodies in our patient cohort **(Table 3.4)**. Cognitive impairment was present in almost all patients (92%), as previously described [90-97% through (Irani et al. 2011; Irani et al. 2010; Aurangzeb et al. 2017; van Sonderen, Schreurs, et al. 2016; Finke et al. 2017)]. FBDS were present at rates similar to those reported previously (van Sonderen, Schreurs, et al. 2016; Irani et al. 2011; Finke et al. 2017), whilst NMT and Morvan's syndrome were rare (8% and 3%, respectively). Only 5% (5/107) of our cohort had a clinical syndrome of isolated NMT **(Figure 3.1H; Table 3.1)**. These observations are consistent with previous studies, which found NMT in 2-4% of those with LGI1-autoantibodies, with a clinical syndrome of isolated NMT in 0-2%, and Morvan's syndrome in 2-4% (Binks et al. 2018; Irani et al. 2010).

The proportion of patients within our cohort with peripheral nerve involvement including neuropathic pain or peripheral neuropathy was 18%. Other studies indicate frequencies of neuropathic pain ranging from 7% (Binks et al. 2018) to ~20% (Gadoth et al. 2017; Ramanathan et al. 2021). Of those with neuropathic pain and available neurophysiological studies, abnormalities including peripheral neuropathy or peripheral nerve excitability were detected in 42% **(**8/19; **Figure 3.5F)**. Others have shown similar cumulative nerve conduction or EMG abnormality rates of around 35% (Gadoth et al. 2017; Ramanathan et al. 2021) – typically normal nerve conduction studies – with reduced intraepidermal nerve fibre densities (Ramanathan et al. 2021). Isolated peripheral nerve syndrome was rare, present in 3% (3/107) of our patient cohort **(Figure 3.1H; Table 3.1)**.

Sleep disorders were surprisingly common, present in 70%, with insomnia as the most frequent sleep complaint, found in around half (55%). Contrastingly, previous studies report presence of any sleep disturbance in only 20-48% of patients with LGI1-autoantibodies (Irani et al. 2010; Irani et al. 2011; Finke et al. 2017; Gadoth et al. 2017), and insomnia in only 7-29% (Irani et al. 2010; Gadoth et al. 2017; Blattner 2019). Others found yet lower frequencies of any sleep disorder in as little as 7%, and insomnia in 4% (Klein et al. 2013). Only two studies report presence of a sleep disorder comparable to our determined rate (86%; 6/7 and 93%;

14/15), albeit from smaller series focused on the sleep abnormalities in these patients (Blattner 2019; Cornelius et al. 2011). In contrast, insomnia is present in most (90%) CASPR2-autoantibody patients (Irani et al. 2012). Patients with LGI1-autoantibody encephalitis show prominent sleep fragmentation on polysomnography, with reductions in sleep efficiency, REM sleep and sleep spindles, and even their complete absence (Serdaroglu et al. 2018; Blattner 2019; Cornelius et al. 2011; Barone & Krieger 2016). Those with secondary bilateral hippocampal damage show reduced slow-wave sleep on polysomnography compared to controls, which is hypothesised to in turn affect memory consolidation during sleep, and may be contributory to the amnestic cognitive impairments seen in these patients (Spanò et al. 2020). Interestingly, 11% of our patient group suffered parasomnias, a feature recently associated with LGI1-autoantibody encephalitis (Blattner 2019).

Psychiatric features were also common, with anxiety in 42/107 (39%), psychosis in 38/107 (36%), and depression in 29/107 (27%), seen at any point in their illness, including more significant manifestations such as suicidal ideation and attempts in a few patients (6/107; 6%). Dysautonomia was found in 25% of patients; previous studies report rates of 15-23% (Irani et al. 2010; Irani et al. 2011; Gadoth et al. 2017). Finally, approximately one quarter of our cohort sustained falls, and 36% of those with falls sustained significant injuries including bone fractures or dislocations, or head injury. Importantly, the commonest association of falls was FBDS (84%, 21/25; **Table 3.4**). Indeed, patients with FBDS were at significantly higher risk of falls, since 34% (21/61) sustained falls, compared to only 9% (4/46) of patients without FBDS (p=0.0023**; Fisher's exact test; **Figure 3.6**).

Table 3.4 Frequency of Clinical Characteristics in LGI1-Autoantibody Disease

Clinical Feature	**Present n (%)**
Cognitive impairment	99/107* (92%) *Absent in 8/107 (NMT n=3, LE n=2, FBDS only n=2, PN n=1)
FBDS	61/107 (57%)
NMT	9/107 (8%)

Morvan's syndrome	3/107 (3%)
Sleep disorder - Insomnia - Hypersomnolence - Sleep-wake reversal - Parasomnia	75/107 (70%) 59[$] (55%) 34 (32%) 19 (18%) 12[ƒ] (11%) [$] 9/59 insomnia not attributed to disease (steroid-insomnia n=5, pain-related n=3, nocturnal seizures n=1) [ƒ] parasomnia consisting acting out dreams (n=8) and/or nightmares/ vivid dreams (n=7)
Psychiatric manifestations - Anxiety - Psychosis / mania / hallucinations / paranoia - Depression - Suicidal ideation	63/107 (59%) 42/107 (39%) 38/107 (36%) 29/107 (27%) 6/107 (6%) [‡] [‡] suicidal attempts n=2, n=1 plotted to kill spouse
Behavioural change Emotionality, irritability, aggression (verbal/ physical), coprolalia, impulsivity, apathy, disinhibition	80/107 (75%)
Dysautonomia Hyperhidrosis, erectile dysfunction, urinary retention, diarrhoea, tachy-/bradycardia, facial flushing, temperature dysregulation	27/107 (25%)
Neuropathic pain/ peripheral neuropathy	19/107 (18%)
Movement disorder Akathisia, parkinsonism, upper limb tremors and myoclonus associated with NMT, chin tremor	5/107 (5%)
Falls or gait problems	28/107 (26%) **Falls 25/107 (23%): with significant injury sustained in 9/25 (36%)**:

	- Fractures or dislocations n=5 (hip, wrist, vertebral, ankle, shoulder dislocation) - Head or facial injury n=5 (scalp laceration/ haematoma/ periorbital haematoma) - 1 patient suffered scalp laceration plus fracture **Imbalance** 3/107 (3%)
Complex motor behaviours	5/104[¥] (5%) [¥] n=3 unknown; no available data
Other: significant weight loss (2-3 stones) in the absence of tumour	3/107 (3%)

Summary of various clinical features characterised in LGI1 autoantibody patients and their frequency across our patient cohort.

NMT, neuromyotonia; LE, limbic encephalitis; FBDS, faciobrachial dystonic seizures; PN, peripheral neuropathy

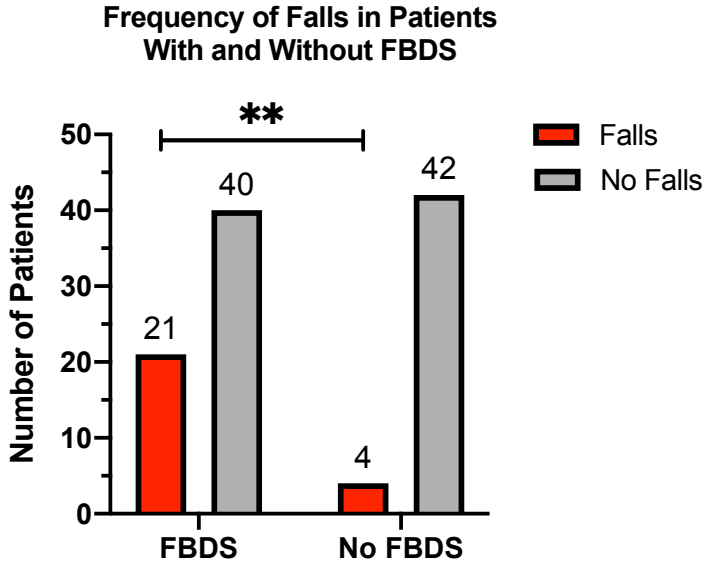


Figure 3.6 Frequency of Falls in Patients with and without FBDS

Data from LGI1-autoantibody patient cohort (n=107) plotted. 21/61 patients with FBDS sustained falls (red), compared to only 4/46 of patients without FBDS (34% versus 9%, respectively; p=0.0023**, Fisher's exact test).

3.2.8.2 Novel Clinical Features

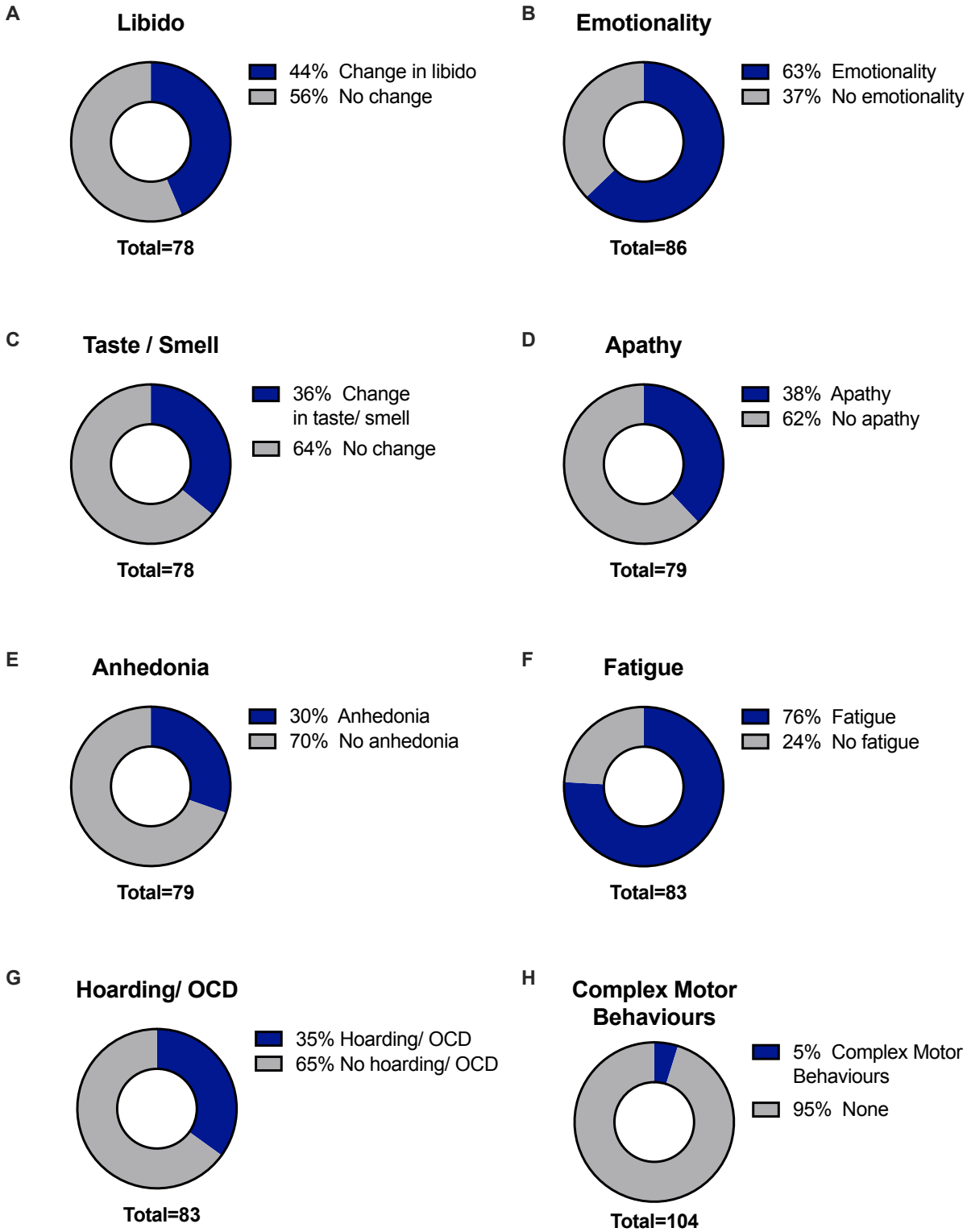
To establish whether the novel clinical features observed in select patients **(Sections 3.1.1 and 3.1.2)** were present across the wider cohort of LGI1-autoantibody patients, we conducted clinical interviews with patients and their relatives which included a specific set of questions inspired by our clinical observations.

Almost half of patients with available data (34/78; 44%) reported change in libido **(Figure 3.7A)**. Most reported loss of libido (31/34), 2/34 reported increased libido, and 1/34 experienced both (initial loss at disease onset with subsequent hypersexuality). Nearly two thirds of patients experienced significant emotionality (54/86; 63%) from disease onset, which persisted for several years at time of latest follow-up **(Figure 3.7B)**. Examples include crying with innocuous stimuli, most commonly with patients becoming tearful whilst watching television programmes such as DIY shows, football, news, X-factor, animal programmes and Disney movies. The emotionality was a profound feature, to the degree that some clinicians described it as 'emotional incontinence'. 28/78 (36%) experienced a change in taste or smell **(Figure 3.7C)**: 17/28 developed a sweet food preference, 8/28 suffered anosmia or dysgeusia, and 3/28 reported both sweet food preference and loss of taste or smell. 30/79 (38%) displayed apathy **(Figure 3.7D)**, and 24/79 (30%) anhedonia **(Figure 3.7E)**. A high proportion of patients (63/83; 76%) suffered fatigue that considerably impacted day-to-day activities, and persisted in the majority for several years at latest follow-up **(Figure 3.7F)**. Some patients reported fatigue as moderate or severe even years from disease onset.

Over one third of patients (29/83; 35%) exhibited new hoarding, or obsessive-compulsive, ritualistic behaviours, either self-reported, or reported by relatives **(Figure 3.7G)**. 22/29 displayed hoarding or obsessive-compulsive tendencies, and 10/29 exhibited new overspending; 3/29 patients had both features. Detailed review of the new, obsessive traits revealed examples such as obsessively stacking plates, and repeated checking of household appliances. Compulsive cleaning or tidying was common, found in 8 of the 20 with obsessive-compulsive behaviours. Rituals included the need to purchase a new plastic toothbrush during the weekly supermarket shopping trip. Punding behaviours were evident in several patients, including repeated, purposeless alignment of household items. One patient was

reported to spend at least thirty minutes assigning numbers to vegetables and meats on their plate, and repeated arrangement of the food items – a ritual which became necessary prior to consumption of any meal. Hoarding was pronounced in several patients, including hoarding of tools, cutlery, or unnecessary gadgets. Specific examples include the hoarding of excessive amounts of fishing equipment, which filled an entire garden shed in one patient. In another, all bedroom drawers were filled with hundreds of packs of makeup pads. Another patient hoarded various items under the household sofa, including hundreds of collected lottery scratch cards, CDs, DVDs, and various items found on the street. The patient also began shoplifting, specifically for chocolates and biscuits, and secretly binged on these items at home, in order to satisfy his sweet food cravings (this patient was one of the seventeen with new sweet food preference). Another patient reportedly repeatedly 'stole' items from the ward during his inpatient admission. Overspending was marked, typically with impulse buying of unnecessary items, or purchasing lavish gifts for relatives. An extreme example included one patient who had invested nearly £200,000 in wine.

Figure 3.7 Novel Clinical Features in LGI1-Autoantibody Patients



A) Proportion of patients with change in libido (loss, gain, or both). **(B)** Proportion of patients with emotionality. **(C)** Percentage of patients reporting change or loss in taste and/ or smell, and/ or new sweet taste preference. Percentage of patients affected by apathy **(D)**, anhedonia **(E)**, and fatigue **(F)**. Proportion of patients in whom hoarding or obsessive-compulsive traits **(G)** and complex motor behaviours **(H)** were observed.

OCD; obsessive-compulsive disorder

3.2.8.3 Complex Motor Behaviours: a novel seizure semiology or sleep disorder?

3.2.8.3.1 Index Case: Initial Observations

We report an index case of a 70-year-old male (JR037) with new-onset LGI1-autoantibody encephalitis who presented with novel, unusual complex manual behaviours. The events were carefully documented by the patient's family, including sequential videos. Attacks typically occurred during sleep or periods of relaxed wakefulness, for instance whilst the patient was sat on his sofa, or during short naps. They consisted of the patient acting out intricate manual behaviours using imaginary objects, for example drinking from a cup **(Figure 3.8A)**, writing **(Figure 3.8B)**, winding a piece of string around the fingers **(Figures 3.8 C and D)**, or counting and subsequent ingestion of tablets **(Figure 3.8E)**. The "pill-taking" events were stereotyped, only taking the form of one of the four aforementioned behaviours. There was associated partial loss of awareness and confusion, with occasional references to his deceased cat. There were temporally associated jerks of the lower limbs, consistent with FBDS. The events were followed by a short period of confusion lasting several minutes, and occurred at a frequency of 2-3 per day at onset of LGI1-autoantibody encephalitis. At the time of presentation, immediately prior to his first hospital admission, the episodes increased in frequency to 2-hourly, and one stereotyped event immediately preceded his first generalized tonic clonic seizure (GTCS). Notably, the complex motor behaviours occurred alongside multiple seizure semiologies, including FBDS, GTCSs, and brief dizzy episodes with associated aphasia, orofacial automatisms including chewing, and confusion lasting several seconds, consistent with focal seizures with loss of awareness.

3.2.8.3.2 Index Case: Investigations and Progress

Patient JR037 underwent several EEGs, including prolonged recording, which revealed evidence of global cortical dysfunction with slowing at 7-8Hz, but no epileptiform features. No electrographic correlate was captured during the complex manual automatisms, nor during episodes of brief lower limb dystonic jerking, consistent with FBDS. He was treated with immunomodulatory therapy including intravenous immunoglobulin (IVIG), high dose intravenous methylprednisolone (IVMP) with oral prednisolone taper, and later, mycophenolate 1g twice daily. Additionally, he was treated with the ASMs levetiracetam 1.5g

twice daily and phenytoin 300mg nocte. He showed excellent response with rapid resolution of all seizure types, and the unusual complex motor behaviours, within 3 weeks of simultaneous initiation of immunomodulatory and ASM therapy.

3.2.8.3.3 Identification of Complex Motor Behaviours Across Cohort

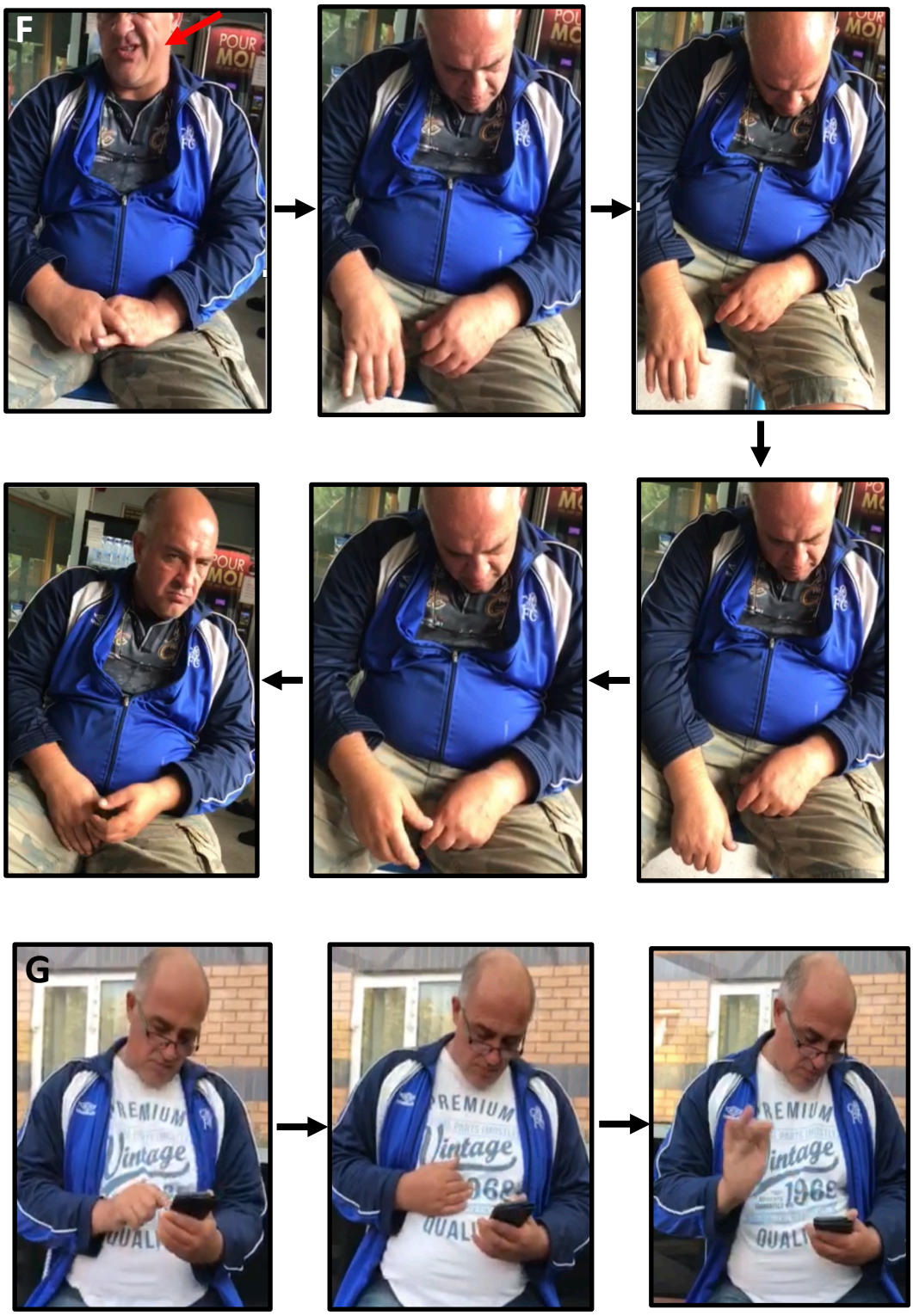
We identified a similar phenotype of unusual complex motor behaviours in another 50-year-old male patient with new-onset LGI1-autoantibody encephalitis (JR932). Events typically occurred whilst the patient was sitting in a relaxed state, and consisted of complex manual movements where he appeared to be manipulating non-existent string. The events were associated with confusion, with references made to dwarves. One such episode is depicted in **(Figure 3.8F)**, where he appears to experience a left sided FBDS, shortly followed by loss of awareness or sleep. Subsequently, he begins to mimic pulling and winding of imaginary string over 60 seconds. There appear to be associated jerks of both upper limbs, following which he regains awareness. He exhibited separate episodes consisting repetitive, habitual scrolling through his mobile phone without purpose multiple times daily which were associated with unusual upper limb movements and coprolalia **(Figure 3.8G)**. These phenomena occurred immediately prior to his initial presentation. Interestingly, the episodes fully resolved within a few days of treatment with lamotrigine, prior to initiation of any immunotherapies.

Given the observed striking similarities in these two patients, we investigated whether these unusual, intricate 'complex motor behaviours' were present across the wider LGI1-autoantibody cohort **(Section 3.1.2; Aims)**. Of patients with available data from clinical notes or patient and/or spouse interviews, 5 of 104 (5%) had evidence of 'complex motor behaviours' **(Figure 3.7H; Table 3.4)**. Their particular 'task imitation' descriptions and individual associations are summarised in **Table 3.5**. In all 5 patients, onset of complex motor behaviours occurred early in disease, typically within weeks 1-2 of symptom onset; in patient JR022, the recurrence of such behaviours occurred in week 1 of a relapse. Other similarities observed across the 5 patients include that the behaviours were highly stereotyped, consisting only 1-3 forms, short-lived (between 30-60 seconds), and associated with a loss of awareness or a relaxed/ sleep state. Patients 1 (JR037) and 3 (JR932) shared striking similarities since they both exhibited episodes where they appeared to manipulate string

through their fingers during apparent sleep, and episodes were associated with FBDS. Patients 1 (JR037) and 2 (JR022) shared similar behaviours including frantically searching for apparently missing items under couch cushions. All patients had high number of other seizure semiologies, ranging from 3-5 (median 4 seizure types), with complex motor behaviours included as a presumed seizure semiology. Also, all patients had a sleep disorder, all exhibited new obsessive-compulsive tendencies or overspending, and all had visual hallucinations.

Figure 3.8 Novel Complex Motor Behaviours in LGI1-Autoantibody Patients





Illustrative stills taken from video footage of patients with novel, unusual manual complex motor behaviours. The index case, a 70-year-old male (JR037) is shown carrying out specific tasks using imaginary objects. These consisted drinking from a cup **(A)**, writing **(B)**, winding string around the fingers **(C and D),** and counting imaginary tablets in the palm of his hand followed by their subsequent ingestion over 45 seconds **(E)**. Patient JR932 displayed almost identical behaviours, with one such episode depicted in **(F)**, which began with left sided FBDS (red arrow, top left image), followed by manipulation of non-existent string over 60 seconds prior to regaining awareness (bottom left image). Patient JR932 demonstrated further unusual repetitive manual behaviours whilst using his mobile phone device **(G)**.

Table 3.5 Features of Novel Complex Motor Behaviours

Clinical Characteristic	Patient 1 JR037	Patient 2 JR022	Patient 3 JR932	Patient 4 JR853	Patient 5 JR1142
Age and sex at onset	70 M	70 M	50 M	72 M	81 F
Syndromic diagnosis	LE plus FBDS; relapsing	LE; relapsing	LE plus FBDS; monophasic	LE plus FBDS; monophasic	LE plus FBDS; monophasic
Complex Motor Behaviour Description	1. Counting and ingestion of imaginary tablets from the palm of hand 2. Drinks water from an imaginary cup 3. Manipulation of imaginary string through fingers	1. Repeated opening and closing of window at home or freezer door at supermarket Recurrence at relapse: 1. imitates eating imaginary food 2. imitates repair of imaginary engines with screws and screwdriver	1. Manipulation of imaginary string – almost identical to patient 1 2. Unusual repetitive manual behaviours whilst using mobile phone with coprolalia	Repeatedly hooking coat (true object) on and off wall hook.	Suddenly looks up, raises right arm, points index finger and begins to imitate conducting an orchestra in 'image of 4'.
Duration	45 seconds	30-60 seconds	60 seconds	60 seconds	60 seconds
Frequency	Twice weekly clusters of 2-3/day increasing to 2-hourly immediately preceding first GTC	1-2/day at onset 3/day at relapse for 1 week	1/week	2 isolated episodes	2-3/week
Timing in relation to disease	Onset (weeks 1-10)	Onset (weeks 1-3) and at relapse 9 years post onset	Onset (weeks 2-6)	Onset (weeks 1-4)	Onset (weeks 6-26)
Treatment response (of complex motor behaviours)	Complete cessation over a few days post IVIG and IVMP (week 10 post onset)	Spontaneous resolution at week 3 without treatments	Resolution within few days of lamotrigine therapy; no immunotherapy	Spontaneous resolution by week 4 without treatments	Complete resolution after day 1 PLEX (week 26 post onset); limited effect of levetiracetam at week 4 and IVMP at week 16
Associated loss of awareness?	Yes – typically appears to fall asleep; occasionally partial loss of awareness - vacant and staring but able to interact.	Yes – appears dazed but able to interact.	Yes	Yes	Yes – vacant and staring
Associated with sleep/	Yes – whilst sat on couch.	Yes – sat on couch.	Yes – sat in chair. Appears to fall asleep.	No	Yes – sat in chair.

relaxed state?	Appears to fall asleep.				
Other associated features during episodes	References to his deceased cat Frantically searches for missing tablets under couch cushions Frequently associated with lower limb FBDS; 1 episode immediately preceded first GTC	"I'm looking for a datum point" Reports flashing neon lights Frantically searches for missing screws under cushions – remarkably similar to patient 1	References to gnomes Frequently associated with FBDS	Odd monologue: "It's all down to the Mad Hatter, you know".	-
Seizure semiologies	**5** 1.FBDS 2.GTCs 3.focal temporal lobe seizures with loss of awareness 4.focal aware seizures 5. complex motor behaviours	**3** 1.Thermal seizures 2.focal temporal lobe seizures with loss of awareness 3.complex motor behaviours	**3** 1.FBDS 2.absences 3.complex motor behaviours	**5** 1.FBDS 2.GTCs 3.focal aware with loss of awareness 4.focal sensory seizures 5.complex motor behaviours	**4** 1.FBDS 2. absences 3.GTCs 4.complex motor behaviours
Associated tumour	No	Bowen's disease (squamous cell skin carcinoma in situ) at relapse	No	No	No
Presence of movement disorder	No	No	No	No	No
Presence of sleep disorder	Yes: profound daytime hypersomnolence. New-onset vivid dreams	Yes: sleep-wake reversal with insomnia and profound daytime hypersomnolence	Yes: sleep-wake reversal with insomnia and profound daytime hypersomnolence	Yes: marked sleep-wake reversal with insomnia and daytime hypersomnolence	Yes: sleep-wake reversal with insomnia and daytime hypersomnolence. New onset vivid dreams (improved post PLEX)
Visual hallucinations	Yes: saw young nieces and nephews in house; saw deceased cat and neighbour from childhood 60 years ago; retrograde	Yes: water all over carpet, spiders and ants	Yes: gnomes	Yes: small animals seen	Yes: deceased father (deceased 50 years previously). References to retrograde memories of school friends and deceased neighbour from 30 years ago
New obsessive compulsive/ ritualistic behaviours/ overspending	From onset and persistent at 4 years: 1.new compulsion to buy new plastic toothbrush during	From onset and persistent at 9 years: new hoarding of tools and cutlery	From onset new overspending: Purchasing lavish gifts for loved ones; hundreds of pounds worth	From onset new overspending and impulse buying (e.g. expensive Rolls Royce spares, concert tickets with no	From onset and persistent at 2 years: 1.new rituals - assigns numbers to all food items on plate and

	weekly supermarket shop 2.obsessively stacking plates at home; during inpatient admission, collected plates of other patients and stacked on hospital trolley			intention of attendance, etc).	meticulously arranges foods in specific orders for up to 2 hours before consumption (e.g. "this broccoli is number 17") 2.collects excessive food items including sandwiches and chocolates for storage in her bedside cupboard
MRI brain	T2W signal change in mesial temporal lobes bilaterally	T2W signal change and swelling in mesial temporal lobes bilaterally R>L (amygdala and hippocampus) 9 years post onset, at relapse: right mesial temporal lobe atrophy; subtle increased signal change L hippocampus	Normal	T2W signal change hippocampi bilaterally L>R	T2W signal change left temporal lobe
EEG	Standard and prolonged EEGs: encephalopathy (global cortical dysfunction; slowing at 7-8Hz). No electrographic correlate during complex manual automatisms of right hand or lower limb FBDS	**Nadir:** right temporal slow waves and runs of rhythmic theta; transient right temporal sharp waves **1 week post relapse onset:** encephalopathy (generalised slowing 7-8Hz); focal intermittent right temporal sharp waves associated with episodes of confusion	Standard EEG normal	Encephalopathy (slowing with excess intermittent theta activity)	Encephalopathy (excess theta activity bilaterally); focal slow waves over left temporal region
Occupation	Vodafone warehouse manager; welder	Car engineer	Security guard	Accountant; New Zealand lamb carcass packager for 10 years	Housewife

Features and associations of novel complex motor behaviours observed in 5 patients.

IVIG, intravenous immunoglobulin; IVMP, intravenous methylprednisolone; PLEX, plasma exchange; T2W, T2-weighted

3.2.9 Clinical Outcomes

Next, across our cohort, we examined cognitive outcomes by Addenbrooke's Cognitive Examination - Revised (ACE-R) scores, and functional level of disability by Modified Rankin Scale (MRS) scores. These outcome measures were chosen since they were most widely used in the LGI1-autoantibody encephalitis literature.

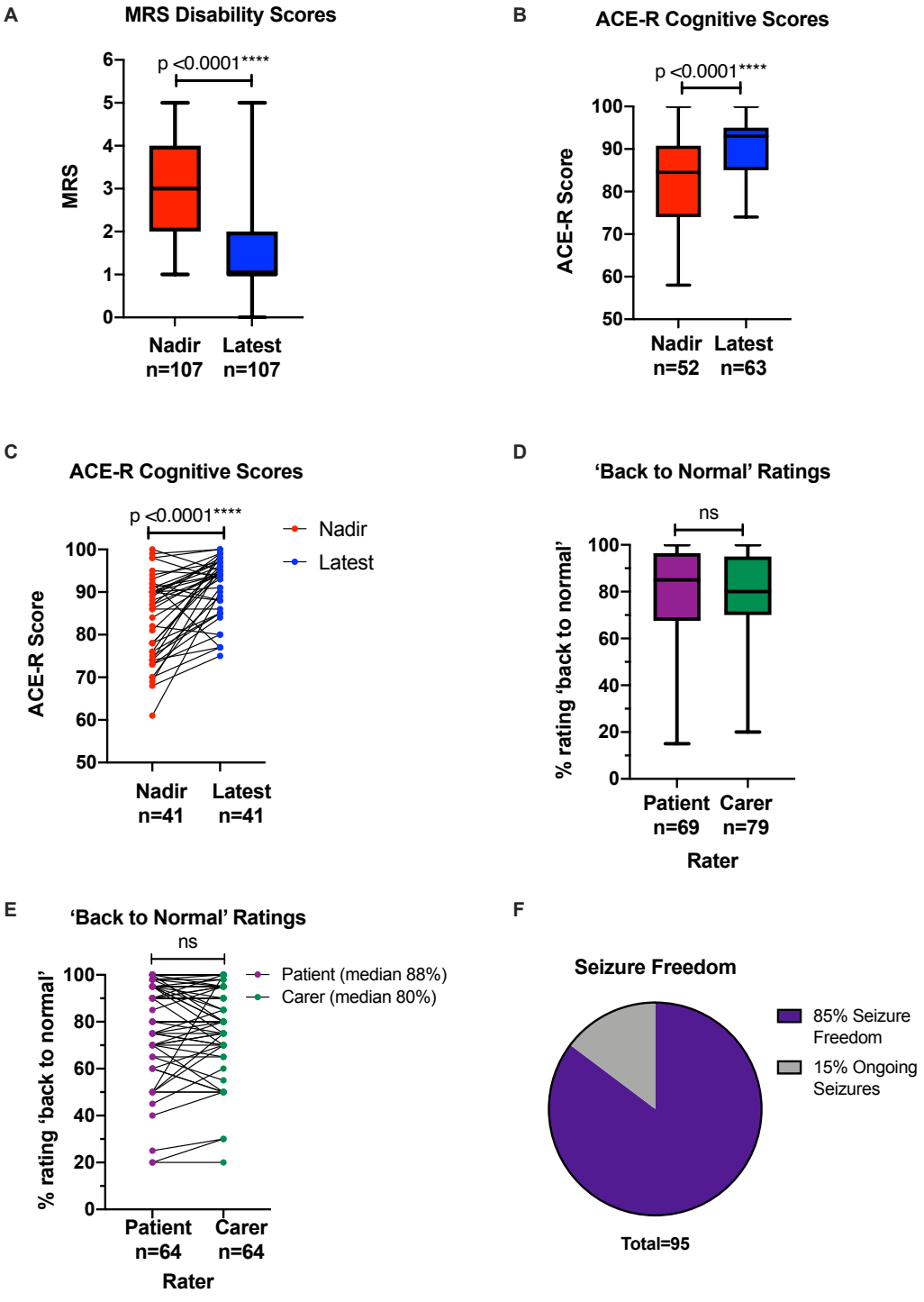
Median MRS scores were 3 at nadir, and 1 at latest follow-up **(Figure 3.9A)**, comparable to a recent study (Muñiz-Castrillo 2021). Over 107 patients, there was significant improvement in level of disability at latest clinical follow-up ($p<0.0001$****; Wilcoxon matched-pairs signed rank test); **(Figure 3.9A)**. Overall, ACE-R scores were significantly worse at nadir (median 85/100, range 58-100, n=52), compared to latest follow-up (median 93/100, range 74-100, n=63); $p<0.0001$****; Mann-Whitney test; **(Figure 3.9B)**. Paired ACE-R scores showed significant improvement at latest follow-up compared to nadir in n=41 patients with available data ($p<0.0001$****; Wilcoxon matched-pairs signed rank test); **(Figure 3.9C)**.

We explored subjective perceptions of 'percentage back to normal' ratings of patients, where 100% was equivalent to the functional baseline prior to disease onset. Overall, self-reported 'percentage back to normal' ratings by patients (n=69) and carers (n=79) at latest follow-up were comparable (median 85%, range 15-100% versus median 80%, range 20-100, respectively; p=0.87, Mann-Whitney test); **(Figure 3.9D)**. Similarly, whilst patients tended to perceive their progress more positively compared to their spouse or carer (median 88% versus median 80%), matched patient versus carer 'percentage back to normal' ratings for n=64 patients were non-significantly different (p=0.90; Wilcoxon matched-pairs signed rank test); **(Figure 3.9E)**.

Of the 98 patients with seizures, 3 lacked follow-up data for seizure frequency. 81/95 (85%) patients with seizures and available data showed seizure freedom at latest follow-up **(Figure 3.9F)**. 2 of the 81 patients with seizure freedom were treated with anti-seizure medications alone and did not receive immunotherapies.

5/107 patients (5%) died, but none as a direct result of either LGI1-autoantibody encephalitis or immunosuppressive treatment. Causes of death were as follows: anterior horn cell disease (n=1), cerebral abscesses (n=1), bowel perforation (n=1), unknown (n=2), although in these two cases seizure cessation was achieved 18 months and 10 years prior to death, somewhat excluding sudden unexpected death in epilepsy.

Figure 3.9 Patient Outcomes



(A) MRS scores of n=107 patients at nadir and latest follow-up. **(B)** ACE-R cognitive scores at nadir (n=52) and latest follow-up (n=63). **(C)** Matched nadir versus latest ACE-R scores in n=41 patients. **(D)** Self-reported 'percentage back to normal' ratings by patients (n=69) and carers (n=79) at latest follow-up, where 100% is equivalent to functional baseline prior to disease onset. **(E)** Matched patient versus carer 'percentage back to normal' ratings for n=64 patients. **(F)** proportion of patients with seizure freedom at latest follow-up. Data shown for n=95 patients with seizures with available data.

MRS, Modified Rankin Scale MRS; ACE-R, Addenbrooke's Cognitive Examination - Revised

3.3 Discussion

3.3.1 Clinical Features and Seizure Semiologies Associated with LGI1-Autoantibody Disease

In this chapter, we characterize and expand the clinical phenotypes of one of the largest series of LGI1-autoantibody patients thus far. There are only two other studies including more than one hundred patients (Gadoth et al. 2017; Muñiz-Castrillo 2021).

As expected, our cohort demographics matched those of previous studies (Irani et al. 2010; Irani et al. 2011; Gadoth et al. 2017; Finke et al. 2017; Binks et al. 2018; Thompson et al. 2017), with predominant male gender (68%), median onset age of 63 years, and almost exclusive White British or European ethnicity. Of all patients with LGI1-autoantibodies, 6% had additional CASPR2 autoantibodies. Within LGI1-autoantibody cohorts, double-positive rates are between 4-9% (Binks et al. 2018; Gadoth et al. 2017; Irani et al. 2011). The most common clinical syndrome was of LE plus FBDS in 50%. The pathognomonic FBDS were present in 57% of our cohort. Cognitive impairment was highly prevalent (92%). Morvan's syndrome, NMT, and other peripheral nerve manifestations were rare. Seizures were present almost universally (92%), with patients manifesting up to 5 seizure semiologies, and significantly more seizure types even compared to a large pharmacoresistant epilepsy cohort. Indeed, 89% of patients with LGI1-autoantibody encephalitis have at least one or more seizure types, comprising autonomic or dyscognitive subtypes (van Sonderen, Thijs, et al. 2016). We classified seizure semiologies into 11 distinct subtypes specific to LGI1-autoantibody encephalitis, and showed that in patients with seizures, 62% exhibited FBDS, 7% had seizures with piloerection, and 8% displayed the recently described paroxysmal dizzy spells (PDS),

occurring in 14% of other cohorts (Gadoth et al. 2017). We also highlight the rare but potentially life-threatening ictal neurocardiac syndromes in 2%, of which clinicians should be aware. Notably, patients trialled up to 7 ASMs, the most commonly used being levetiracetam in 61%.

Whilst the determined frequencies of many clinical features were similar to those from previous reports, some unexpected findings were observed. Behavioural changes were present in 75%, and psychiatric manifestations in 59%, with suicidal attempts in 2 patients. Such serious psychiatric manifestations and risks have not been previously reported in association with LGI1-autoantibody encephalitis. Additionally, our data show that presence of sleep disorders, including insomnia, appear to be much higher than previously reported, and that 11% of patients suffer parasomnias. One quarter of patients suffered falls, and more than one third sustained significant injuries. Falls were almost always caused by FBDS, and we showed that presence of FBDS poses significantly higher risk of both falls and subsequent significant injury. These findings suggest that care of LGI1-autoantibody patients may benefit from multidisciplinary input including Neuropsychiatry, Sleep Specialists, and Occupational Therapists for home modifications. Finally, we found that presence of hyponatraemia or abnormal EEG at initial investigation significantly associated with clinical severity. These findings suggest that routine, simple investigations may aid decisions for initiation of aggressive immunotherapy regimes in select patients.

3.3.2 Potential Triggers

We explored the potential associations of various triggers, to ascertain whether there might be an obvious trigger or prodromal event to LGI1-autoantibody encephalitis. Surprisingly, nearly one quarter of patients had prodromal infections occurring at a median time of 4 weeks prior to LGI1-autoantibody onset. Similarly, we found that nearly one fifth of patients received vaccinations at a median time of 3.5 weeks prior to disease onset, almost always influenza vaccinations. One major limitation of this study is the lack of control group, comparing any statistically significant differences in the proportion of age- and sex-matched individuals suffering infections and receiving vaccinations. The close temporal relationships of infections and vaccinations with disease onset, however, may suggest their relevance to

disease pathogenesis. For instance, prodromal infections and vaccinations may cause polyclonal immune activation, thus expanding B cell populations, and perhaps serum levels of pre-existing autoantibodies. Our finding of significant stress in 12% of patients 0-8 weeks prior to onset may be relevant through similar mechanisms. This polyclonal expansion could potentially precipitate disease in susceptible patients, such as those with genetic risk factors.

Whilst the relevance of specific organisms remains unknown in LGI1-autoantibody encephalitis, the majority of prodromal infections affected the upper or lower respiratory tract, although causative organisms were unknown. Interestingly, one patient had epididymo-orchitis at onset, and another had shingles of the trigeminal nerve (V1). Scrotal hydrocoele drainage has been shown to associate with development of CASPR2-autoantibody associated Morvan's syndrome (Irani et al. 2012; S. Sharma & P. Sharma 2013), and epididymo-orchitis with meningoencephalitis are described during treatment with immune checkpoint inhibitors (Quach et al. 2019). 42% of our patients had a prior history of HSV1 or VZV infection. Whilst herpes viruses are ubiquitous pathogens, their association with other neurological diseases is intriguing. For example, HSV1 infection and carriage of type 4 allele of apolipoprotein E gene confer increased risk of Alzheimer's disease (Itzhaki 2018), and HSV1 encephalitis can precipitate secondary NMDAR-autoantibody encephalitis (Prüss et al. 2012). In individuals with HSV1 encephalitis, Toll-like receptor 3 (TLR3) mutations causing subsequent type 1 interferon (IFN-1) deficiency is a postulated mechanism of infection (Mørk et al. 2015). Together, these observations suggest neuronal surface antibodies can arise after viral-induced neuronal damage, particularly in genetically predisposed individuals, and that herpes viruses may be particularly relevant. Additionally, the reactivation of such viruses after a latent period may be an important factor, as evidenced by our patient with first manifestation of HSV1-associated oral cold sores after 10 years of remission at simultaneous onset of both LGI1-autoantibody encephalitis and Graves' disease. Thus, our observations of shingles and epididymo-orchitis may be relevant immunisation mechanisms, providing clues to the pathogenesis of some forms of autoimmune encephalitis.

13% of our cohort had tumours, with well-recognised associated thymomas accounting for 14% of all tumours. It is possible that other tumour types found in our series, particularly the oral and penile carcinomas (both types of squamous cell carcinoma), may be less recognised

disease triggers, although these are not rare in the general population. The association of squamous cell carcinomas and LGI1-autoantibody encephalitis was highlighted recently (Virupakshaiah et al. 2021), with further corroboration evidenced by strong LGI1 expression within both squamous cell lung carcinomas (Virupakshaiah et al. 2021) and early oral squamous cell carcinomas (Fialka et al. 2008). Further evidence for LGI1 as a tumour suppressor gene is provided by studies which demonstrate downregulation or complete lack of expression of LGI1 in brain tumours, secondary to LGI1 mutations (Chernova et al. 1998).

We show strong association with other autoimmune diseases in one third of LGI1-autoantibody patients, some with multiple autoimmune conditions. In particular, simultaneous, *new* onset of other autoimmune diseases concurrent with LGI1-autoantibody encephalitis, albeit in only 5%, provides insights into a widespread loss of immune tolerance, including shared HLA associations amongst the conditions (e.g. IgG4-related disease; (Huijbers et al. 2015).

Potential occupational exposures were found in 19% of patients. Whilst the major limitation is lack of a control group to compare the frequency of potentially relevant exposures, woodwork and pesticide exposures were potentially important. The abattoir workers from this cohort may have had occupational triggers to their disease, since aerosolised brain tissue can provoke immunisation against VGKC in exposed subjects (Meeusen et al. 2012).

Finally, whilst we demonstrate little evidence for role of the gut microbiome (3% of cohort), it is intriguing that one of only two patients with abrupt dietary change (50%) had detectable serum LGI1-specific IgA autoantibodies, which were found in only 3/30 (10%) patients tested.

In summary, we show evidence suggesting a potential role for several triggers to LGI1-autoantibody encephalitis.

3.3.3 Relapse Characteristics and Potential Precipitants

Relapse data in LGI1-autoantibody patients are limited. Our results showed that relapses were surprisingly common, in 36% (39/107). Reported relapse rates are variable, ranging from

14% (Irani et al. 2011; Shin et al. 2013); (4/29 and 2/14, respectively), 18% (6/33) (Lai et al. 2010), 25% (2/8) (Li et al. 2018), 27% (13/48) (Ariño et al. 2016), 29% (30/103) (Thompson et al. 2017), 33% (12/36) (Sola-Valls et al. 2020), and 35% (van Sonderen, Thijs, et al. 2016), to as high as 59% (36/61) (Gadoth et al. 2017), although these were generally derived from smaller cohorts. Perhaps more surprisingly, 36% (14/39) of relapsing patients in our study had multiple relapses. In fact, some (21%; 8/39) had up to 3 clinical relapses. One study found that, similarly, 25% of their relapsing patients showed multiple relapses (Gadoth et al. 2017).

Relapse patterns indicated that the most common precipitant was weaning of prednisolone therapy in 47%. Moreover, another third (32%) occurred following a median of 4 months since prednisolone cessation, although it is unclear whether this is causative or incidental. Others have shown that rapid prednisolone tapering by 20-30mg over 3 months can trigger relapse (Irani et al. 2011), suggesting a steroid sensitivity in these patients. Correspondingly, Gadoth and colleagues showed that in 92% of relapsing patients, the relapse was precipitated by immunosuppressant discontinuation or reduction (Gadoth et al. 2017). In our cohort, this rate was equivalent to 86% (prednisolone reduction or discontinuation 79%; weaning or discontinuation of other immunosuppressants 7%), and thus analogous. As mentioned earlier, 90% of the cohort received oral prednisolone therapy, and were subject to similar effects of weaning and/ or cessation. Steroid-dependency alone is unlikely to be the only factor predisposing to relapse, since 3% of relapses occurred in immunotherapy-naïve patients, and many showed multiple relapses. Additionally, FBDS appeared to associate with risk of relapse, since they significantly associated with relapsing compared to non-relapsing patients. Interestingly, LLPCs are refractory to deletion by cyclophosphamide {Cupps:1982tm}, which suggests that the patient who relapsed despite cyclophosphamide therapy may have a predominantly LLPC-driven disease. Collectively, these findings indicate that other commonalities may predispose to relapse, particularly underlying immune pathways.

3.3.4 Novel Clinical Manifestations: First Reports of Probable Agrypnia Excitata and Hoarding Behaviours in LGI1-Autoantibody Encephalitis

We describe several novel clinical features herein. The most striking, perhaps, is that of the novel ‘complex motor behaviours’, observed in five patients. The behaviours shared several

common features across all five patients. First, they occurred in the acute phase (within the first 8 weeks, or at onset of relapse). Second, the motor behaviours were exclusively manual, and consisted task imitation of recognisable behaviours, such as drinking from a cup, using imaginary, non-existent objects. Third, the episodes typically displayed associated loss of awareness, and occurred in patients with encephalitis consisting multiple other seizure semiologies (3-5; with inclusion of complex motor behaviours as a potential seizure type). Finally, all patients had associated visual hallucinations, sleep disorder, and new hoarding, ritualistic, or overspending behaviours. Whilst we established a frequency of 5% for complex motor behaviours within the cohort, these symptoms may have been under-recognised/under-reported as they have not previously been described. The highly unusual events occurred very early in disease (typically at onset, prior to any formal diagnosis) increasing the chances of their under recognition. In patients living alone, it is possible that such events were missed completely. Additionally, this study is limited by recall bias in a retrospective study of a cohort of patients with cognitive difficulties.

The mechanism underlying this phenomenology is intriguing. In order to ascertain whether these motor features are best categorized as a seizure phenomenon or sleep disorder, we shall examine several features of these behaviours. The features supportive of seizure include their stereotypy, duration, associated confusion and reduced awareness, and the strong temporal relationship of events with immediate precipitation of GTCs (Patient 1). Although the complex manual motor behaviours captured by electroencephalogram in Patient 1 did not demonstrate any electrographic correlate, this does not exclude seizures. Indeed, <20% of FBDS show epileptic correlates (Irani et al. 2011). These patients had multiple other seizure semiologies, thus complex motor behaviours may represent one of an array of seizure types. Treatment response remains unclear and thus unhelpful in the differential diagnosis given such a small number of patients. Two of the five showed apparent excellent response to immunotherapy, with rapid resolution of all seizure types and complex motor behaviours within three weeks. However, one patient showed resolution with lamotrigine, prior to the initiation of any immunotherapy. Two patients (Patients 2 and 4) showed spontaneous resolution prior to the initiation of *any* treatments. Thus, it remains unclear whether the complex motor behaviours indeed responded to the immunomodulatory therapies, or the

anti-seizure medication, or if they would have otherwise resolved spontaneously without treatment, as with Patients 2 and 4.

Whilst some correlations may indicate an epileptic basis, other possible aetiologies include agrypnia excitata (Guaraldi et al. 2011), and complex visual hallucinations (Manford & Andermann 1998). Agrypnia excitata is a sleep disorder seen in Morvan's syndrome, limbic encephalitis, or those with familial fatal insomnia (Guaraldi et al. 2011; Cornelius et al. 2011). The characteristic oneiric stupor, or 'dream enactment', comprises motor gestures which mimic daily-life activities. Typical examples include repeated opening and closing of doors, mimicking eating and drinking, and manipulation of non-existent objects such as electrical wires (Guaraldi et al. 2011). All of these behaviours bear striking resemblance to the gestures described in our patients. Additionally, oneiric stupor typically occurs nocturnally during sleep, or during relaxed wakefulness, analogous to our patients. The constellation of signs displayed by our five patients, including insomnia, confusion, and complex motor behaviours, likely to be oneiric stupor, could be compatible with the syndrome of agrypnia excitata. Although agrypnia excitata seen in Morvan's syndrome occurs in association with CASPR2 autoantibodies, all five patients were negative for this autoantibody. Nevertheless, it may be that there is an overlap of features between LGI1- and CASPR2- autoantibody encephalitis owing to common pathophysiological mechanisms, predominantly of thalamo-limbic dysfunction. Whilst our series appears to be the first report of agrypnia excitata in patients with LGI1-autoantibodies, it is possible that historical reports of patients with positive VGKC autoantibodies and agrypnia excitata (Guaraldi et al. 2011; Cornelius et al. 2011) had LGI1-autoantibodies, particularly given the described 'myoclonus' (often confused with FBDS). Finally, the lack of EEG correlate from the complex manual movements in Patient 1 may further favour agrypnia excitata over epilepsy, particularly considering that almost all (94%) non-FBDS seizures in LGI1-autoantibody encephalitis, including motor focal seizures, show electrographic ictal epileptiform activity (van Sonderen, Schreurs, et al. 2016).

Rapid eye movement (REM) sleep behaviour disorder (RBD) has been described in VGKC-autoantibody encephalitis (Iranzo et al. 2006), proposed to be due to limbic dysfunction and secondary dysregulation of brainstem REM sleep structures. Lesions within the pontine reticular formation can cause sleep disturbances and abnormal processing of sensory

afferents (Manford & Andermann 1998). The association of parasomnia in 11% of our patients, consisting new vivid dreams, and or acting out dreams, is supportive of associated sleep disorders in our patient group and similar mechanisms. These observations may be explained by LGI1 expression within hypothalamus, thalamus and raphe nuclei (Irani et al. 2012). Unusual sleep disorders consisting purposeful gestures resembling those observed in our patients are common in both DPPX-autoantibody encephalitis (Tobin et al. 2014; Hara et al. 2017) and IgLON5 disease (Sabater et al. 2014; Högl et al. 2015; Gaig et al. 2017). The described tau deposition within the brainstem tegmentum and hypothalamus of IgLON5 patients (Sabater et al. 2014) may provide insights into the pathophysiological networks implicated in our LGI1-autoantibody patients.

Finally, complex visual hallucinations, which can occur secondary to focal seizures (Manford & Andermann 1998), should be considered as a final differential diagnosis. The content of such hallucinations is typically dramatic, which could be the basis for the unusual, intricate behaviours displayed by our patients, such as use of their mobile phone and taking pills. Furthermore, animals are often seen (Manford & Andermann 1998). Indeed, in our series, Patients 1 and 4 experienced visual hallucinations consisting cats and animals, and the references to gnomes by Patient 3, and Mad Hatter by Patient 4 during the episodes may reflect visual hallucinations. Complex visual hallucinations caused by focal epilepsy are thought to arise from a direct irritative (excitatory) process of visual cortical areas such as posterior parietal or temporal association cortex. Other postulated mechanisms include neurochemical imbalance with increased cortical serotonergic activity (Manford & Andermann 1998). Indeed, the temporal lobes are highly implicated in LGI1-autoantibody encephalitis, and it is possible that autoantibodies lead to neurotransmitter imbalance. It is plausible that the complex motor behaviours observed in our patients represent complex visual hallucinations secondary to focal seizures.

The new features observed in our patients comprising obsessive compulsive-type behaviours, compulsive shopping, and punding are reminiscent of impulse control disorders observed in Parkinson's disease. The punding behaviours in particular, consisting repeated, purposeless arrangement of objects, are incredibly alike (Kelly et al. 2020). These new rituals were profound in one patient, since the assignment of numbers to individual food items and their

specific arrangement lasted up to two hours prior to each meal, markedly impacting on daily function. Additionally, compulsive shopping led to shoplifting in several patients. In Parkinson's disease, use of dopamine agonists associates with increased risk of these behaviours (Kelly et al. 2020). Thus, dysregulated dopaminergic pathways, potentially mediated by autoantibody effects, may represent a possible mechanism in LGI1-autoantibody patients. Finally, the emotionality detected in two thirds of patients appears to represent pseudobulbar affect observed in various dementia disorders, and Parkinson's disease (Ahmed & Simmons 2013), and may indicate disordered serotonergic and or glutamate pathways in LGI1-autoantibody encephalitis.

3.3.5 Clinical Outcomes

Overall, we found favourable clinical outcomes in our cohort, with median MRS of 1, median ACE-R of 93, and self-reported 'back to normal' ratings of 85% at latest clinical follow-up. Despite this, it is evident that many of our patients had persistent cognitive deficits, since follow-up ACE-R scores ranged from 74-100. Additionally, patients and/ or their spouses reported persistent long-term symptoms such as fatigue and cognitive impairment which impacted their quality of life, including employment. Consistently, others report severe, persistent verbal and visuospatial memory deficits in association with marked hippocampal atrophy in these patients (Finke et al. 2017), and mesial temporal sclerosis in up to half (van Sonderen, Thijs, et al. 2016). The hippocampal volume loss appears focused around CA3 (Finke et al. 2017; Miller et al. 2017), which associates with severe episodic but not semantic amnesia for postmorbid autobiographical events. Early initiation of immunotherapy has been shown to favourably influence cognitive outcomes in those with FBDS (Thompson et al. 2017). Similarly, in non-selected LGI1-autoantibody cohorts, latency between disease onset and initiation of immunotherapy significantly correlates with memory deficits and CA3 atrophy (Finke et al. 2017; Miller et al. 2017). These data demonstrate the variation in outcome measurement between studies, with some measuring hippocampal atrophy, and others reporting defined specific cognitive scales, or disability scales. These illustrate the need for a harmonised assessment of these patients across centres.

Finally, whilst our fatality rate across the entirety of longitudinal follow-up was 5% (and likely unrelated to disease), some studies report seemingly high two-year case fatality rates of 19%,

some pre-treatment, and others as direct consequences of steroid treatment (van Sonderen, Thijs, et al. 2016).

3.4 Conclusions

Through this work, we have gained significant clinical experience with autoimmune encephalitis patients within our specialist centre. Herein, we provide one of the most detailed analyses to date of one of the largest internationally reported LGI1-autoantibody cohorts, along with a large, robust pharmacoresistant epilepsy control group.

Whilst we confirm the established patient demographics and other associations of these patients, we present several novel findings. These include the significant association of multiple seizure semiologies in this patient group, which appears to be exclusive to patients with LGI1-autoantibodies; the predictive value of certain investigations, including serum sodium; the risk of falls and subsequent injury conferred by presence of FBDS; that infections or vaccinations may act as triggers to LGI1-autoantibody disease, likely in genetically predisposed individuals; that relapses are common, occurring in over one third of patients. Rapid weaning or cessation of steroid therapy was the most common precipitant of relapse. Additionally, we report presence of co-existent autoimmune diseases including IgG4-RD, and that sleep disorder appears to be more prevalent than previously reported. Finally, we expand the clinical phenotypes, and describe novel associations such as hoarding and agrypnia excitata, using case notes and video footage analysis to present the phenotypes. These findings expand the clinical phenotypes of this relatively new disease entity, aiming to increase clinical recognition of these rare disorders, with ultimate aim of consequential expedited immunotherapies and improved outcomes in these patients.

CHAPTER 4. GERMINAL CENTRES:

Potential Contributions of Germinal Centre Reactions to LGI1 and CASPR2-Autoantibody Production

4.1 Introduction

4.1.1 Mechanisms and Sites of Autoantibody Production and Maintenance

In autoantibody-mediated neurological syndromes, a key question is the mechanism underlying autoantibody production and maintenance. Data suggest that ongoing germinal centre (GC) reactions likely make a contribution to autoantibody production in NMDA receptor (NMDAR)-antibody encephalitis (Makuch et al. 2018). Autoantigen-specific IgMs were detected in 60% of patients after at least 6 months (in some cases years) following disease onset – far greater than the 5-day half-life of IgM. The authors took this to indicate ongoing *new* autoantibody production rather than persistence. Additionally, circulating patient B cells (differentiated into antibody-secreting cells *in vitro*) produced NR1-IgG and -IgM across disease durations, suggesting that ongoing GC reactions may account for NMDAR-specific IgM/IgG production in patients with NMDAR-antibody encephalitis. Moreover, Chemokine C-X-C Motif Ligand 13 (CXCL13), a chemokine which serves as a biomarker of GC-reactions (Havenar-Daughton et al. 2016), is elevated in serum and CSF of patients with NMDAR-antibody encephalitis (Liba et al. 2016). An overview of GC reactions is provided in **Introduction (Chapter 1, Section 1.6.6)**. Intriguingly, a separate observation found that in neuromyelitis optica (NMO), aquaporin-4 (AQP4) autoantibodies can derive from naïve pre-GC B cells (Wilson et al. 2018) suggesting an early, pre-GC loss of tolerance contributes to the generation of AQP4-autoantibodies.

Hence, GC reactions and early loss of immune tolerance appear to be relevant in autoantibody-mediated neurological diseases. Since our main research focus was to understand the mechanisms behind autoantibody production and maintenance in patients with LGI1- and CASPR2-antibody encephalitis, the following aims were formulated.

4.1.2 Project Aims:

1. To ask whether GC reactions are a potential mechanism of autoantibody production in LGI1- and CASPR2-antibody syndromes. To investigate this, we sought presence of CASPR2 and LGI1 IgMs, and examined CXCL13 levels, alongside clinical correlations.

2. To longitudinally examine specific epitopes of LGI1-autoantibodies as a method to infer the dynamics of autoantibody subpopulations, in patient blood and CSF.

4.1.3 Methodology

1. Presence of CASPR2-IgMs was sought by live cell-based assay (CBA) in 51 serum samples from n=25 CASPR2-antibody patients, n=35 HCs, and n=44 disease controls (n=24 AQP4- and n=20 LGI1-antibody patients). The live CASPR2 IgM CBA was developed, optimized, and correlated with flow cytometry **(Methods Chapter 2, Section 4.2)**.
2. Presence of LGI1-IgM and -IgAs was sought by live CBA in 120 IgG-depleted serum samples (70 LGI1-antibody patients, 30 HCs, and 20 NMDAR-antibody patients; **Methods Chapter 2, Section 4.3)**.
3. LGI1-autoantibody epitope specificities: end-titrations against full-length LGI1, and the two major LGI1 domains, LRR and EPTP, were quantified by live-CBA for 102 sera and 11 CSF samples from 38 LGI1-antibody patients **(Methods Chapter 2, Section 4.4)**.
4. CXCL13 levels were examined by ELISA in n=142 LGI1-, n=63 CASPR2- and n=19 LGI1/CASPR2-antibody patient sera, n=37 HCs, n=227 pharmacoresistant epilepsy patients, and n=55 seizure-free controls. Additionally, n=60 CSF samples were tested. Clinical correlates and longitudinal CXCL13 trends were explored in LGI1-, CASPR2-, and LGI1/CASPR2 -antibody patients **(Methods Chapter 2, Section 4.5)**.

4.2. Results: CASPR2 IgM CBA Development and Optimization with Flow Cytometry Comparison

4.2.1 CASPR2 IgM Screening

Initial CASPR2 IgM screening showed high frequencies across all groups (83% in CASPR2-antibody patients, 60% in LGI1-antibody patients, 80% in HCs); for matched LGI1-antibody patients, only 40% possessed disease-specific LGI1 IgM antibodies **(Figure 4.1A)**. CASPR2 IgM scores were significantly higher in CASPR2-antibody patients compared to HCs (mean score 2 versus 1, respectively; p=0.0268*, Mann Whitney); **(Figure 4.1B)**. However, the detection rate in HCs was surprisingly high, almost equal to that of CASPR2-antibody patients, likely indicating that the CASPR2 IgM live CBA required optimization. Additionally, surprisingly, in the matched n=10 LGI1-antibody patients, a greater proportion (60%) were positive for CASPR2 IgM than for LGI1 IgM (40%).

4.2.2 CASPR2 IgM Live CBA Optimization: Variation of Primary and Secondary Antibody Dilutions, and Temperature

Upon variation of secondary antibody dilution **(Figure 4.1C-E)** from 1:500 to 1:2000 reduced cell surface signal, and at 1:4000 substantial signal loss caused overlap between HCs and patients. Hence, secondary antibody dilution was set at 1:500 since this yielded greatest distinction between patients and HCs **(Figure 4.1C)**. For positive samples, lower CASPR2 IgM cell surface signal scores were observed after incubation of cells with IgM at 37℃ compared to 4℃ **(Figure 4.1F+I)** – this prompted the question of whether CASPR2-IgM was being internalised, a process favoured at 37℃, and was explored elsewhere. A primary antibody cut-off was initially set as 1:400 based upon end-point dilutions of CASPR2 IgM in n=15 HCs **(Figure 4.1G)**, determined as HC mean+3SD [160+(3x82.8)]. Using this 1:400 serum dilution, n=106 samples (blinded and with repeats) showed good inter-assay reliability **(Figure 4.1H)**, however CASPR2 IgMs were still detectable in 11% of HCs and 30% of LGI1-antibody patients **(Figure 4.2B)**. Thus, further assay optimization was undertaken. End-titrations of more HC subjects with detectable CASPR2 IgM were performed, with primary antibody cut-off now at 1:600 **(Figure 4.2A)**. The final optimized CASPR2 IgM CBA using the new primary antibody cut-off of 1:600 (with incubation for 1 hour at 4℃, and secondary antibody dilution 1:500) was

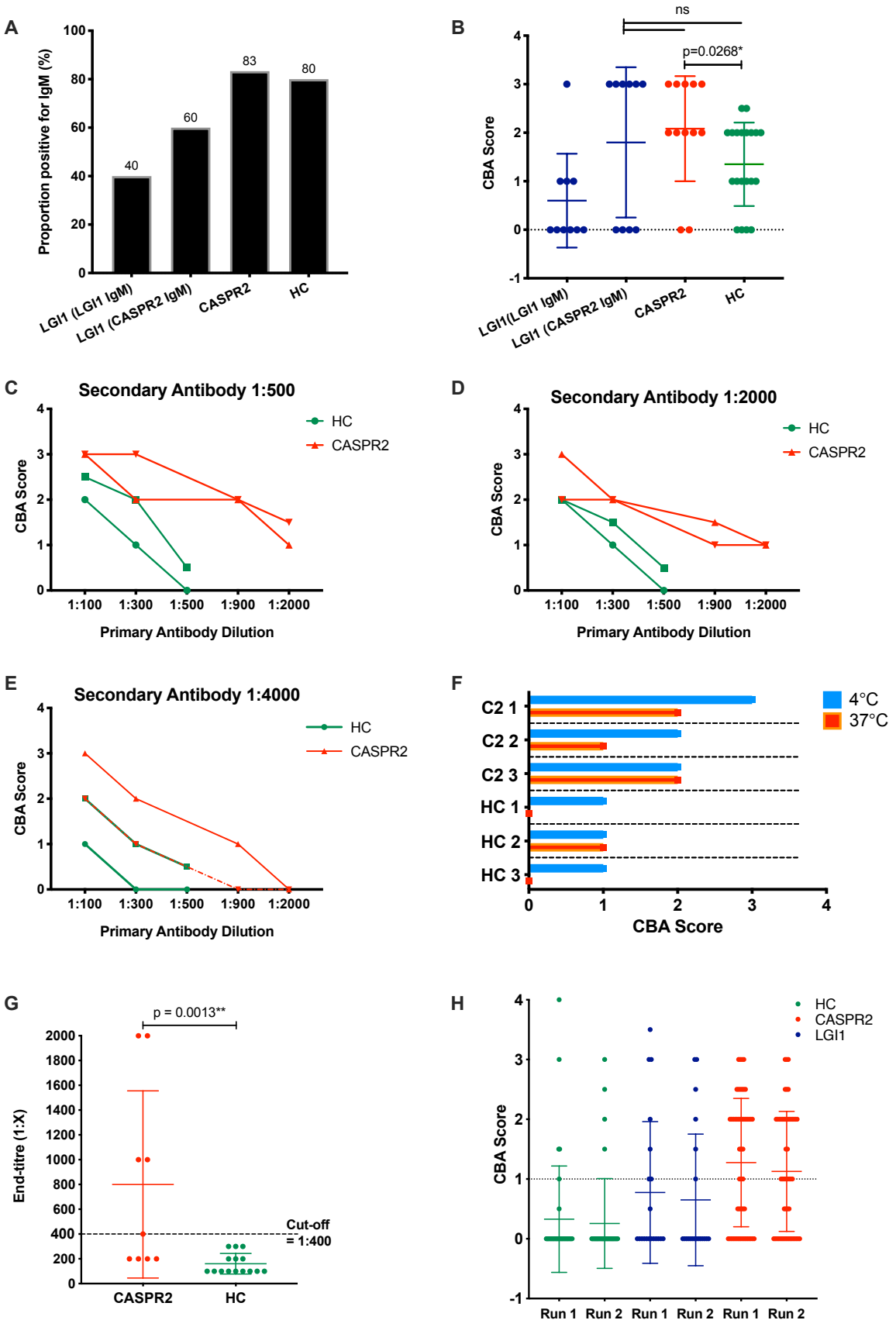
run blindly on n=130 samples **(Figure 4.2I)**. This more robustly eliminated HC reactivity, further reducing it to 6% (CASPR2 IgM positive rate HC 6% versus CASPR2-antibody patients 24%; p=0.0006***, Fisher's exact test); **(Figure 4.2B)**. Additionally, using this set of conditions, none of n=24 AQP4-antibody patients - included as a second disease control group - demonstrated CASPR2 IgM reactivity. These optimizations facilitated development of the optimal parameters for testing as above.

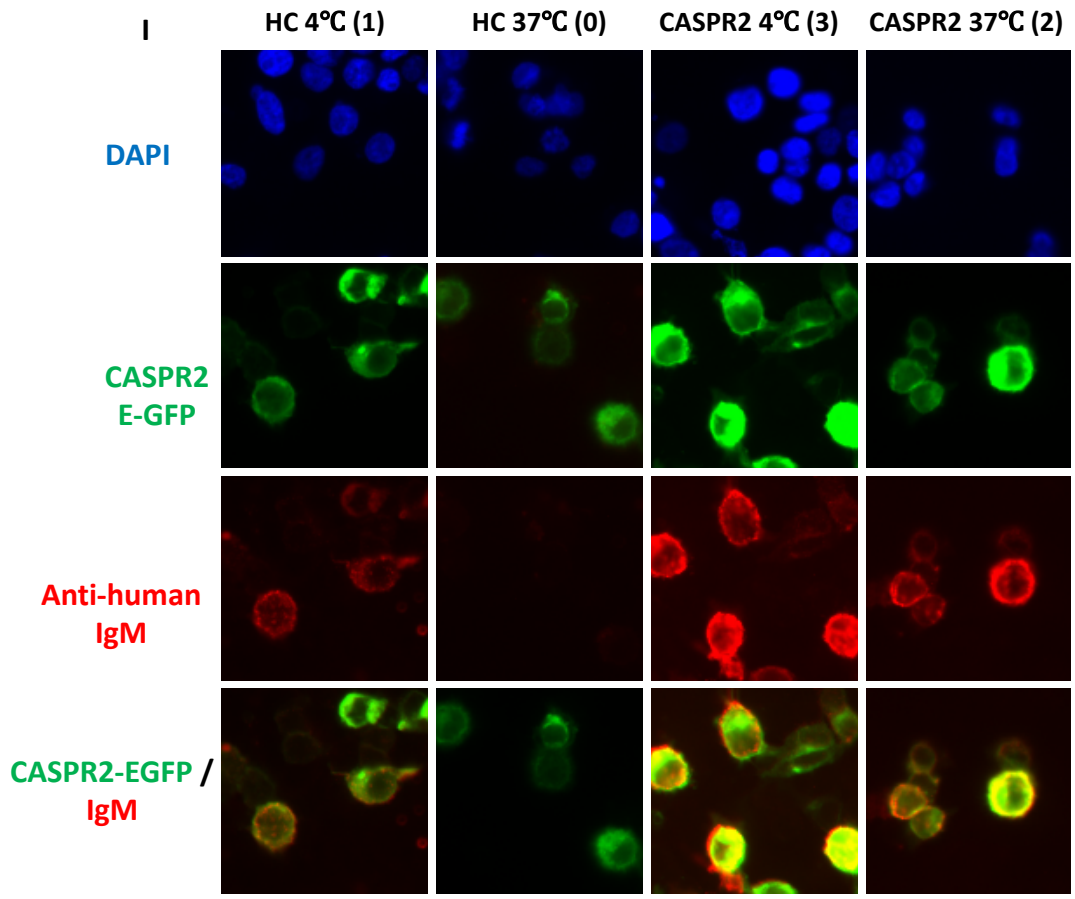
4.2.3 Frequency of CASPR2 IgM Antibodies by Optimized CBA Combined with Flow Cytometry is 14% in CASPR2 Patients and 3% in Healthy Controls

Next, in order to validate the optimized CASPR2 IgM CBA, the n=130 cohort was quantified for CASPR2 IgM by FACS **(Figure 4.2C-G)**. Fluorescence intensity of samples was standardized using beads with fixed antibody binding capacities (ABC), to generate ΔABC values **(Chapter 2, Methods; Section 4.2.2)**. Threshold for CASPR2 IgM positivity by FACS was initially determined as 21342 ΔABC, based upon mean+3SD of n=10 HCs proven to lack CASPR2 IgM reactivity by CBA **(Figure 4.2D)**. Following unblinding for HC vs patient status, ΔABC cut-off was recalculated as 44753 from n=33 HCs. Using this cut-off, CASPR2 IgM antibodies were identified in 15/51 (29%), 4/20 (20%), 1/24 (4%), and 2/35 (6%) of samples with CASPR2-antibodies, LGI1-antibodies, AQP4-antibodies and HCs, respectively **(Figure 4.2E+H)**. From the same samples, comparison of CBA and FACs results revealed comparable CASPR2 IgM detection rates by both techniques **(Figure 4.2H),** at 7/51 (14%) by both CBA and FACS from 4/25 (16%) CASPR2-antibody patients. Median sample time was ~10.5 months from onset, versus ~25 months in CASPR2 IgM negative samples **(Table 4.1)**.

Individual clinical correlations with CASPR2-IgM status did not reveal any significant associations between presence of CASPR2-IgMs and male gender, presence of tumours, relapses, or particular clinical syndromes **(Table 4.1)**.

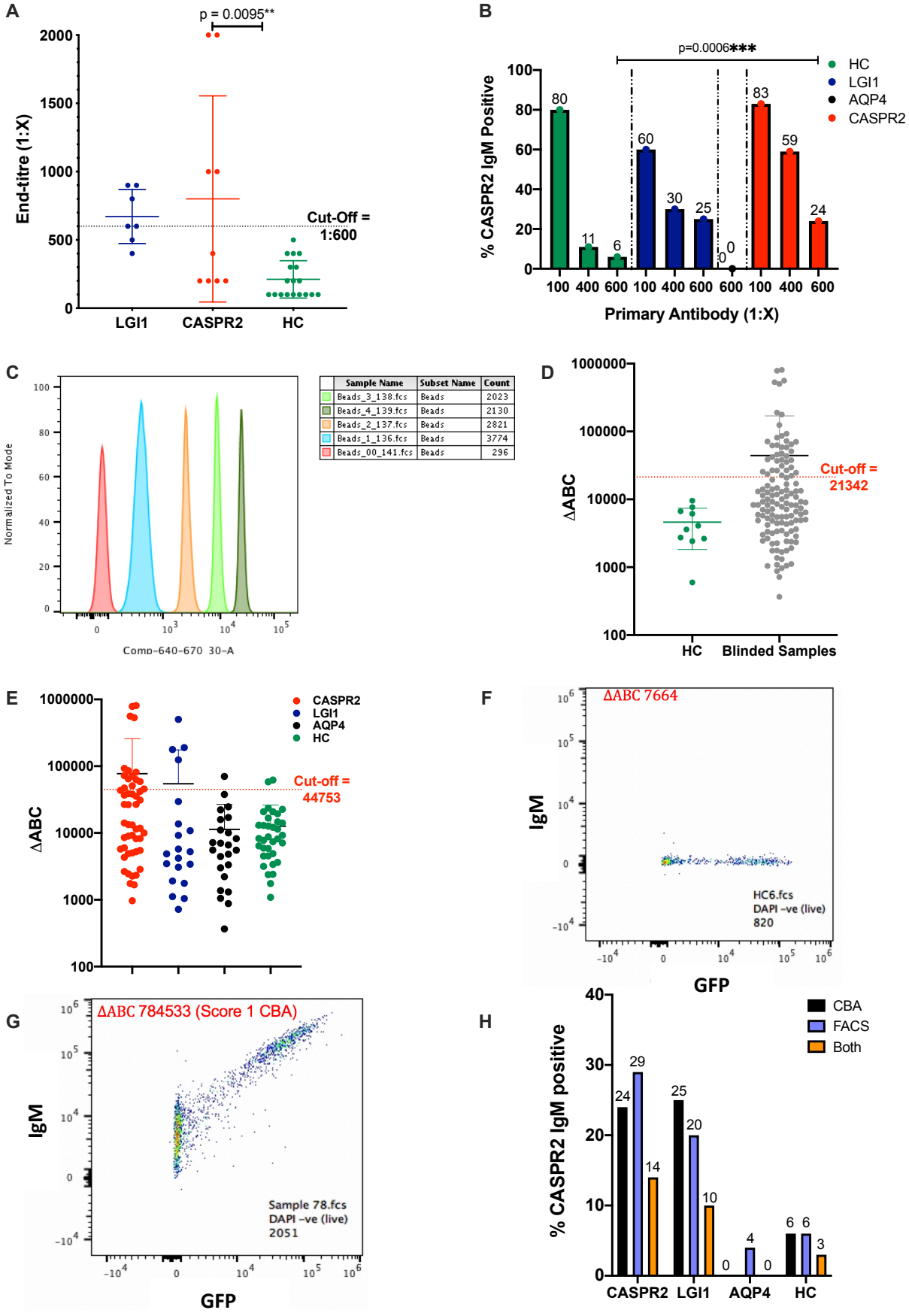
Figure 4.1 Frequency of CASPR2 IgM Across Patient Groups and CASPR2 IgM Live Cell-Based Assay Optimization

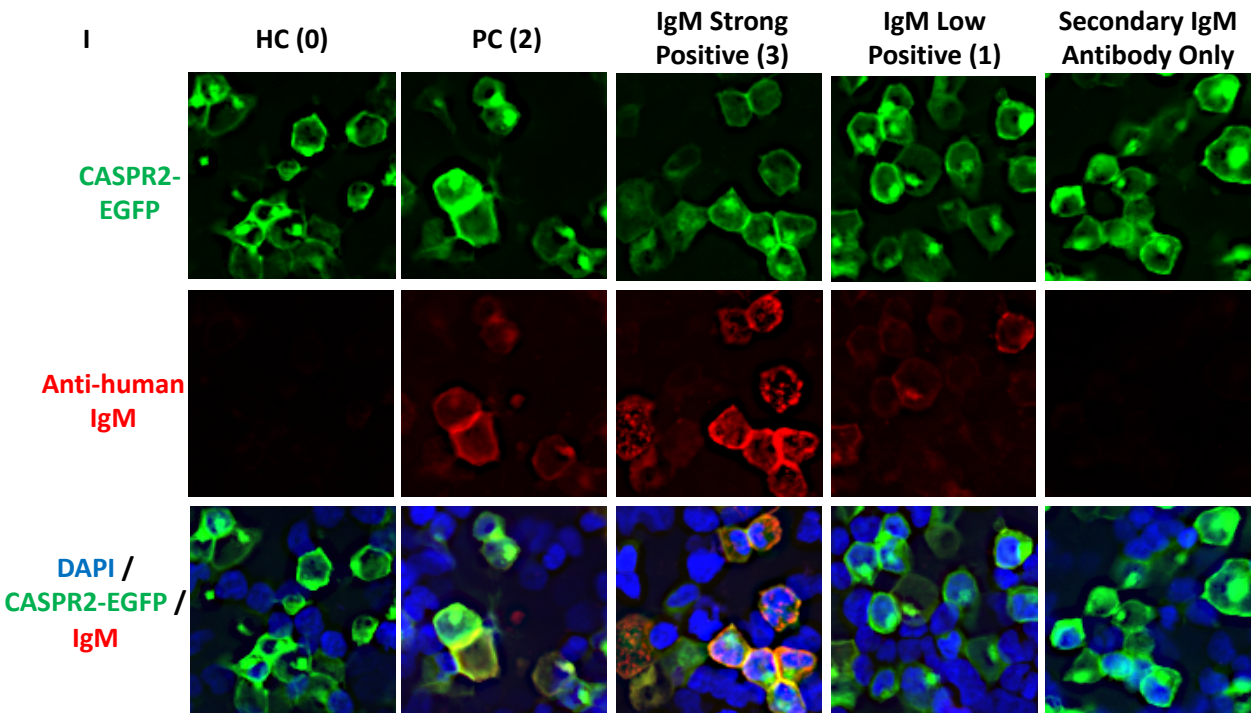




(A) Frequency of LGI1 IgM antibodies in LGI1 IgG-positive patients (n=10) and CASPR2 IgM in the same LGI1 IgG-positive patients, CASPR2 IgG-positive patients (n=12), and HCs (n=20). **(B)** CBA scores of IgM positive samples. CASPR2 patients had significantly higher CASPR2 IgM CBA scores than HCs (p=0.0268*, Mann Whitney). Mean CASPR2 IgM scores of LGI1 patients were non-significantly different to CASPR2 patients or to HCs. **(C-E)** Effect of variation of primary and secondary antibody dilution on CASPR2 IgM CBA score shown for n=2 CASPR2 patients (red) and n=2 HCs (green). CASPR2 patients showed higher CASPR2 IgM scores than HCs, and in both groups, CBA scores reduced with increasing primary antibody dilution. When secondary antibody dilution was varied, CBA scores between CASPR2 patients and HCs were most distinct at 1:500 **(C)**, became closer at 1:2000 **(D)**, and overlapped at 1:4000 **(E)**. **(F)** Effect of temperature shown for three CASPR2 patients (C2 1-3) and HCs (HC 1-3) with detectable CASPR2 IgM. Lower CBA scores were observed at 37°C compared to 4°C in 2/3 (67%) CASPR2 patients and 2/3 (67%) HCs. CBAs performed with standard 1 hour incubation of primary antibody (1:100); secondary antibody 1:500. **(G)** Quantification of samples with detectable CASPR2 IgMs by end-point dilutions, defined as the last dilution with a CBA score of 1, but not 0.5. End-titres were significantly different between HCs (n=15) and CASPR2 patients (n=9); p=0.0013**, Mann-Whitney test. Primary antibody cut-off was determined as 1:400 based on HC mean+3SD [160+(3x82.8)]. **(H)** Optimized CASPR2 IgM assay (primary antibody 1:400) run with n=106 samples (n=35 HCs, n=20 LGI1 as DCs, n=51 CASPR2). Samples were tested twice on different days (run 1 and run 2), and scored blindly. There were 7 discrepancies in CBA scores across 106 samples. **(I)** 2 left panels: HC with low-positive (score 1) CASPR2 IgM (red) at 4°C and complete loss of surface signal at 37°C (score 0). 2 right panels: CASPR2 patient with strong-positive (score 3) CASPR2 IgM at 4°C and reduction to score 2 when tested at 37°C; x40 magnification.

Figure 4.2 CASPR2 IgM Validation and Quantification by Flow Cytometry





(A) Further assay optimization and derivation of new primary antibody cut-off. CASPR2 IgM end-titres across patient groups shown (LGI1 n=7, mean 1:671; CASPR2 n=9, mean 1:800; HCs n=18, mean 1:211). End-titres were significantly different between HCs and CASPR2 patients (p=0.0095**, Mann-Whitney). A new primary antibody cut-off of 1:600 was derived from HC mean+3SD [211+(3x136.7)]. **(B)** Frequency of CASPR2 IgM detection across assay optimization process, from primary antibody 1:100, to 1:400, to final optimized dilution of 1:600. With initial 1:100 dilution, CASPR2 IgMs were detected in 60% of LGI1 patients (DCs) and 80% of HCs. With optimization, these reduced to 25% and 6%, respectively. Final optimized CBA run blindly for n=130 samples (n=35 HCs, n=20 LGI1 and n=24 AQP4 as DCs, n=51 CASPR2).
(C) MFI of beads used for flow cytometry standardization shown. No antibody binding capacity (ABC; beads 0, red), and incrementally increasing amounts of ABC for beads 1-4. **(D)** Summary data showing ΔABC for n=10 known HCs without CASPR2 IgM by CBA, and n=130 blinded samples. Cut-off for CASPR2 IgM positivity via FACS was determined as a ΔABC of 21342 based upon mean+3SDs ΔABC of n=10 HCs. **(E)** Breakdown of ΔABC values by patient group. Example of negative **(F)** and positive **(G)** CASPR2 IgM flow cytometry plot, in a HC and CASPR2 patient, respectively. **(H)** Rate of CASPR2 IgM positivity by CBA, FACS, and by both detection techniques, per patient group, with largely comparable rates found between techniques; a true positive CASPR2 IgM sample was regarded as positive by both techniques, but not either alone. **(I)** CASPR2 IgM CBA images at final optimization (1:600); x40 magnification. HC (left panel) with no IgM binding to green surface-expressed CASPR2-EGFP; 2 positive CASPR2 patients (panels 3 and 4) with strongly-positive IgM (red) binding to the cell surface (score 3, panel 3) and membrane co-localization (orange) with surface CASPR-EGFP, compared to low-positive signal (score 1, panel 4). No background signal was observed when secondary anti-human IgM antibody was used alone, without primary antibody/serum (right panel).

MFI, median fluorescence intensity; ABC, antibody binding capacity; ΔABC, delta (difference) in antibody binding capacity.

Table 4.1 Clinical Correlations of CASPR2 IgM-positive Patients

Patient Characteristic	**CASPR2 IgM +ve patients (n=4)**	**CASPR2 IgM -ve patients (n=21)***	**P values**
Median time of sample from disease onset (days)	321 (111-2551) (n=7 samples)	760 (23-4824) (n=44 samples)	-
Male sex	100%	89%	>0.9999
Median onset age	68	69	-
Tumour presence	25%	5%	0.3241
Relapse (ever occurred)	0%	21%	>0.9999
Syndrome			
LE	75%	53%	0.6036
NMT	25%	11%	0.4529
Morvan's	0%	11%	>0.9999
Unusual syndrome (LE+ataxia; LE+chorea; Morvan's+propriospinal myoclonus)	0%	16%	>0.9999
Unrelated	0%	11%	>0.9999

Comparison of clinical characteristics of CASPR2 IgM-positive and -negative CASPR2-antibody patients, deemed as positive only if determined positive by both optimized CBA (1:600) and flow cytometry. Data from n=51 samples from 25 patients. Fisher's exact test.

*2/21 IgM-negative patients lacked available clinical data; proportions indicated based upon n=19 patients.

LE, limbic encephalitis; NMT, neuromyotonia

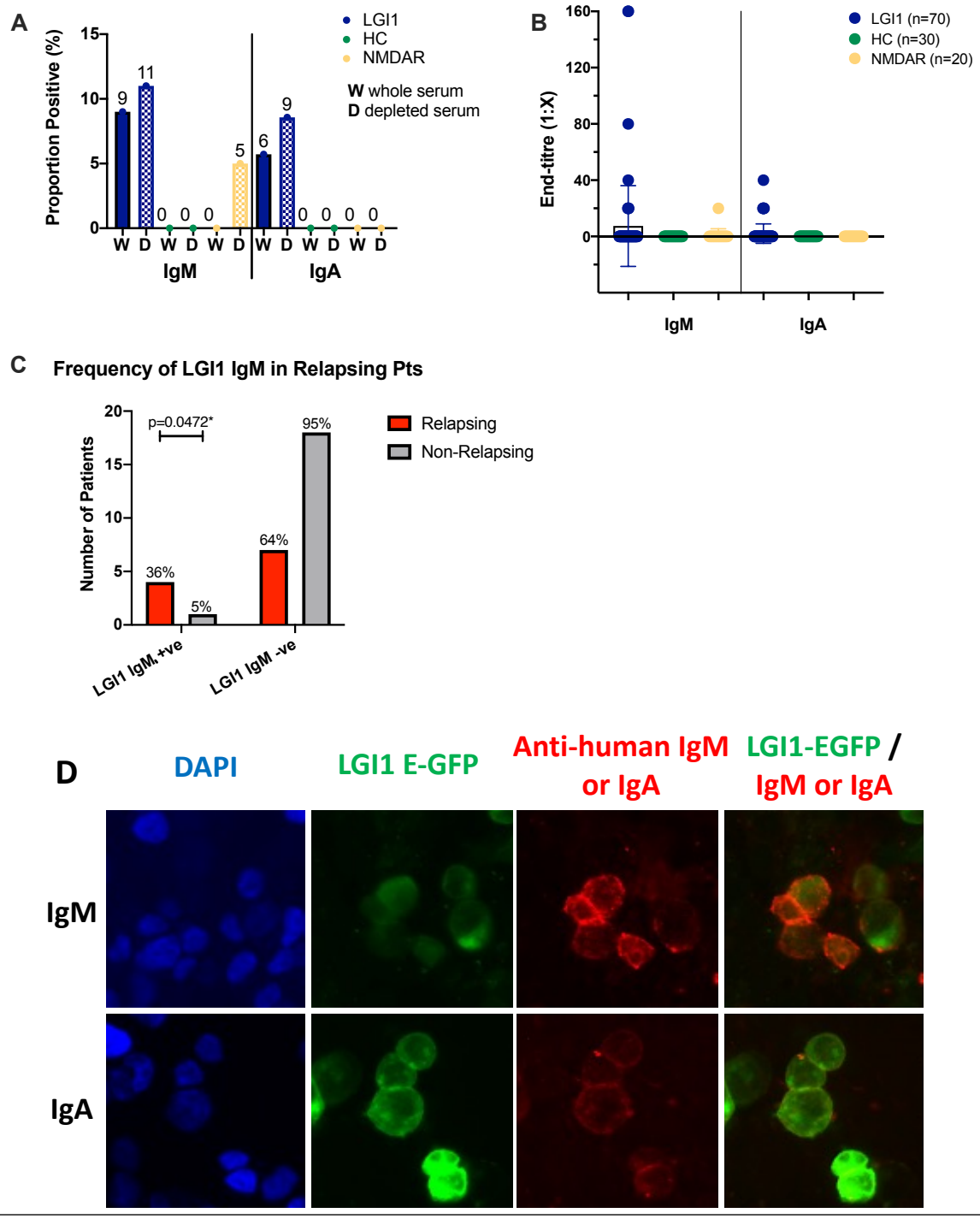
4.3 Results: Frequency of LGI1 IgM and IgA Antibodies in IgG-Depleted Sera

Next, as for CASPR2 IgM studies, we sought presence of LGI1-specific IgM and IgA antibodies in LGI1-antibody patients using live CBA in whole sera and IgG-depleted sera. IgG depletion was undertaken to ensure likely higher affinity IgGs did not inhibit binding of IgMs. 6/70 (9%) LGI1-antibody patient sera were positive for IgM in unfractionated serum. After IgG depletion, LGI1-antibody patient sera had absence of detectable LGI1 IgG antibodies, likely confirming complete depletion, and 8/70 (11%) sera were IgM-positive with end-titres ranging from 1:20-1:160 **(Figure 4.3 A+B)**. 4 of the 6 (67%) samples that had LGI1-IgM in undepleted serum also showed LGI1-IgM after IgG-depletion.

LGI1-IgMs were tested in 70 sera from 30 patients, including 11 relapsing patients. LGI1 IgM were positive in 8/70 (11%) IgG-depleted sera from 5/30 (17%) patients. 7/8 (88%) were from 4 relapsing patients, and 1/8 (12%) was from a non-relapsing patient sampled 1 month post disease onset. Overall, 4/11 (36%) relapsing patients had detectable LGI1 IgMs, compared to only 1/19 (5%) of non-relapsing patients (p=0.0472*, Fisher's exact test); **(Figure 4.3C)**.

4/70 (6%) were positive for LGI1-IgA in whole serum, and 6/70 (9%) were LGI1-IgA-positive in IgG-depleted sera at very low end-titres (range 1:20-1:40), including all 4 (100%) samples that were IgA-positive in whole serum. No HC sera (n=30) were positive for LGI1-specific IgM or IgA. 1/20 (5%) NMDAR-antibody patients had detectable LGI1-specific IgM from IgG-depleted serum, just reaching detection threshold (end-titre 1:20). Overall, LGI1-antibody patients showed LGI1-specific IgM and IgA antibodies at rates of ~10% **(Figure 4.3D)**.

Figure 4.3 LGI1 IgM and IgA Antibody Frequencies



(A) Frequencies of LGI1-specific IgM and IgA antibodies in whole and IgG-depleted sera by live CBA. LGI1-specific IgMs were detected in 6/70 (9%) LGI1 patient samples in whole serum, and 8/70 (11%) IgG-depleted sera. 0/30 HCs were positive, but 1/20 (5%) NMDAR-antibody patient had detectable LGI1-IgM on IgG-depleted serum. LGI1-specific IgAs were found in 4/70 (6%) and 6/70 (9%) LGI1 patient whole and IgG-depleted sera, respectively. No HCs or NMDA-antibody patients had LGI1-specific IgA antibodies. **(B)** In LGI1 patients, LGI1 IgM end-titres ranged from 1:20-1:160, and LGI1 IgA end-titres were 1:20-1:40. The LGI1-specific IgM found in the NMDAR-antibody patient reached 1:20 end-titre. **(C)** LGI1 IgM were present more frequently in relapsing compared to non-relapsing patients (4/11, 36%, versus 1/19, 5%; p=0.0472*, Fisher's exact).
(D) LGI1-specific IgM (top row) and IgA (bottom row) antibodies found in LGI1 patients. Anti-human IgM or IgA (red) can be seen co-localizing with surface-expressed LGI1-EGFP (green) on HEK cells. DAPI, blue; images taken at x40 magnification.

4.4 Results: LGI1-Autoantibody Epitope Specificities

Additionally, we explored patient serum binding patterns to the two major LGI1 domains (LRR and EPTP) to examine whether LGI1-antibodies were restricted to one particular LGI1 domain or both over time. Findings were correlated with potentially influencing factors including patient-specific or disease timepoint. End-point titrations against full-length LGI1, LRR- and EPTP-domains (Yamagata et al. 2018) were quantified by live-CBA for 102 sera and 11 CSF samples from 38 LGI1-antibody patients.

4.4.1 LGI1 Patients Show Serum Autoantibodies to Both EPTP and LRR Domains in Equal Proportions Throughout Disease

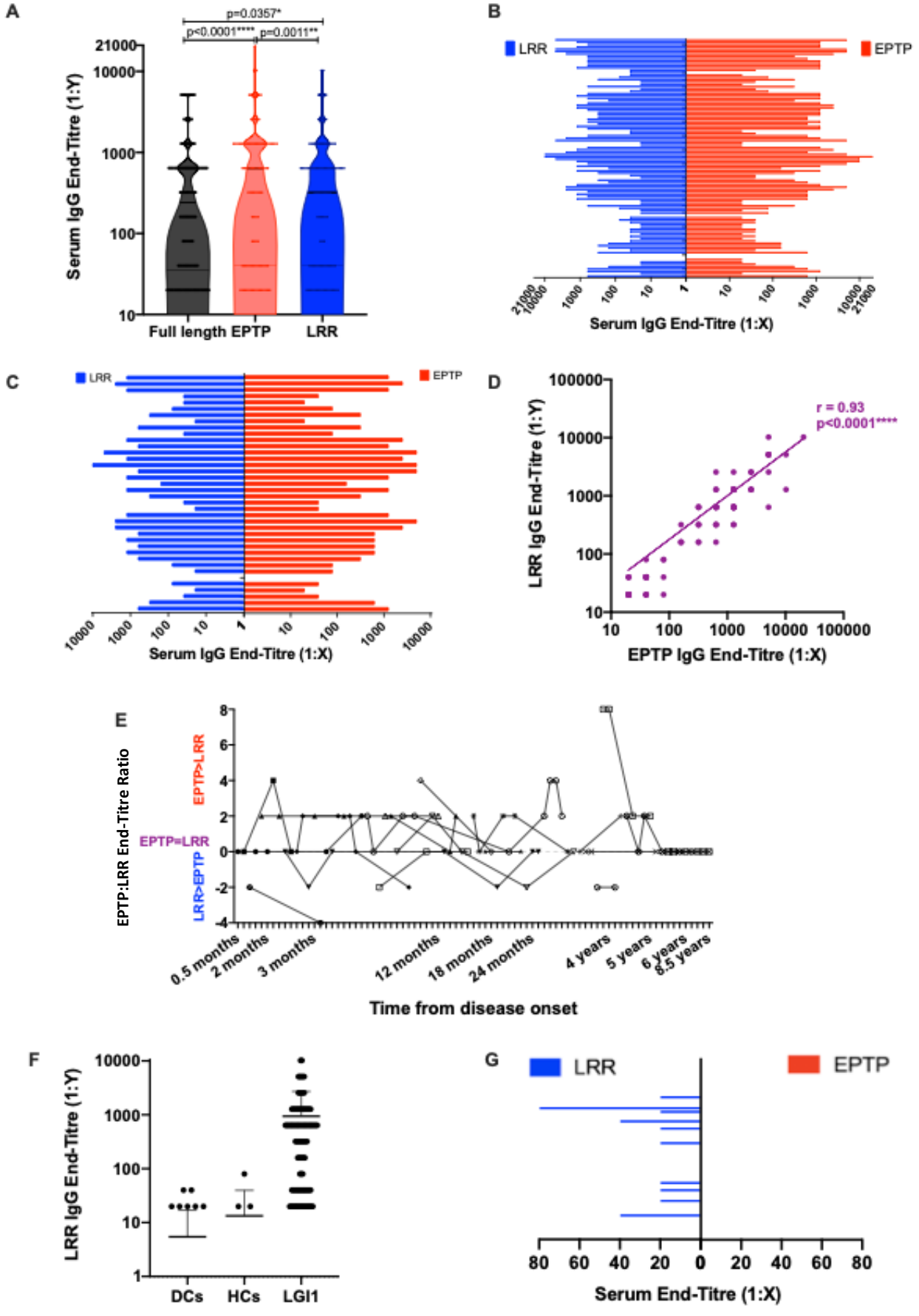
Overall, serum EPTP domain end-titres were higher than end-point dilutions for the LRR domains or full-length LGI1 **(Figure 4.4A)**. Of 102 sera from patients with LGI1-antibody encephalitis, 2 samples (from the same patient) were negative on all three constructs. Of the remaining 100 sera, only 3 lacked reactivity to any of the three constructs: one bound full-length and LRR, but demonstrated no reactivity to EPTP, one bound full-length and EPTP but not LRR, and another bound full-length at low titre (1:20) but neither EPTP nor LRR **(Figure 4.4B)**. Overall, EPTP and LRR end-titres showed strong correlations within individual samples (Spearman r = 0.9257, p<0.0001****, n=102; **Figure 4.4B+C+D)** and full-length LGI1-antibody serum end-titres also correlated strongly with EPTP- and LRR-domain end-titres (Spearman r 0.9221 and 0.8965, respectively, p<0.0001****).

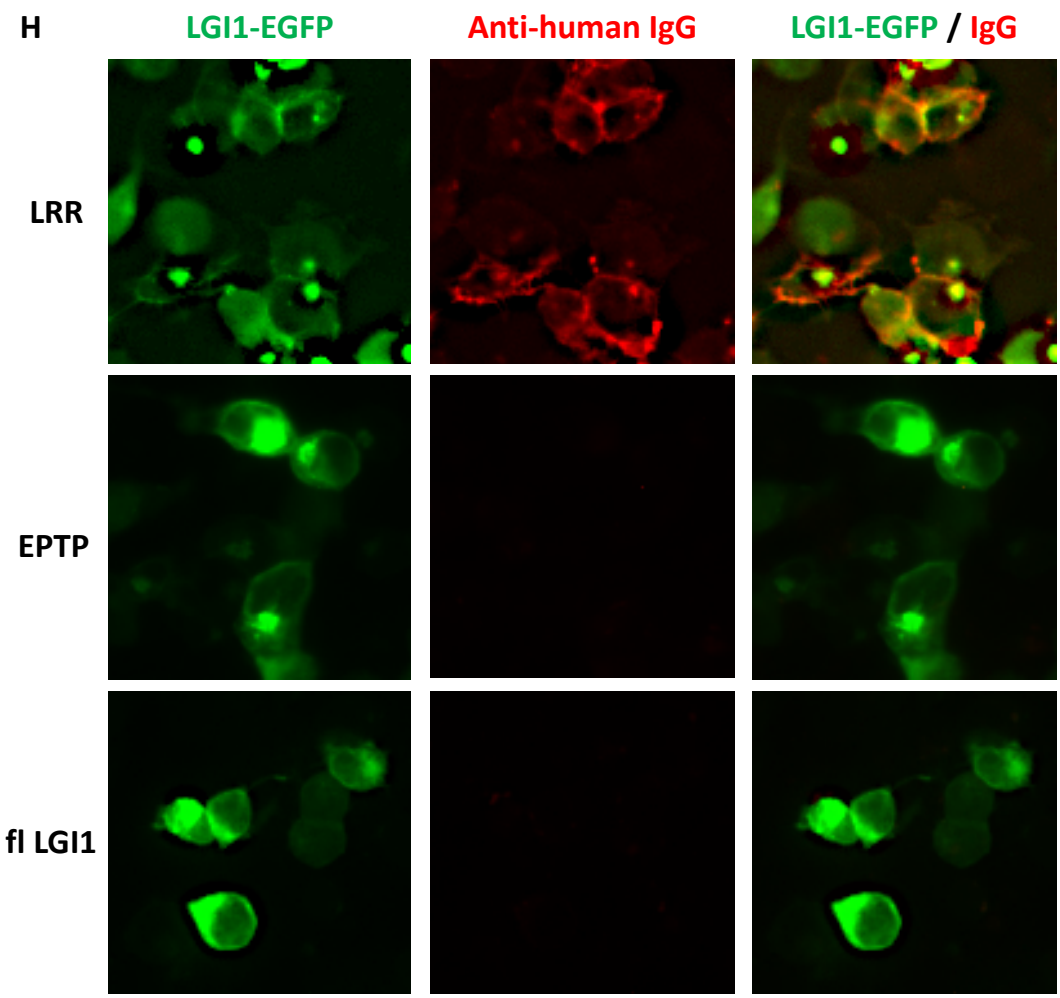
EPTP:LRR end-titre ratios were examined longitudinally in n=19 patients with available serum samples, to ask whether relative epitope shifts were observed during the disease course, representing putative ongoing GC activity. Overall, patients showed constant EPTP:LRR ratios between first and last sample (p=0.32; Wilcoxon test**; Figure 4.4E**), although there was some skewing towards EPTP early, particularly within the first year. By 5 years post onset, ratios appeared equal in all patients. The only clear exception was an 8-fold higher EPTP to LRR ratio from a LGI1/CASPR2 double-positive, relapsing patient.

4.4.2 Control Patient Sera Bind the LRR LGI1 Domain

Unexpectedly, 33% (3/9) of HCs and 21% (7/33) of DCs showed serum reactivity to LRR **(Figure 4.4 F, G+H)**. No HC or DC sera showed reactivity to EPTP-domain **(Figure 4.4G)** or full-length LGI1. Maximum end-titre reached 1:80 in HCs, and 1:40 in DCs **(Figure 4.4F)**. All reactive DC sera were from MS patients.

Figure 4.4 Patient Serum Antibody Binding Patterns to LGI1 EPTP and LRR Domains





(A) End-titres for n=102 sera tested by live CBA for LGI1 constructs (full-length LGI1 black, EPTP red, LRR blue). Mean end-titre: EPTP 1:1298, LRR 1:946, full-length 1:815. Comparison of matched serum end-titres (EPTP versus fl p<0.0001****; EPTP versus LRR p=0.0011**; LRR versus fl p=0.0357*; Wilcoxon signed rank test).
EPTP and LRR end-titre comparison for matched sera for n=102 samples **(B)** and first sample (closest to disease onset) for n=38 patients **(C)**. Correlation for serum EPTP and LRR end-titres shown for n=102 samples; some points overlapping **(D)**.
(E) Longitudinal trends in EPTP:LRR end-titre ratios over disease course (81 samples available from 19 patients). Sera with equal EPTP:LRR ratios are plotted along Y=0, those with LRR>EPTP in the negative Y-axis region, and EPTP>LRR in the positive Y-axis region.
(F) HC and DC reactivities to LRR-domain. Sera from 3/9 (33%) HCs and 7/33 (21%) DCs bound to LRR (end-titres 1:20-80 and 1:20-40, respectively). **(G)** EPTP versus LRR end-titre comparison for matched sera of HCs and DCs; no HC or DC sera reacted to full-length LGI1 or EPTP.
(H) Images of an MS patient serum binding to LRR (1:40) with co-localization of anti-human IgG (red) with surface-expressed EGFP-tagged LRR (green). No reactivity is seen to EPTP or full-length (fl) LGI1. x40 magnification

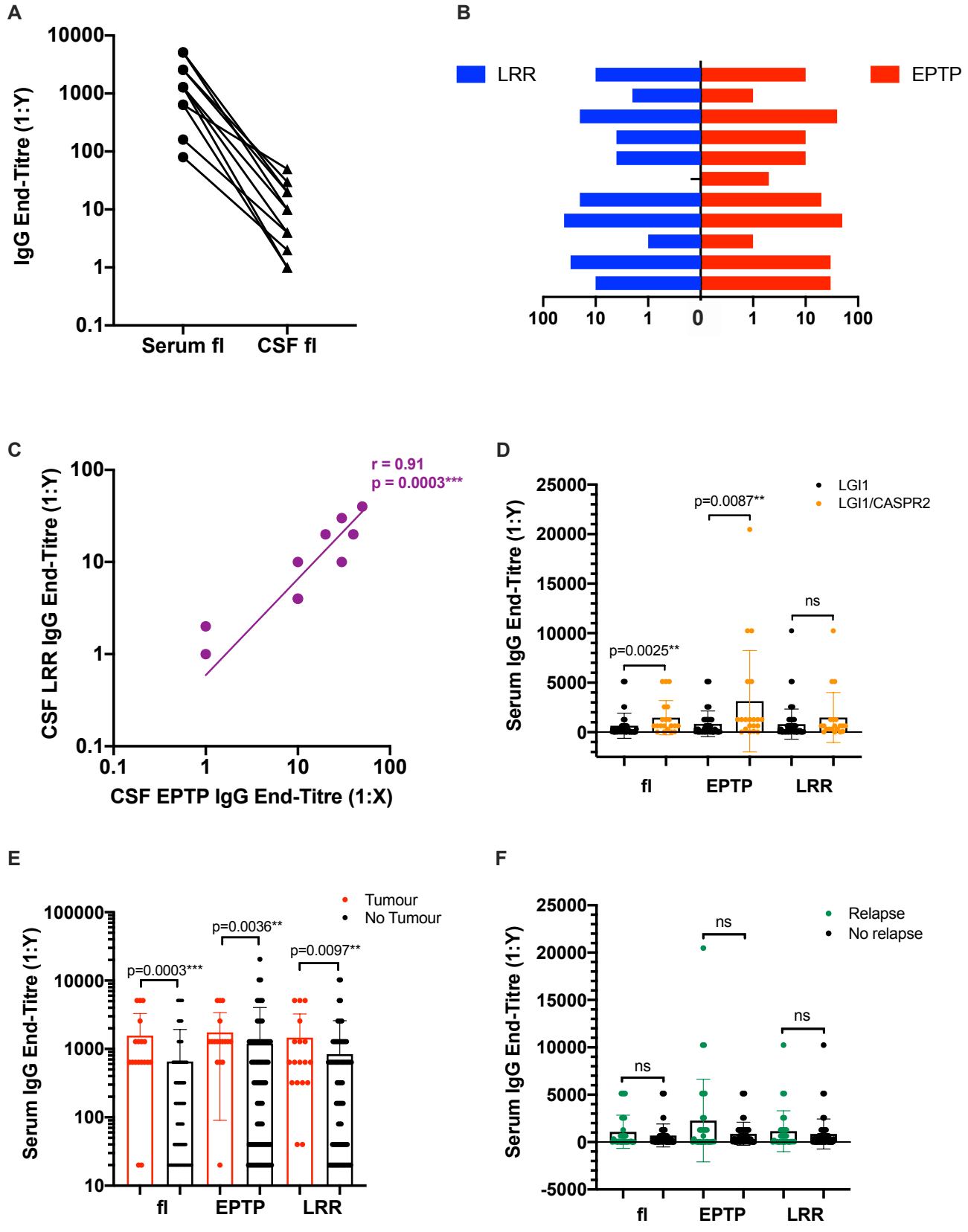
4.4.3 Patient CSF Binding Patterns are Similar to Serum: LGI1 Autoantibodies to both EPTP and LRR domains are found in Equal Proportions

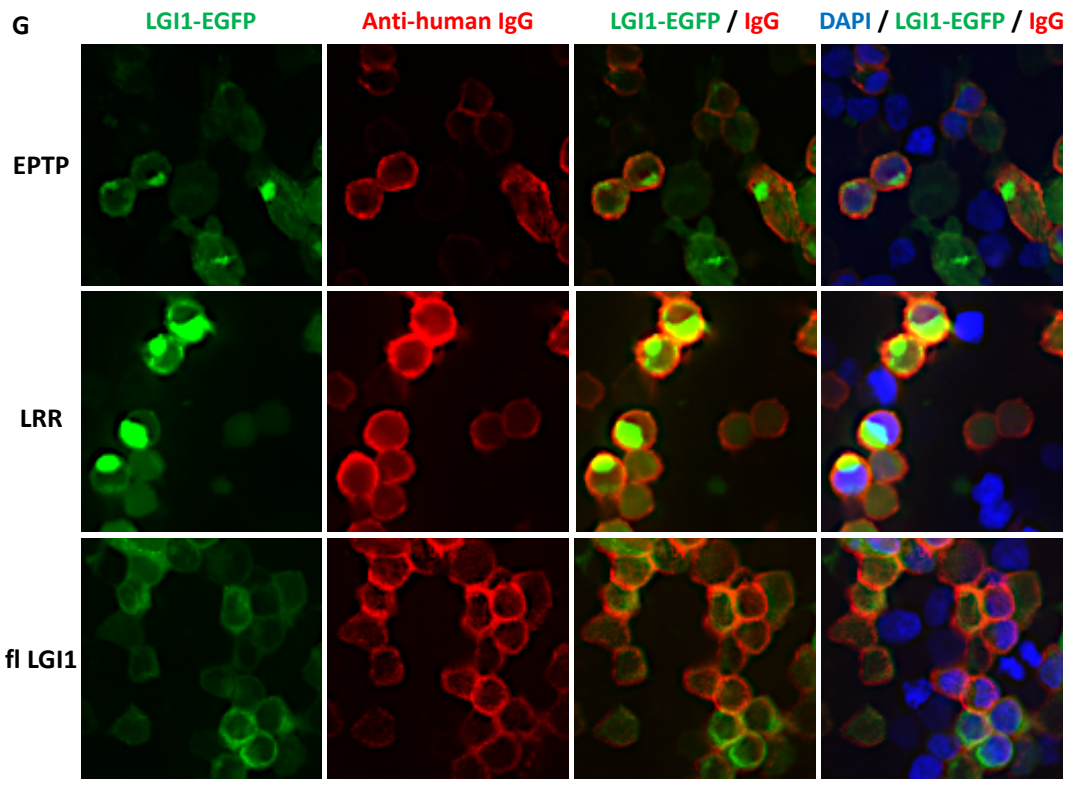
Next, CSF LGI1 epitope binding was examined. 11 of 13 (85%) CSF samples from n=10 patients were positive for LGI1 antibodies and quantified by end-titre. When paired sera and CSF were compared (at matched disease timepoints), serum full-length LGI1 antibody levels were up to 1280-fold (mean 313-fold) higher than CSF **(Figure 4.5A)**. 10/11 (91%) CSF samples had antibodies to both LGI1 domains, and only 1 patient showed selective binding to EPTP only **(Figure 4.5B, G+H)**. As with serum, CSF EPTP end-titres were similar or higher than LRR **(Figure 4.5B)** and these correlated strongly (Spearman r = 0.9072, p=0.0003***, n=11); **(Figure 4.5C)**.

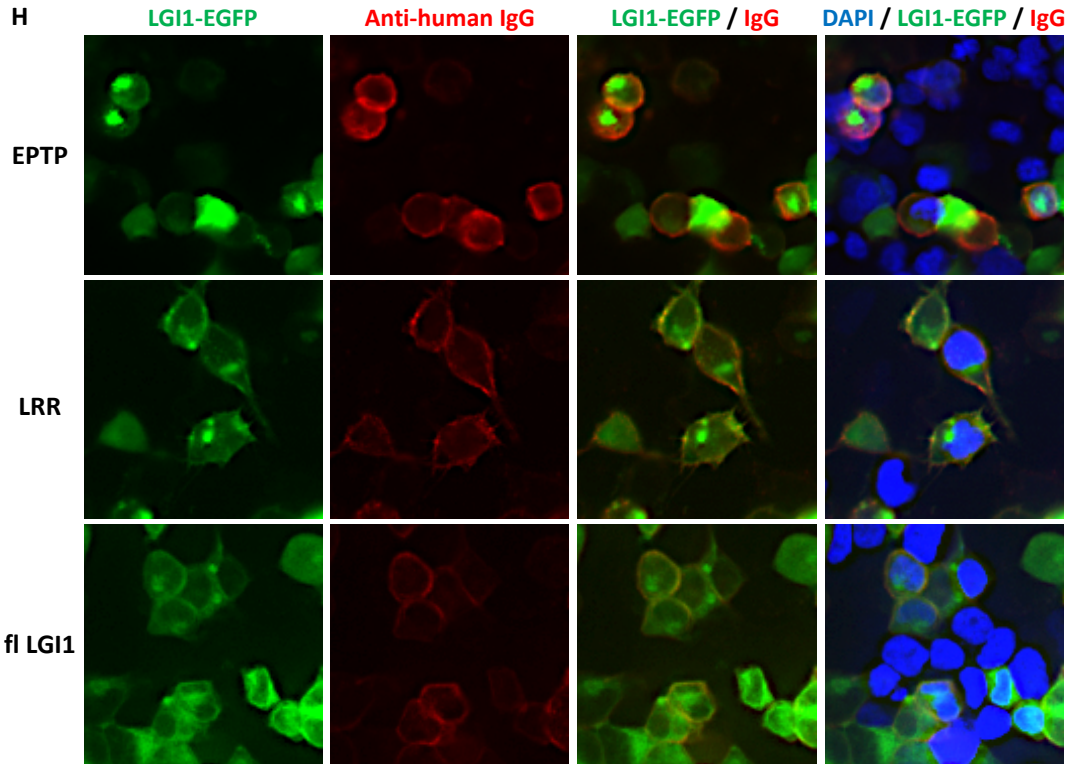
4.4.4 Presence of Tumour or Additional CASPR2 Autoantibodies May Influence LGI1 Autoantibody Production, but not Epitope Specificities

LGI1-, EPTP- and LRR-antibody end-titres were higher in LGI1/CASPR2 double-positive patients (n=20 sera from 6 patients) compared to LGI1-antibody only patients (full-length p=0.0025**, EPTP p=0.0087**, LRR p=0.1262 ns; Mann Whitney test); **(Figure 4.5D)**. Similarly, end-titres were significantly higher in samples from patients with tumours (n=18 samples from 4 patients) compared to those without (full-length p=0.0003***; EPTP p=0.0036**; LRR p=0.0097**; Mann Whitney test); **(Figure 4.5E)**. No significant differences were found in antibody levels in samples from patients with ever-relapse versus non-relapsing patients **(Figure 4.5F)**.

Figure 4.5 Patient CSF Antibody Binding Patterns to LGI1 Epitopes and Clinical Associations




G
LGI1-EGFP
Anti-human IgG
LGI1-EGFP / IgG
DAPI / LGI1-EGFP / IgG
EPTP
LRR
fl LGI1


H
LGI1-EGFP
Anti-human IgG
LGI1-EGFP / IgG
DAPI / LGI1-EGFP / IgG
EPTP
LRR
fl LGI1

(A) Serum versus CSF IgG end-titre comparison shown for full-length (fl) LGI1. Lines link n=11 paired samples at matched timepoints in disease. Serum LGI1 antibody levels were mean 313-fold higher than CSF (range 12.8-1280).
(B) Comparison of EPTP and LRR end-titres in matched CSF samples shown (n=11). 10/11 CSF samples had antibodies to both LGI1 domains. Only 1 patient lacked reactivity to LRR. CSF EPTP end-titres were similar or higher than LRR (EPTP range 1:1-50, LRR 0-1:40).
(C) Correlation for CSF EPTP and LRR end-titres for n=11 samples; some points overlapping.
(D) End-titre comparison for LGI1-antibody (n=82 samples from 32 patients) and LGI1/CASPR2 double-positive (n=20 samples from 6 patients) patient sera shown for all three LGI1 constructs. Mean end-titres were higher in double-positive compared to LGI1-antibody only patients (fl 1: 1462 versus 657; EPTP 1: 3128 versus 852; LRR 1: 1479 versus 816, respectively; significant for fl and EPTP).
(E) End-titre comparison for tumour (n=18 samples from 4 patients) versus non-tumour samples (n=84 samples from 34 patients) found significantly higher antibody levels in tumour patients for all 3 constructs. Mean end-titre in tumour versus non-tumour group, respectively: fl 1: 1567 versus 654; EPTP 1: 1743 versus 1203; LRR 1: 1462 versus 836.
(F) 31 sera from 8 patients with relapses were compared to 71 samples from 30 patients without relapse. No significant difference was observed in mean end-titre (relapse versus no relapse, respectively: fl 1: 1081 versus 699, p=0.60; EPTP 1: 2272 versus 873, p=0.90; LRR 1: 1155 versus 855, p=0.91). Mann-Whitney; two-tailed P value used for statistical analyses **(D-F)**.
Double-positive LGI1/CASPR2-antibody patient CSF demonstrating reactivity to all LGI1 epitopes from neat **(G)** and remaining substantially positive (CBA score 2) at 1:20 dilution **(H)**; images x40 magnification.

4.5 CXCL13 as a Biomarker of Disease Perpetuation in LGI1- and CASPR2-Autoantibody Syndromes

4.5.1 Evidence for CXCL13 as a Biomarker for Germinal Centre Activity

CXCL13 is a potent B cell chemoattractant secreted by stimulated antigen presenting cells and some T cells. CXCL13 acts via its receptor, chemokine receptor 5 (CXCR5) on B cells, triggering B cell homing from the blood to GCs within lymph nodes. Monitoring of GC activity is crucial for measuring antibody responses, particularly in vaccine medicine. Although this was traditionally only possible by direct assessment of lymphoid tissue, one pivotal study showed that plasma CXCL13 levels are a useful biomarker of GC activity (Havenar-Daughton et al. 2016). In HIV-infected humans, and immunized mice and macaques, plasma CXCL13 levels correlated with levels of HIV-specific broadly neutralizing antibodies, number of GCs detected in lymph nodes, and importantly, the magnitude of antibody responses post immunizations in humans. Moreover, consistent correlations between CXCL13 levels and GC activity were found across a variety of immune-activation settings, including HIV infection, HIV and yellow-fever vaccines, immunizations containing either aluminum or other ligand adjuvants, and acute viral infections. This supports the validity of CXCL13 as biomarker of GC activity in the context of antibody responses generated by various mechanisms, including autoimmunity. Indeed, high CXCL13 levels are detected in the synovium of patients with rheumatoid arthritis, and in the salivary glands of patients with Sjögren's syndrome (Finch et al. 2013), implicating CXCL13 in examples of autoimmune diseases.

In neurological autoantibody-mediated diseases, increased serum CXCL13 levels are found in myasthenia gravis (Shiao et al. 2010), and CXCL13 levels are elevated in the CSF of patients with NMDAR-antibody encephalitis (Leypoldt et al. 2015; Liba et al. 2016; Kothur et al. 2016), and NMO (Zhong et al. 2011). Moreover, CSF CXCL13 levels are elevated in other neuroinflammatory disorders such as multiple sclerosis (MS) (Khademi et al. 2011), ADEM (Kothur et al. 2016), paediatric opsoclonus-myoclonus syndrome (OMS) (Pranzatelli et al. 2013), and neuroborreliosis (Wutte et al. 2011; Hytönen et al. 2014). Intriguingly, histological studies have detected CXCL13 in active MS lesions (Krumbholz et al. 2006). Finally, in MS, CXCL13 levels directly correlate with intrathecal immunoglobulin production and number of CSF B cells and plasma cells (Krumbholz et al. 2006; Lepennetier et al. 2019). These collective

findings indicate a role for CXCL13 in recruitment of peripheral B cells to the CSF compartment.

4.5.2 Results: CXCL13 in LGI1- and CASPR2-Autoantibody Syndromes

At time of performing the experiments, CXCL13 had not been studied in LGI1- or CASPR2-antibody diseases. The objectives were to perform a cross-sectional study to examine CXCL13 concentrations in LGI1- and CASPR2-antibody patients, to infer GC activity.

4.5.2.1 Validation Studies

Serum CXCL13 Cut-Off for Positivity is 145 pg/ml and the CXCL13 ELISA Assay Shows Strong Inter-Assay Reliability

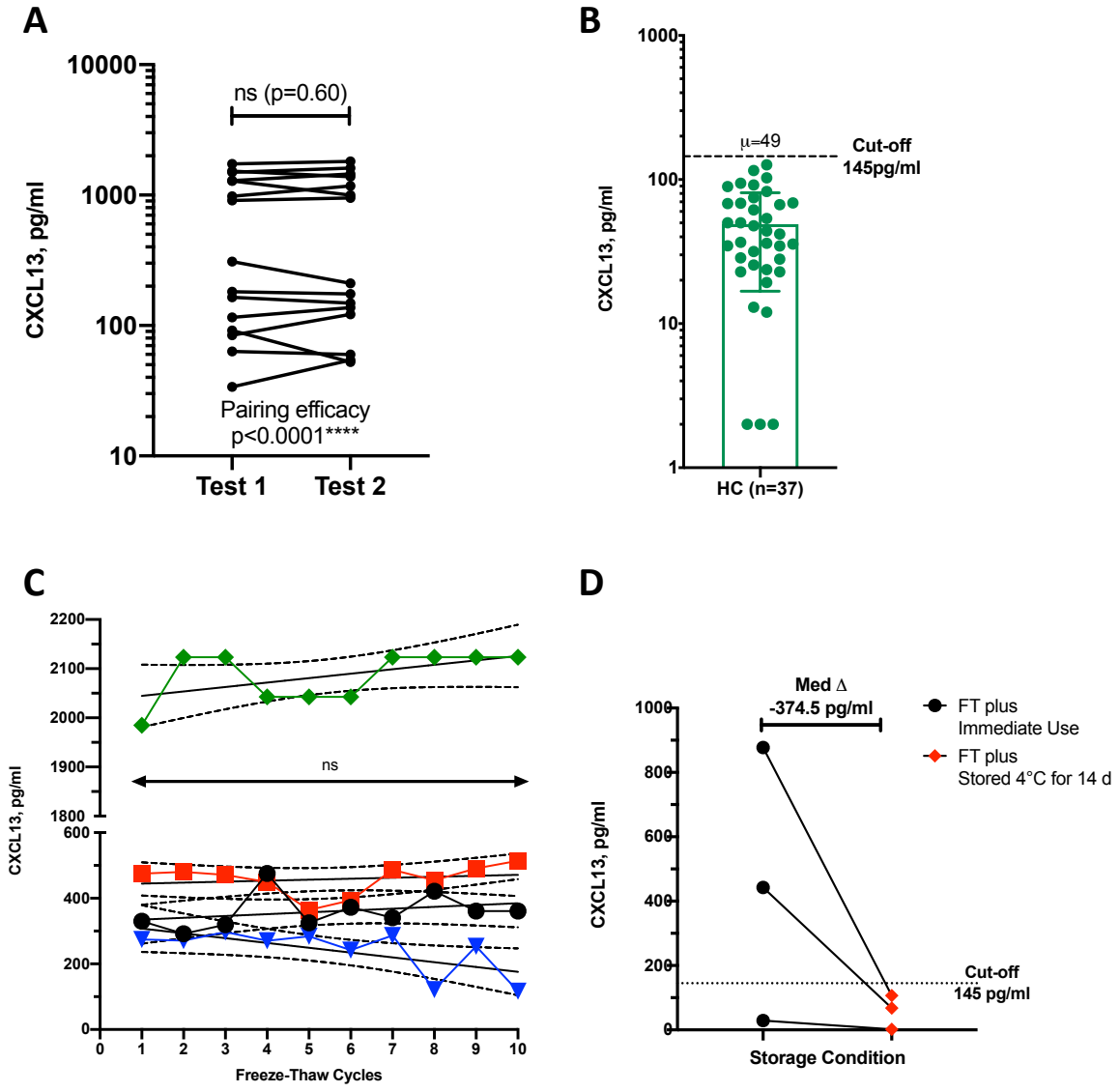
Serum CXCL13 levels were determined by sandwich ELISA **(Methods, Chapter 2, Section 4.5)**. A serum CXCL13 cut-off for positivity of 145 pg/ml was determined as mean+3SD of the n=37 HCs **(Figure 4.6B)**. No outliers were identified from the HC values when analyzed by the non-linear regression ROUT outlier analysis method (Q=1%). HC serum levels (median 42 pg/ml, mean 49 pg/ml; **Table 4.2)**, were comparable to those reported by others [median 64 pg/ml from n=300 HCs (Wutte et al. 2011) and mean 105 pg/ml from n=233 HCs (Shiao et al. 2010)]. When 15 sera were compared on separate assays, no significant differences in the measured CXCL13 level of individual samples were found (p=0.60), confirming inter-assay reproducibility **(Figure 4.6A)**.

Sample CXCL13 Levels are Affected by Prolonged Storage at 4°C but not Multiple Freeze-Thaw Cycles

Some of our samples were analysed after varied numbers of freeze-thaw cycles. Thus, I sought to ascertain whether the freeze-thaw process reduced CXCL13 levels. Freeze-thawing did not affect serum CXCL13 concentrations, with no significant differences detected in 4 sera over 10 freeze-thaw cycles (p>0.36); **(Figure 4.6C)**. Prolonged sample storage at 4°C for 14 days, however, dropped the CXCL13 levels in 3 sera (median change -374.5 pg/ml) compared to immediate testing following thawing **(Figure 4.6D)**. Moreover, in 2 samples originally

identified as positive, the CXCL13 level dropped to below cut-off with prolonged storage at 4°C.

Figure 4.6 CXCL13 Validation Studies: Inter-Assay Reliability, Derivation of Serum Cut-Off level, and Impact of Freeze-Thaw



(A) CXCL13 concentrations of 15 sera tested twice on separate assays on separate days. CXCL13 levels were non-significantly different between assays (p=0.60; Wilcoxon matched-pairs signed rank test), and pairing was significantly effective (p<0.0001****; Rs 0.9643, Spearman). **(B)** Derivation of serum CXCL13 cut-off level from mean+3SD of n=37 HCs. **(C)** Effect of freeze-thaw on CXCL13 concentration was assessed by measurement of CXCL13 concentrations in 4 serum samples across 10 freeze-thaw cycles; samples compared on the same assay. No significant differences in CXCL13 level observed over 10 freeze-thaw cycles (green p=0.36, red p=0.40, black p>0.99, blue p=0.40; Runs test). Interpolation of standard curve line plotted. **(D)** Impact of sample storage on CXCL13 level. Fresh serum samples were obtained from n=3 patients, aliquoted into 2 vials, and cryopreserved immediately at -80°C. 1 vial was thawed only on the day of testing ('FT plus immediate use'). The 2nd vial was thawed and stored at 4°C for 14 days until testing ('FT plus stored 4°C for 14 d'). Median change in detectable CXCL13 level was -374.5 pg/ml with storage at 4°C. In 2/2 (100%) of samples originally detectable as positive, the level dropped to below cut-off with prolonged storage at 4°C.
FT, freeze-thaw

4.5.2.2 Serum and CSF CXCL13 Levels Across Autoantibody Targets

Serum CXCL13 levels were determined in 543 samples from 398 subjects: LGI1 (n=142 from 48 patients), CASPR2 (n=63 from 31 patients), double-positive LGI1/CASPR2 (n=19 from 4 patients), pharmacoresistant epilepsy patients (n=282), and healthy controls (HC, n=37 from 33 participants).

Additionally, 60 CSF samples were tested: n=31 HCs, n=3 DCs (n=1 headache, n=1 GAD epilepsy, n=1 drug resistant epilepsy, DRE), n=16 from 12 LGI1-antibody patients, n=7 from 6 CASPR2-antibody patients, and n=3 from 2 double-positive LGI1/CASPR2 patients. The patient groups evaluated and CXCL13 values are summarised in **Table 4.2**.

Table 4.2 Summary CXCL13 Sample Groups and Concentrations

Sample Type	Group	Sample n (subjects)	Mean CXCL13, pg/ml	Median (range) CXCL13, pg/ml	CXCL13 Positive n (%)
Serum	HC	37 (33)	49	42 (2-127)	0/37 (0)
	Drug resistant epilepsy	227 (227)	85	41 (2-1706)	29/227 (13)
	Seizure-free epilepsy controls	55 (55)	70	29 (2-837)	5/55 (9)
	LGI1	142 (48)	338	144 (3-3415)	70/142 (49)
	CASPR2	63 (31)	157	69 (17-822)	17/63 (27)
	Double-positive LGI1/CASPR2	19 (4)	740	666 (36-2740)	18/19 (95)
CSF	HC	31 (31)	1.7	1.0 (1.0-10.3)	1/31 (3)
	DC*	3 (3)	1.7	2.0 (1.0-2.0)	0/8 (0)
	LGI1	16 (12)	3.2	2.0 (2.0-13.6)	1/16 (6)
	CASPR2	7 (6)	13.6	2.0 (2.0-52.2)	2/7 (29)
	Double-positive LGI1/CASPR2	3 (2)	2.0	2.0 (2.0-2.0)	0/3 (0)

Table 4.2 summarises the CXCL13 groups by antibody/ disease and indicates mean CXCL13 concentrations in serum and CSF samples. For each group, the proportion regarded as positive based upon assay cut-off of mean+3SD is indicated.

*DCs n=3 (headache n=1, GAD epilepsy n=1, drug resistant epilepsy n=1).

Serum CXCL13 Levels Are Significantly Elevated in the Presence of LGI1-Antibody

Significant variation in median CXCL13 levels was observed across groups (p<0.0001****, Kruskal-Wallis ANOVA) with non-significant differences in median CXCL13 serum level between the following groups: DRE versus seizure-free, HC versus DRE, and HC versus seizure-free (p>0.999, Dunn's multiple comparisons); **(Figure 4.7A)**. CASPR2-antibody patients had mean CXCL13 serum levels of 157 pg/ml **(Table 4.2),** which were significantly higher than HCs, DRE, and seizure-free patients (p=0.0341*, p=0.0011** and p=0.0007***, respectively); **(Figure 4.7A)**. However, LGI1-antibody patients revealed the highest serum CXCL13 levels (mean 386 pg/ml) and highly significant differences across all groups (LGI1 versus DRE, seizure-free, and HCs p<0.0001****; LGI1 versus CASPR2 p=0.0015**); **(Figure 4.7A)**. Moreover, when patients with LGI1-antibodies were subdivided into those with LGI1-only and those with additional CASPR2-antibodies (double-positive patients), three observations emerged **(Figure 4.7B)**. First, LGI1-only antibody patients persisted to show significantly higher CXCL13 levels than all other groups (LGI1 versus HCs, DRE, and seizure-free p<0.0001****; LGI1 versus CASPR2 p=0.0175*), and reached levels of 3415 pg/ml. Second, double-positive patients also demonstrated significantly higher serum CXCL13 levels (mean 740 pg/ml) compared to all other groups (LGI1/CASPR2 versus CASPR2, HCs, DRE, and seizure-free p<0.0001****). Finally, no significant differences were observed in serum CXCL13 levels between LGI1-only antibody versus LGI1/CASPR2 double-positive patients (p=0.0527). Taken together, these findings indicate that patients with LGI1- (and CASPR2-) antibodies show an elevated serum CXCL13 level.

CSF CXCL13 Levels Are Elevated in LGI1- and CASPR2-Antibody Patients

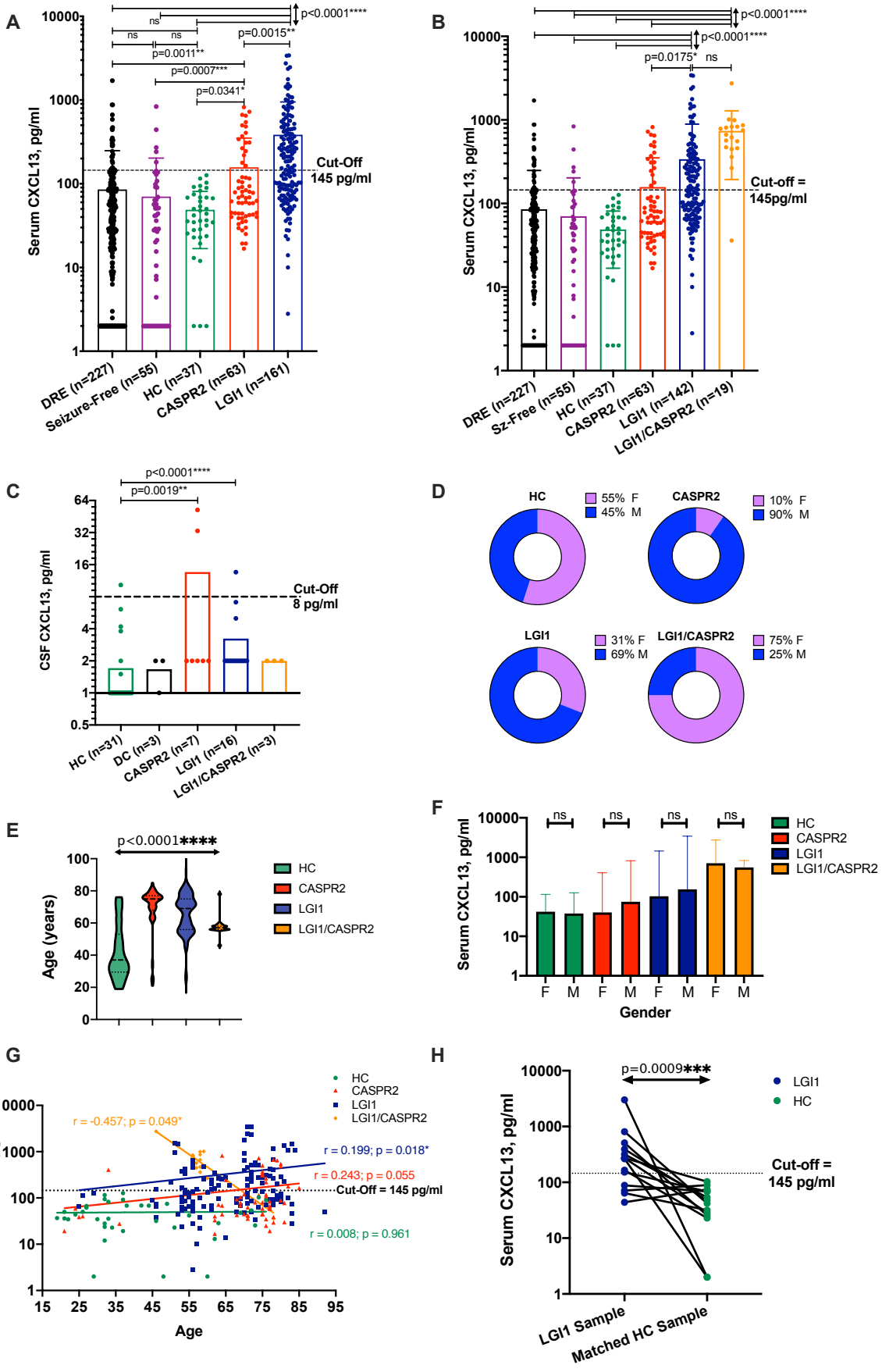
CSF cut-off was set as 8 pg/ml based on mean+3SD of n=31 HCs **(Figure 4.7C)**. Highest CSF CXCL13 levels were found amongst CASPR2-antibody patients (mean 13.6 pg/ml; **Table 4.2**). Kruskal-Wallis one-way ANOVA revealed significant differences in CSF CXCL13 levels across groups (p<0.0001****). CSF CXCL13 levels in CASPR2- and LGI1-antibody patients were significantly higher than HCs (p=0.0019** and p<0.0001****, respectively; Dunn's multiple comparisons test); **(Figure 4.7C)**. For 26 matched patient samples, serum CXCL13

concentrations were higher than CSF (mean 225 pg/ml versus 6 pg/ml; p<0.0001****, Wilcoxon matched pairs signed rank test); **(Figure 4.7I)** with serum:CSF CXCL13 ratios a mean of 108-fold higher in serum (range 0.3-547).

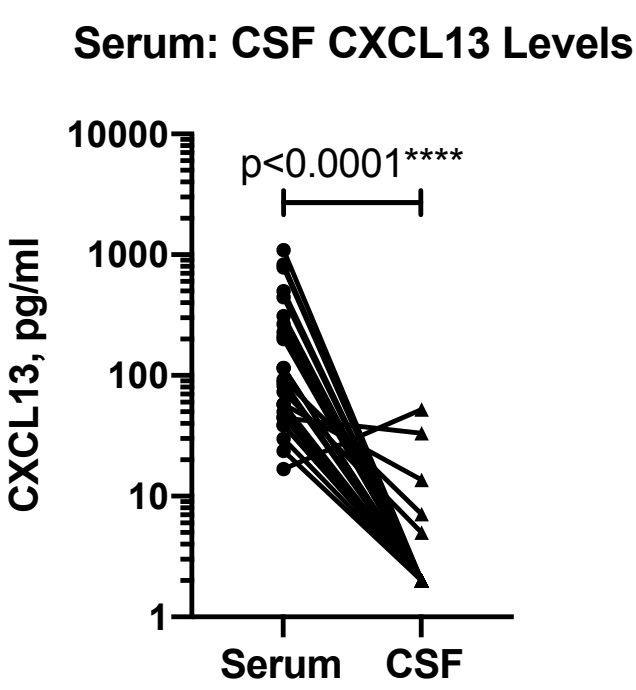
CXCL13 Serum Levels Are Not Influenced by Gender but may be Partially Influenced by Age

Next, given the known predilection for LGI1-antibody disease in older males, we sought to explore whether increased age and male gender could contribute to CXCL13 levels. Cohort demographics revealed even gender representation within HCs, and as expected, LGI1 and CASPR2 groups were skewed towards male gender, whereas double-positive LGI1/CASPR2 patients skewed towards female gender **(Figure 4.7D)**. However, across all groups, no significant differences were found between male and female serum CXCL13 levels **(Figure 4.7F)**. Age significantly differed between groups (p<0.0001****, Kruskal-Wallis); median age in years at sample (range): HC 37 (19-76); CASPR2 75 (21-85); LGI1 69 (25-92); LGI1/CASPR2 57 (46-78); **(Figure 4.7E)**. Significant correlations were found between age and CXCL13 level in LGI1- and LGI1/CASPR2-antibody patients (Spearman r=0.199, p=0.018*; r= -0.457, p=0.049*, respectively) but not in HCs or CASPR2-antibody patients (p=0.961; p = 0.055, respectively); **(Figure 4.7G)**. However, it can be seen that CASPR2- and LGI1-antibody patients across extremes of age (from the second to ninth decade) exhibit CXCL13 levels both above and below cut-off, and double-positive LGI1/CASPR2 patients demonstrate almost universally elevated CXCL13 levels. Yet, HCs spanning 19-76 years show CXCL13 levels <145 pg/ml. Moreover, LGI1/CASPR2-autoantibody patients showed an *inverse* association between age and CXCL13 levels. Yet, this group of patients exhibit some of the cohort's highest CXCL13 levels. Finally, comparison of CXCL13 levels between LGI1-antibody patients and age- and sex-matched HCs confirmed significantly higher levels in LGI1-antibody patients (p=0.0009***, Wilcoxon matched-pairs signed rank test; **Figure 4.7H**). Thus, any effect of age can only partly explain the markedly elevated CXCL13 levels observed in patients with LGI1 antibodies. Taken together, these findings indicate that the older age and male gender preponderances of patients with LGI1-antibodies do not appear to influence the CXCL13 level. Rather, antibody status and/ or disease appears to account for the elevated CXCL13 concentrations seen in LGI1-antibody syndromes.

Figure 4.7 Serum and CSF CXCL13 Concentrations Across Autoantibody Groups and Influence of Age and Gender on CXCL13 Level



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(A) Serum CXCL13 concentrations in drug resistant epilepsy (DRE) patients, seizure-free epilepsy controls, healthy controls (HC), and patients with CASPR2- and LGI1-antibodies. Scatter plot; mean+SD shown. **(B)** Serum CXCL13 concentrations across groups. Patients with LGI1-antibodies subdivided into LGI1-only (blue) and those with additional CASPR2 antibodies (LGI1/CASPR2; orange). **(C)** CSF CXCL13 levels across patient groups. CSF cut-off derived from mean+3SD of n=31 HC values. Adjusted p values derived from Dunn's multiple comparisons test **(A-C)**. **(D)** Donut chart representation of gender distribution within groups. **(E)** Violin plot of age distribution amongst patient groups for all samples; median ages (dotted line). **(F)** Influence of gender on serum CXCL13 level; median with range plotted. Female versus male median CXCL13 levels (HC p=0.65, CASPR2 p=0.23, LGI1 p=0.31, LGI1/CASPR2 p=0.56; Mann-Whitney). **(G)** Influence of age on CXCL13 level across groups. Correlations (Spearman r, p values) shown. Data for n=261 samples **(E-G)**. **(H)** Comparison of CXCL13 levels between LGI1 patients (n=14) and age- (+/- 3 years) and sex-matched HCs (n=14). **(I)** Comparison of CXCL13 concentrations in serum and CSF for n=26 samples at matched timepoints. **(H-I)** Wilcoxon matched pairs signed rank; lines indicate matched samples.

4.5.2.3 Clinical Correlations and Serum CXCL13

CXCL13 Levels in LGI1-Antibody Patients Decrease Over the Disease Course

No correlation was found between serum CXCL13 level and serum LGI1-antibody IgG end-titre (Spearman r = 0.008; p=0.920); **(Figure 4.8A)**. This finding was consistent when first samples (n=50) were correlated (Spearman r= -0.039; p=0.789), and when divided into LGI1-only and LGI1/CASPR2 samples (Spearman r= -0.129; p=0.147 and r=0.028; p=0.910, respectively); data not shown.

In patients with LGI1, CASPR2, or both antibodies, the serum CXCL13 level showed a significant inverse correlation with time from disease onset (Spearman r= -0.211; p=0.002**); **(Figure 4.8B)**: these decreases in CXCL13 level over time were statistically significant only in LGI1-only antibody samples (Spearman r= -0.253, p=0.002**); **(Figure 4.8C)**.

Serum CXCL13 Levels Strongly Associate with Relapses in LGI1 Patients but not Seizure Frequency or Presence of Tumour

Looking for other clinical correlations which might reflect a role of GCs (Makuch et al. 2018), no association was found between presence of tumour and serum CXCL13 level **(Figure 4.8D):** median CXCL13 levels were non-significantly different in samples from patients without tumour versus with a tumour at any stage (CASPR2 p=0.938, LGI1 p=0.215, LGI1/CASPR2 p=0.592; Mann Whitney test).

Of LGI1-antibody patients with available clinical data, 17/49 (35%) had clinical relapses. Serum CXCL13 levels from all available samples of patients with 'ever relapse' were compared to those from non-relapsing patients **(Figure 4.8E)**. Serum CXCL13 levels of all patients with an LGI1 antibody with 'ever relapse' were significantly higher (mean 522 pg/ml) compared to those without relapses (mean 242 pg/ml; p<0.0001****, Mann Whitney). When subdivided into patients with LGI1-only antibody and those with additional CASPR2 antibodies, LGI1-only antibody patients with relapses showed significantly higher CXCL13 levels (mean 486 pg/ml) than those without (mean 210 pg/ml; Mann Whitney p=0.001**). There were no differences in CXCL13 levels between relapsing (mean = 664 pg/ml) versus non-relapsing double-positive

LGI1/CASPR2-antibody patients (mean = 1388 pg/ml; p>0.9999, Mann Whitney), although numbers were small. Patients with relapses were significantly more likely to have a positive CXCL13 level ≥ 145 pg/ml across their disease course [59/84 (70%) of samples] compared to non-relapsing patients [28/74 (38%) of samples] (p<0.0001****, Fisher's exact test; **Figure 4.8I**). Overall, in the n=17 relapsing patients, CXCL13 concentrations of relapse samples (n=18; obtained +/- 4 weeks of clinical relapse) were significantly higher compared to non-relapse samples (n=66), (median 749 versus 269 pg/ml, respectively; p=0.018*, Kolmogorov-Smirnov test); **(Figure 4.8F)**. Finally, no differences were found between the *initial* serum CXCL13 level of n=17 relapsing versus n=32 non-relapsing patients (median 246 versus 101 pg/ml, respectively; p = 0.098, Mann Whitney; data not shown), suggesting that the *initial* absolute CXCL13 level may not serve as predictive of relapse.

To ask about disease severity, we explored CXCL13 levels against varying seizure frequencies. Seizure frequency data were available in 47/52 patients with LGI1-antibodies (including double-positive LGI1/CASPR2 patients). The serum CXCL13 level from the first available sample was correlated with the maximum seizure frequency (for those with multiple seizure semiologies, the semiology with the maximal seizure frequency was selected). The initial CXCL13 level showed no association with maximal daily seizure frequency (Spearman r=0.072; p=0.633); **(Figure 4.8G)**. Indeed, a patient without seizures shows the highest CXCL13 levels (2740.3 pg/ml), whilst at the other end of the spectrum, some patients with as many as 360 seizures/day have levels of only 46.1 pg/ml. No differences were found in initial CXCL13 level when peak daily seizure frequency was subdivided into patients with 0-10, 11-100, and >100 (median 122, 93, and 185 pg/ml, respectively; p=0.813, Kruskal Wallis test); **(Figure 4.8H)**.

CXCL13 Levels May More Accurately Predict Relapses Than LGI1 IgG Titre in Some Patients

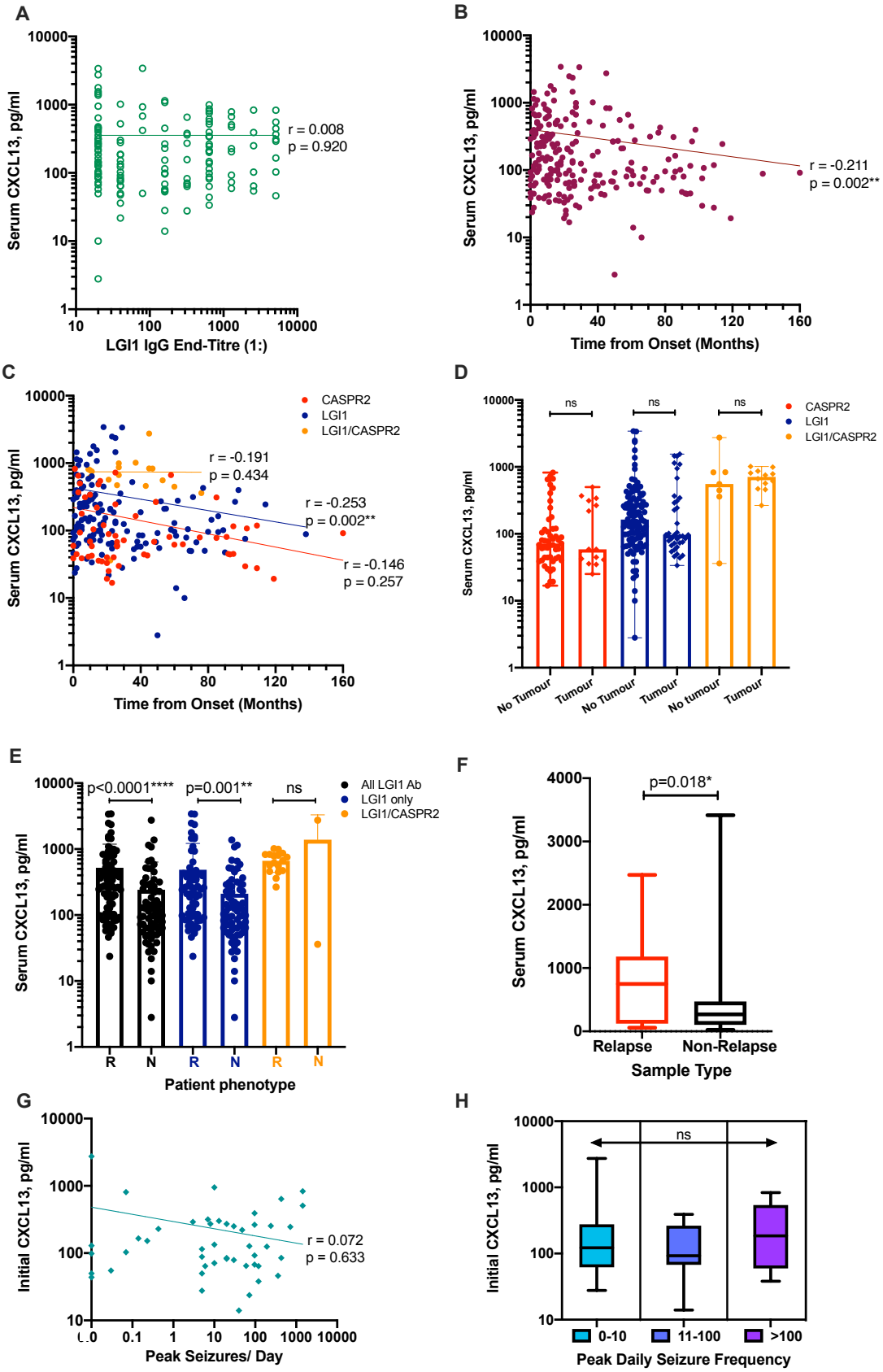
Analysis of individual patient serum CXCL13 trends was performed in patients with available ≥3 longitudinal samples. This revealed dynamic fluctuations in serum CXCL13 level over time **(Figure 4.9)**. Strikingly high CXCL13 levels are seen in LGI1- (n=18) and LGI1/CASPR2-antibody patients (n=2), with sustained levels above cut-off **(Figure 4.9A)**. The highest level of 3415 pg/ml was detected in an LGI1-antibody patient, and only 3/18 (17%) of LGI1-antibody patients had control-range CXCL13 levels throughout their illness. In contrast, the majority of

CASPR2-antibody patients (n=8) show CXCL13 levels below cut-off. Only 2/8 (25%) demonstrate elevated CXCL13 levels, both of whom had the worst clinical outcomes (MRS 6, death), and were the only CASPR2-antibody patients who died due to their disease. CXCL13 trends of one of these patients reveal gradual reduction following immunotherapies, reaching levels below cut-off around 15 months after disease onset **(Figure 4.9B)**. At 21 months, the serum CXCL13 is seen to rise steadily, although remained below cut-off at last available sample, one month prior to death.

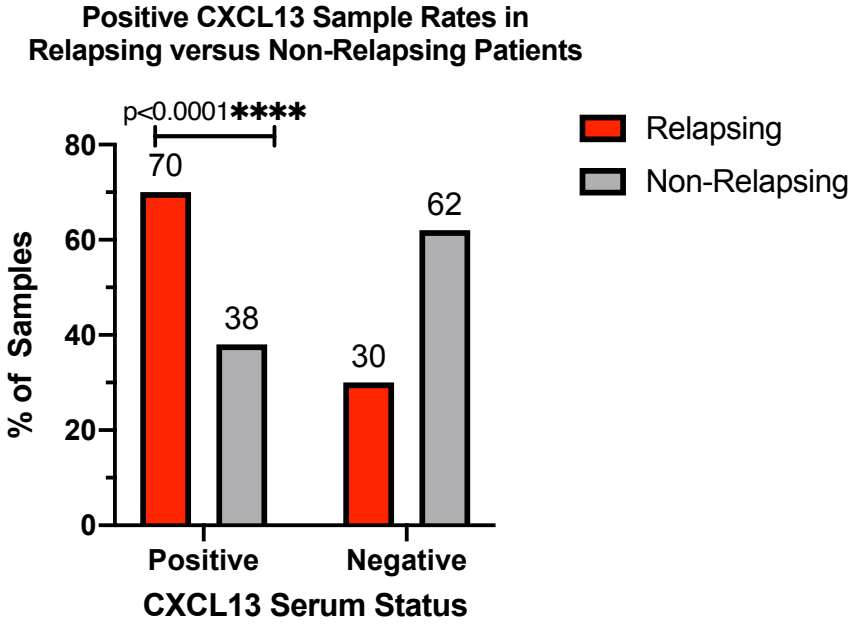
In a double-positive LGI1/ CASPR2-antibody patient with refractory Morvan's syndrome and metastatic thymoma, both serum CXCL13 and LGI1-antibody levels appear to respond to Rituximab therapy, but not earlier forms of immunotherapy, including thymectomy **(Figure 4.9C)**. At 49 months, the serum CXCL13 reaches its lowest level (265.4 pg/ml) in conjunction with clinical response where the patient became asymptomatic.

Examples of two LGI1-antibody patients revealed dramatic falls in serum LGI1-antibody levels in response to immunotherapy, which remain persistently low and even become undetectable, despite clinical relapses **(Figure 4.9D and E)**. Moreover, in both patients, the serum CXCL13 level, but not the LGI1-antibody level, is seen to rise prior to the onset of subsequent relapse(s). Other LGI1-antibody patients demonstrate disparate patterns in CXCL13 trends and clinical parameters. One subject with previously elevated CXCL13 levels showed concentrations which remained in the negative range despite two subsequent severe clinical relapses **(Figure 4.9F)**. Another exhibited favourable response to immunotherapy, with seizure cessation, and decline in CXCL13 levels to below cut-off over time. However, the LGI1-antibody level is shown to persist long-term, beyond disease remission **(Figure 4.9G)**. All of these cases represent examples where monitoring of the serum CXCL13 level in addition to the LGI1-antibody level may prove clinically useful. In some patients, the CXCL13 levels may predict relapses, typically more accurately than the LGI1 IgG end-titre.

Figure 4.8 Serum CXCL13 Clinical Correlations



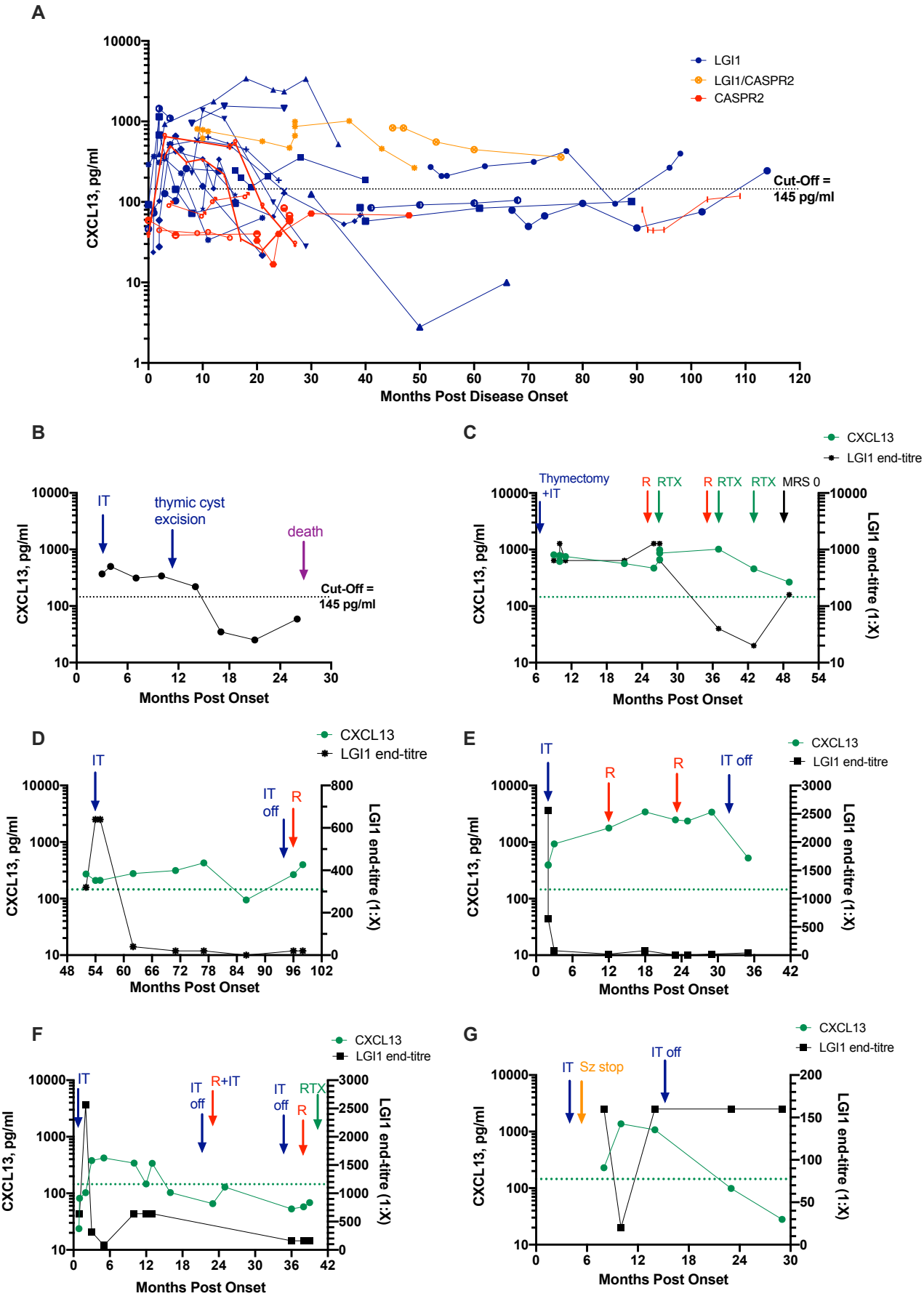
I



(A) Correlation between serum CXCL13 level and serum LGI1-IgG end-titre; n=148 samples from all patients with an LGI1-antibody. **(B)** Serum CXCL13 level correlation with time from disease onset across all antibodies; n=223 samples. Median sample time from onset (months): CASPR2 25, LGI1 16, LGI1/CASPR2 27. **(C)** Analysis by antibody found significant decrease in CXCL13 level over time exclusively in LGI1-only antibody samples (LGI1-only n=142, CASPR2 n=62, LGI1/CASPR2 n=19); Spearman correlation **(A-C)**. (**D)** Median CXCL13 levels (pg/ml) in non-tumour versus tumour samples respectively: CASPR2 p=0.938 (71.6g/ml; n=49 samples from 27 patients versus 58.7pg/ml; n=14 samples from 4 patients); LGI1 p=0.215 (163.2pg/ml; n=105 samples from 40 patients versus 95.9pg/ml; n=37 samples from 8 patients); LGI1/CASPR2 p=0.592 (554.6pg/ml; n=7 samples from 3 patients versus 709.3pg/ml; n=12 samples from 1 patient); Mann Whitney. **(E)** CXCL13 levels in relapsing 'R' versus non-relapsing 'N' patients; Mann Whitney. All patients with LGI1 antibodies: 'R' (mean 522 pg/ml, n=84 samples from 17 patients) versus 'N' (mean 242 pg/ml, n=74 samples from 32 patients). LGI1-only antibody: 'R' (mean 486 pg/ml, n=67 samples from 15 patients) versus 'N' (mean 210 pg/ml, n=72 samples from 30 patients). LGI1/CASPR2: 'R' (mean 664 pg/ml, n=17 samples from 2 patients) versus 'N' (mean 1388 pg/ml, n=2 samples from 2 patients). **(F)** Comparison of CXCL13 concentrations in relapse versus non-relapse samples of n=17 relapsing patients. Median 749 pg/ml in relapse samples (n=18) versus 269 pg/ml in non-relapse samples (n=66). **(G)** Relationship between initial CXCL13 level and peak seizure frequency (n=47); Spearman correlation. Maximal daily seizure frequency plotted; for status epilepticus, daily frequency determined as 1440 based upon 1 seizure/ minute. **(H)** Initial CXCL13 levels in patients grouped into low to high peak daily seizure frequency; Box and Whiskers with median; Kruskal Wallis. **(I)** Proportion of CXCL13 positive samples in relapsing versus non-relapsing patients; Fisher's exact.

Non-linear regression lines fitted to log-log scale **(A+G)** and semi-log scale [X linear, Y log, **(B+C)**].

Figure 4.9 Individual Longitudinal Patient CXCL13 Levels Give Informative Dynamic Data



(A) Longitudinal patterns of serum CXCL13 levels in CASPR2-, LGI1-, and double-positive LGI1/CASPR2-antibody patients over disease course. All LGI1/CASPR2 and most LGI1 patients show sustained, markedly elevated CXCL13 levels across disease. **(B)** CXCL13 trends in one of only two CASPR2 patients with elevated serum CXCL13 levels. **(C)** Dynamic fluctuations in CXCL13 levels (green) versus LGI1-antibody end-titre (black) in an LGI1/CASPR2 double-positive patient with markedly high CXCL13 levels. Both serum markers appear to respond to Rituximab (RTX) therapy. **(D and E)** LGI1-antibody patients with dramatic drop in serum LGI1-antibody levels in response to immunotherapy (IT). Prior to the onset of subsequent relapse(s), the serum CXCL13, but not the LGI1-antibody level, is seen to increase. **(F)** Longitudinal serum markers in a 53-year-old male with LGI1-antibody encephalitis with no increase in CXCL13 levels above established cut-off despite 2 clinical relapses. **(G)** Patient showing favourable response to immunotherapy, with seizure cessation, and decrease in CXCL13 levels to below cut-off over time. Despite an initial decline following immunotherapy, the LGI1-antibody level is seen to rise, and persists long-term following remission.

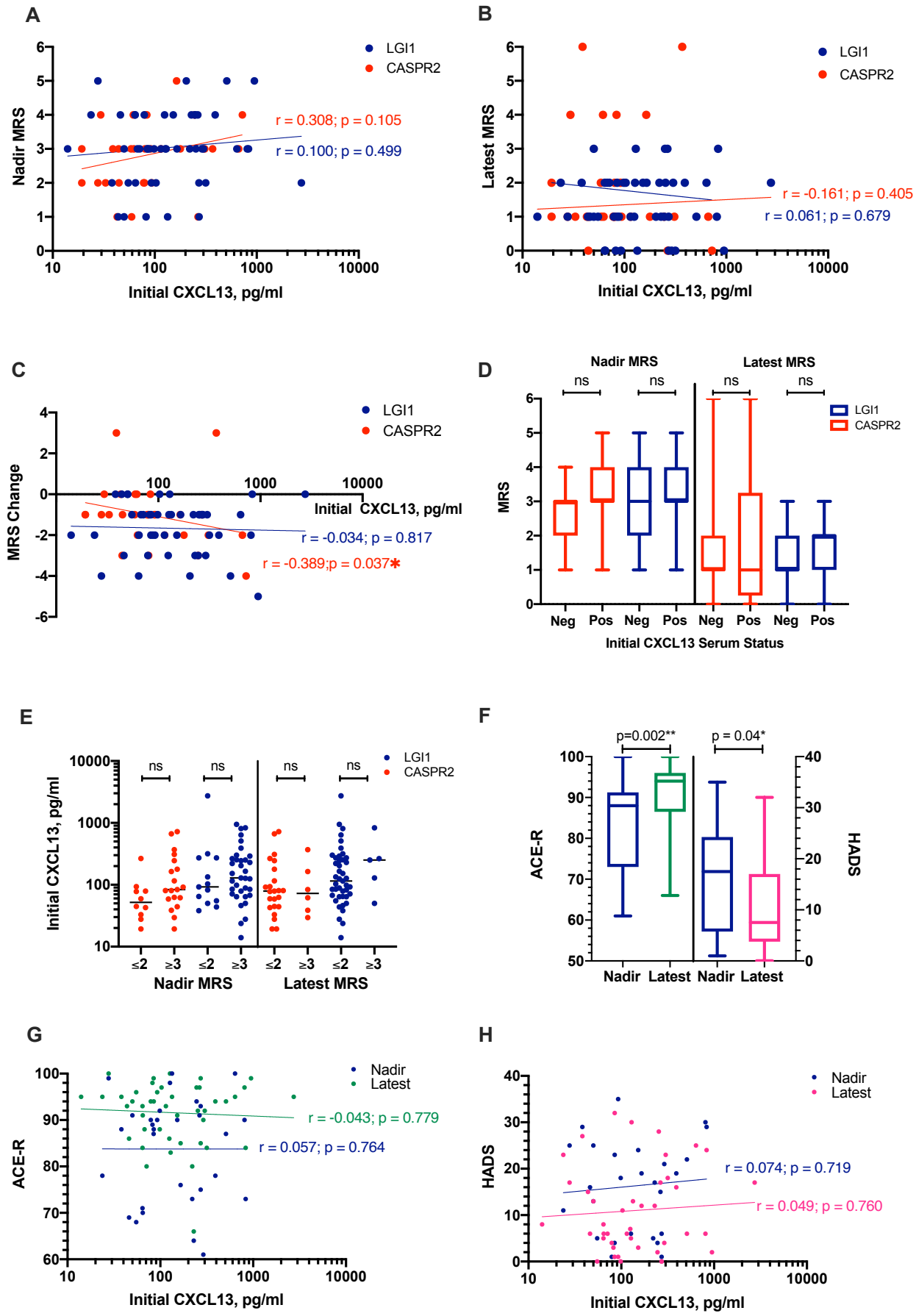
IT, immunotherapy; R, relapse; RTX, Rituximab; MRS, Modified Rankin Score; Sz, seizure

4.5.2.4 Correlations of CXCL13 Serum Levels with Clinical Outcomes

Initial serum CXCL13 level did not correlate with nadir or latest MRS in CASPR2-antibody (n=29) or LGI1-antibody patients (n=48): (Spearman r=0.308, p=0.105; LGI1 r=0.100, p=0.499) and (Spearman r= -0.161, p=0.405; LGI1 r=0.061, p=0.679); **(Figure 4.10A-B)**. The initial serum CXCL13 level did however show a modestly significant inverse association with degree of change in MRS (from nadir to latest) in CASPR2-antibody patients (Spearman r= -0.389, p=0.037*); **(Figure 4.10C)**. This may indicate that a high initial serum CXCL13 level may be a predictor of greater functional improvement in CASPR2-antibody patients. No clear trends were observed when patients were classified into groups based upon 'negative' (<145 pg/ml) or 'positive' (>145 pg/ml) initial CXCL13 serum status **(Figure 4.10D)**. Initial CXCL13 level only trended towards being higher in patients with worse MRS at nadir (MRS ≥3) compared to those with MRS ≤2 (CASPR2 median 84 versus 52 pg/ml, p=0.07, n=29; LGI1 129 versus 93 pg/ml, p=0.47, n=48; Mann Whitney). Correspondingly, in LGI1-antibody patients, higher initial CXCL13 levels were found in patients with worse functional outcomes (MRS ≥3 251 versus MRS ≤2 115 pg/ml, p=0.38; Mann Whitney); **(Figure 4.10E)**.

Next, the initial CXCL13 level was compared with nadir and latest ACE-R scores and HADS, where available, for all patients with LGI1-antibodies. As expected, median nadir and latest scores were significantly different for both ACE-R (88 versus 94, respectively; p=0.002**) and HADS (18 versus 8, respectively; p=0.04*; Mann-Whitney test), indicating improvements over time **(Figure 4.10F)**. However, the initial serum CXCL13 level did not show any correlation with either nadir/ latest ACE-R scores **(Figure 4.10G)**, nor with nadir/ latest HADS **(Figure 4.10H)**.

Figure 4.10 Serum CXCL13 and Clinical Outcomes



Correlation of initial serum CXCL13 level with nadir MRS **(A)**, latest MRS **(B)**, and **(C)** change in MRS (nadir to latest) in CASPR2-antibody (n=29) or all LGI1-antibody patients (n=48). **(D)** Median nadir and latest MRS showed no difference when patients were divided into 'negative' (<145 pg/ml) versus 'positive' (>145 pg/ml) initial CXCL13 serum status. CASPR2 p=0.178 and p=0.410; LGI1 p=0.124 and p=0.499; nadir and latest MRS, respectively. **(E)** No significant differences were found in initial CXCL13 levels of patients with worse MRS (≥3) versus MRS ≤2 (nadir MRS ≥3 versus nadir MRS ≤2: CASPR2 p=0.07, LGI1 p=0.47; latest MRS ≥3 versus latest MRS ≤2: CASPR2 p=0.97, LGI1 p=0.38). **(F)** Change in cognitive and mood assessments: nadir versus latest ACE-R scores and HADS. **(G)** Initial CXCL13 level correlation with nadir (n=30), and latest (n=44) ACE-R scores. **(H)** Initial CXCL13 level correlation with nadir HADS (n=26) and latest HADS (n=42).

(A-C; G-H) non-linear regression lines fitted to semi-log scales; Spearman correlation. **(D-F)** Mann Whitney.

MRS, Modified Rankin Scale; HADS, Hospital Anxiety and Depression Scale; ACE-R, Addenbrooke's Cognitive Examination-Revised

4.6 Discussion

CASPR2 IgM

It is possible that the CASPR2 IgMs detected in our study are 'natural' IgM autoantibodies. Natural autoantibodies are a substantial fraction of serum antibodies in HCs reacting to various self-antigens, and their function in health is proposed to be the clearance of apoptotic debris (Avrameas 1991). Indeed, seroprevalence rates of some autoantibodies in HCs and DCs are comparable to our CASPR2 IgMs: the highest against the NMDAR (10%), with CASPR2-antibodies detected in ~1% of individuals (57% IgM) (Dahm et al. 2014). In this paper, which used fixed CBAs, maximum end-titres were 1:100, whereas we found HC end-titres as high as 1:500, perhaps explained by a live cell-based assay system.

It is possible that the some of the HC sera with high CASPR2 IgM levels were sourced from our laboratory, perhaps from individuals working closely with CASPR2, who may have become immunized. Indeed, VGKC-autoantibodies have been detected in abattoir workers (Meeusen et al. 2012), suggesting autoimmunization by inhaled aerosolized brain tissue containing the antigen. This implies that VGKC are highly immunogenic, and this may translate to the CASPR2 complexed with VGKCs. Whether these CASPR2 IgMs are pathogenic, or persist transiently or even lifelong without triggering disease is unclear. Some authors theorize risk of pathogenesis in individuals with compromised blood-brain barrier (Hammer et al. 2014), coupled with immunological activation and polyclonal B-cell expansion in the context of infection or inflammation (Reiber et al. 1998).

Whilst there is paucity of literature studying CASPR2 IgMs in disease (Balint et al. 2013), their detection post assay optimization in 16% patients with CASPR2-antibody syndromes suggests they may be important. Indeed, NMDAR IgMs have been reported to be functionally relevant (Hammer et al. 2014). Moreover, although IgM antibodies typically have lower affinity for antigen than IgG (Mäkelä et al. 1970), low-affinity IgMs still display complement activation capabilities (Murakami et al. 2014). This potentially supports their pathogenic role in CASPR2-antibody diseases, where complement deposition may be a feature (Bien et al. 2012).

Here, CASPR2 IgMs were detectable at as long as 2551 days (7 years) post onset, with a median time of 321 days (~10.5 months). As with NMDAR-antibody encephalitis (Makuch et al. 2018), this suggests that ongoing GC reactions may be responsible for antibody production in some (16%) patients with CASPR2-antibody syndromes. Considering high tumour associations in CASPR2 (Irani et al. 2010; Lai et al. 2010), the tumours themselves may contain GCs and contribute to antibody production. Whilst clinical correlations did not indicate that CASPR2 IgM may be associated with underlying tumours **(Table 4.1)**, it is difficult to draw definitive conclusions in rare diseases. Another limitation is lack of available CASPR2-antibody patient CSFs for IgM studies.

LGI1 Autoantibody Epitope Specificities

Our results show that patient LGI1 IgG-autoantibodies react with both LRR and EPTP LGI1 domains, consistent with findings of others (Petit-Pedrol et al. 2018; Ohkawa et al. 2013), indicating polyclonality. Within individual patients, we demonstrate marked consistency of EPTP:LRR antibody levels over disease course. Our study is novel as it is the first which examines these epitope-reactivities longitudinally and studies CSF-reactivities (Petit-Pedrol et al. 2018). As with sera, CSF EPTP:LRR reactivities showed striking correlations. However, markedly higher serum LGI1 antibody levels compared to CSF supports peripheral generation of the LGI1-antibody. Of potential interest, the patient with 1280-fold higher serum LGI1 antibodies compared to CSF was double-positive, with severe Morvan's syndrome and underlying thymoma. This may suggest peripheral antibody responses are especially marked in: 1. double-positive patients, 2. presence of underlying tumour, 3. certain clinical syndromes, or a combination of all three potentiating factors. Indeed, higher LGI1-antibody levels were seen in double-positive patients, those with tumours, and even relapsing patients.

Several factors may reflect the underlying immunological mechanisms. Some points of interest include the CSF with the highest (3-fold) EPTP:LRR – that of a double-positive, relapsing patient – also showed 8-fold higher serum EPTP:LRR. As previously described (Binks et al. 2018), double-positive patients with available genotyping lacked HLA-DRB1*07:01, highlighting distinct antibody responses and immunogenetic mechanisms in these patients. Finally, the only patient lacking CSF LRR antibodies had a 4.5-year delay from onset to immunotherapy. It is possible that, over time, one epitope becomes predominant which may

differ within immune compartments (periphery, CNS, lymph nodes, bone marrow, or tumours), and that immunotherapy may influence this. However, it is difficult to draw conclusions from one patient. Interestingly, and perhaps more relevant to the differences observed, this was the only LGI1-antibody patient with available HLA-genotyping lacking HLA-DRB1*07:01, and in fact possessed the same HLA-associations carried by LGI1/CASPR2 positive patients [HLA-B*4402, HLA-C*0501, two copies of HLA-DQA1*0301, and HLA-DQB1*0301 (Binks et al. 2018)], but tested negative for CASPR2-antibodies. These findings reinforce the strong influence of HLA alleles on LGI1-peptide presentation to T cells, with distinct manifestations.

Finally, the unexpected control serum binding observed to LGI1-LRR may have several explanations. The LRR motif is present in a vast array of proteins, and chiefly, allows protein-protein interactions, owing to its elongated nature and structural flexibility (Kobe & Kajava 2001). Given its ubiquitous nature, including presence within bacteria (Kobe & Kajava 2001), it is possible that the control binding is due to cross-reactivity to microbes or other proteins with similar peptide sequences to LGI-LRR. It would be intriguing to explore whether this mechanism leads to LGI1-antibody encephalitis through molecular mimicry, analogous to Guillain-Barré syndrome triggered by *Campylobacter jejuni*/GM1 ganglioside mimicry (Gregson et al. 1997).

CXCL13

Our data show that CXCL13 serves as a potential clinical biomarker in LGI1-antibody syndromes. LGI1-antibody patients showed elevated serum CXCL13 levels, which were significantly higher than controls and CASPR2-antibody patients. 49% of samples from LGI1-antibody patients and 95% of samples from LGI1/CASPR2-antibody patients were regarded CXCL13 positive based upon our assay cut-off **(Table 4.2)**. No differences were found between healthy controls, pharmacoresistant epilepsy patients, or seizure-free epilepsy control patients, suggesting limited evidence for GC-reaction contribution to pharmacoresistant epilepsy. This is in-keeping with findings of others, where chronic epilepsy patients with frequent, daily seizures lacked elevation of CSF CXCL13 (Kothur et al. 2019). The differences between LGI1- and CASPR2-antibody patients suggest that GC-reactions may be more

relevant to LGI1-antibody syndromes. This highlights likely disparate mechanisms of disease perpetuation in the closely linked entities of LGI1- and CASPR2-antibody diseases, akin to dichotomous findings of HLA-associations in these patient groups (Binks et al. 2018).

Using a robust assay detection technique, with strong inter-assay reliability, we found serum CXCL13 levels are affected by prolonged storage at 4°C, but not by multiple (up to 10) freeze-thaw cycles. This suggests degradation of the chemokine at 4°C. Our CXCL13 stability and freeze-thaw findings replicate those of others, who show no effect of 5-10 freeze-thaw cycles on CSF CXCL13 levels (Hytönen et al. 2014; Leypoldt et al. 2015). Similarly, Wutte et al (Wutte et al. 2011) found slight decreases in serum CXCL13 levels of samples stored at 4°C for 24 hours (median 98 v 88 pg/ml). In our study, CXCL13 level was more markedly affected, to extent which impacted classification of positivity. This is likely due to longer duration of storage at 4°C in our study (14 days).

Insights to disease processes may be gained by comparison of peripheral and CSF immune compartments. Whilst our CSF CXCL13 data were limited by numbers, DC levels (2 pg/ml) and cut-off for positivity (8 pg/ml) match those reported by others (Lin et al. 2019; Wutte et al. 2011; Leypoldt et al. 2015). LGI1- and CASPR2-antibody patients had significantly higher CSF CXCL13 levels (mean 3.2 pg/ml and 13.6 pg/ml, respectively) compared to controls (mean 1.7 pg/ml). By comparison, mean CSF CXCL13 levels are reported at ~2.2 pg/ml in other LGI1-antibody cohorts (Lin et al. 2019), 9.6 pg/ml in NMDAR-antibody encephalitis (Leypoldt et al. 2015), 44 pg/ml in MS, 83 pg/ml in NMO (Zhong et al. 2011), and 500 pg/ml (median) in neuroborreliosis (Wutte et al. 2011). In our cohorts, serum CXCL13 levels were higher than CSF, suggesting peripheral synthesis. In contrast, CSF levels are greater than serum in conditions such as MS (Krumbholz et al. 2006), suggesting intrathecal production of CXCL13. Intriguingly, ectopic meningeal GCs are described in MS (Serafini et al. 2004). Further, in NMDAR-antibody encephalitis, some studies demonstrate elevated CXCL13 levels *only* within the CSF compartment, exclusively at disease onset (Liba et al. 2016). Moreover, while the CSF CXCL13 level does not correlate with CSF or serum NMDAR-antibody titres, it appears to correlate with the degree of intrathecal synthesis of NMDAR antibodies (Leypoldt et al. 2015). Our data confirmed no correlation between serum CXCL13 level and serum LGI1-IgG antibody titre.

CXCL13 is not *specific* to autoantibody-mediated diseases, and can theoretically be elevated in the context of *any* underlying cause of increased GC activity, such as viral and bacterial infections (Khademi et al. 2011; Wutte et al. 2011; Kothur et al. 2016). However, several observations suggest that the elevated levels we found in LGI1-antibody patients are likely relevant to the disease process. Firstly, the high serum CXCL13 levels detected (up to 3415 pg/ml) exceed those described for any other condition except untreated neuroborreliosis, in which *CSF* levels as high as 150, 000 pg/ml (median ~6000 pg/ml) are reported (Hytönen et al. 2014). In our cohort, mean and median serum CXCL13 levels were 338 pg/ml and 144 pg/ml in LGI1-antibody patients, and 740 pg/ml and 666 pg/ml in LGI1/CASPR2-antibody patients, respectively. In NMDAR-antibody encephalitis, mean serum levels are only 53.1 pg/ml (Leypoldt et al. 2015), and in neuroborreliosis median 114 pg/ml (Wutte et al. 2011). Secondly, we confirmed that the male gender predilection of LGI1-antibody patients does not influence the CXCL13 level. Although younger age is associated with lower serum and CSF CXCL13 levels in similar conditions (Wutte et al. 2011; Leypoldt et al. 2015), we confirmed that any effect of increased age on CXCL13 level from our older cohort is likely to only be partial. Thirdly, disease phase appears to be relevant, since highest CXCL13 levels were found at disease onset, and CXCL13 levels decreased significantly over disease course. Furthermore, and perhaps most importantly, dynamic fluctuations in CXCL13 concentrations were observed over time within individual patients, including exceptionally high levels at onset, reductions following immunotherapy and rises prior to relapses. Taken together, these observations indicate that serum CXCL13 levels in LGI1-antibody patients may represent a biomarker of the underlying immunological disease processes.

Only one other study has investigated serum CXCL13 levels in LGI1-antibody patients (Lin et al. 2019). However, mean concentrations were found to be only 36 pg/ml, nearly 10-fold less than our findings, and control means were 4.5-fold lower. Possible reasons for the discrepancies between our studies include different patient populations (Chinese versus our UK group), sample sizes (Lin et al measured CXCL13 concentrations in 16 samples from 16 patients, whereas we tested 142 samples from 48 patients), time of sampling (not specified by Lin et al) and differences in CXCL13 determination methodology. Whilst Lin et al specify that samples were stored at -80°C, in total, 7 cytokines and chemokines were individually

tested, and it is not known whether samples were stored at 4°C for prolonged periods between testing.

We found that higher serum CXCL13 levels associated with relapses in LGI1-antibody patients. Similarly, others demonstrate that higher CSF CXCL13 levels correlate with increased relapse rates in MS, NMO, and NMDAR-antibody encephalitis (Khademi et al. 2011; Zhong et al. 2011; Leypoldt et al. 2015). In MS, CXCL13 has been proposed as a prognostic marker, since high CXCL13 levels at onset are associated with increased relapse rates (Khademi et al. 2011). Additionally, our data indicate that higher initial CXCL13 serum concentrations *may* predict worse functional outcomes in LGI1-antibody patients. Comparatively, other studies have found that CXCL13 levels correlate with disease severity in MG (Shiao et al. 2010), NMDAR-antibody encephalitis (Liba et al. 2016), NMO (Zhong et al. 2011), and MS (Khademi et al. 2011). In OMS, severe disease associates with high CSF CXCL13, CXCL10, and BAFF, and oligoclonal bands (Pranzatelli et al. 2013). Finally, we observed reductions in serum CXCL13 levels following immunotherapy. Whilst it is not known whether levels drop over time independently of treatment, other studies make similar observations. In MS, CSF CXCL13 levels decrease with effective immunotherapies (Khademi et al. 2011) and intriguingly, children with OMS show significant reductions in both CSF CXCL13 and BAFF with treatments (Pranzatelli et al. 2013).

These findings suggest that CXCL13 is a potential therapeutic target for autoantibody- and B cell-mediated disorders. Humanised monoclonal antibodies against CXCL13 are already available (Klimatcheva et al. 2015), and CXCL13 neutralization shows efficacy in murine models of MS and rheumatoid arthritis (Finch et al. 2013; Klimatcheva et al. 2015) with reductions in antigen-specific B cells within GCs, fewer GCs, and reduced disease severity. Importantly, these animal studies suggest that CXCL13 antibody therapy can modulate pathogenic immune responses whilst maintaining humoral host defence (Finch et al. 2013). Some authors argue that manipulation of the CXCL13-CXCR5 pathway may be particularly useful in MS or NMDAR-antibody encephalitis since it may provide targeted prevention of B cell recruitment to the CNS, rather than systemic depletion of B cells with CD20-targeting therapies (Huber & Irani 2015).

Serum CXCL13 is persistently elevated in LGI1-antibody encephalitis, and may be a useful biomarker. In the absence of available antibody results in the initial phase, it may aid to distinguish from other autoantibody-mediated neurological conditions. Elevated early CXCL13 levels may serve as an early predictor of poor outcome or relapse, and inform immunotherapy regimes. Additionally, longitudinal monitoring of serum CXCL13 may serve to predict or confirm clinical relapses, and aid prompt, anticipatory additions, and or adjustments, to immunotherapies. Prospective studies are required to determine the value of these approaches. Finally, LGI1 IgMs significantly associated with relapses, and 88% positive samples were from relapsing patients, suggesting that LGI1 IgM may also serve as a relapse marker.

4.7 Conclusions

Overall, our data indicate that GC-reactions appear to be more relevant in LGI1-antibody than in CASPR2-antibody syndromes. We examined surrogate markers for evidence of GC reactions, including IgM, epitope binding patterns, and CXCL13. In CASPR2-antibody patients, CASPR2 IgMs were found in only 16%, and only 27% showed elevated CXCL13 serum levels. Additionally, our data suggest limited evidence for GC-reaction contribution to pharmacoresistance in epilepsy. Although LGI1 IgMs were detected in only 17%, they appear to associate with relapses, and we demonstrate evidence for presence of other markers of GC-reactions in a high proportion of LGI1-antibody patients. The markedly elevated CXCL13 levels found exclusively in LGI1-antibody patients is one such example. Our data showing significantly higher serum:CSF antibody and CXCL13 ratios suggest peripheral, GC-driven antibody production in LGI1-antibody syndromes. Both appear to be enhanced by presence of tumour and additional CASPR2 antibody, perhaps explained by the tumours partaking in further GC-reactions, thus contributing to CXCL13 and antibody levels.

In summary, these findings suggest sustained, ongoing GC-reactions operate in LGI1-antibody syndromes, and that serum CXCL13 appears to be a useful biomarker for LGI1. This may provide informative, individual longitudinal trends, which may aid individual patient treatment strategies. Future studies should explore CXCL13 blockade as a potentially beneficial additional therapeutic strategy in LGI1-antibody patients with persistently elevated

CXCL13 levels who appear to be resistant to other immunotherapies. Exploration of the serum and CSF milieu for identification of other relevant cytokines also remains to be studied in these patients. These data inform the molecular basis of the pathogenesis of LGI1- and CASPR2-autoantibody-mediated epilepsies, and indicate that other mechanisms of disease perpetuation should be explored, particularly in CASPR2-antibody syndromes, since GCs likely contribute to autoantibody production in only a small proportion of these patients.

CHAPTER 5. PLASMA CELLS:

Potential Contributions of Bone Marrow-Derived Long-Lived Plasma Cells to LGI1- and CASPR2-Autoantibody Production and Disease Perpetuation

5.1 Introduction

5.1.1 Background and Aims

The above findings suggest GC reactions may account for a proportion of specific-autoantibody production in LGI1- and CASPR2-antibody associated syndromes. However, as summarised in **Chapter 4**, in CASPR2-autoantibody syndromes, CASPR2 IgM autoantibodies are detected infrequently (16% of patients) and, in patients with LGI1-autoantibody syndromes, LGI1 IgM antibodies are seldom present. These findings argue against a dominant role for GC-reactions in these diseases. Whilst serum CXCL13 levels are markedly elevated in LGI1-autoantibody patients, and LGI1-autoantibodies with specificities to both EPTP- and LRR-epitopes are found in equal proportions throughout disease course – both potentially suggestive of ongoing GC-activity – these data do not negate the possibility of additional mechanisms of autoantibody production occurring in parallel. Moreover, as shown in **Chapter 3**, approximately one third of patients with LGI1-autoantibodies relapse despite immunotherapies which are proposed to work via preventing lymphocyte division, and in the absence of any underlying contributory tumour. Collectively, these findings indicate that targeting GC B cells alone may not be a sufficient therapeutic target, and that other mechanisms of autoantibody production and disease perpetuation should be explored.

Since our research group has studied the peripheral blood, cerebrospinal fluid, and lymph nodes of these patients in detail, a natural extension was to investigate the potential role of the bone marrow (BM) compartment in these diseases. The potential contribution of the BM is two-fold: an early, pre-GC loss of tolerance with the production of autoantibodies from naïve cells emigrating from the BM, and/or the BM acting as a major site for long-term autoantibody production through long-lived PCs (LLPCs).

Evidence for the former is indicated by several studies. An observation from our group suggested aquaporin-4 (AQP4) autoantibodies can derive from naïve pre-GC B cells (Wilson et al. 2018), and another found high rates of CASPR2-specific naïve B cells compared to memory B cells (Kienzler and Irani, unpublished). Both suggest an early, pre-GC loss of central tolerance. In healthy controls, 55-75% of antibodies initially emerging from the BM are

autoreactive prior to regulation by immune checkpoints (Wardemann et al. 2003). Thus, if a central loss of tolerance is indeed relevant to LGI1- or CASPR2-autoantibody syndromes, the BM is likely to play a substantial contribution.

The alternative model for the potential contribution of the BM to antibody production by LLPCs seems a more attractive target to explore. It is well established that antigen-specific antibody-secreting cells can persist for decades, residing in the human BM, in the absence of antigen stimulation. This is thought to be a major mechanism by which individuals vaccinated against tetanus (GOTTLIEB et al. 1964) and diphtheria (Kjeldsen et al. 1985) can maintain adequate protective antibody levels for up to 25 years in the absence of boosters. Perhaps the best evidence however comes from the detection of circulating smallpox-specific antibodies in vaccinated individuals over 35 years following the global eradication of smallpox itself (Brynjolfsson et al. 2017). The BM compartment has been studied a little in myasthenia gravis (MG), a similar autoantibody-mediated neurological disorder, where BM-derived lymphocytes – presumed to be PCs – appeared to be responsible for ongoing specific acetylcholine receptor (AChR)-autoantibody production (Fujii et al. 1986). Notably, tetanus-specific LLPCs persist in primate BM for 10 years, despite depletion of memory B cells with Rituximab, an anti-CD20 antibody therapy (Hammarlund et al. 2017). Whilst some early PCs express CD20, LLPCs almost never do, and this is particularly true for BM-derived LLPCs (Halliley et al. 2015; Medina et al. 2002). Hence, LLPCs may account for the 36% relapse rate seen within our LGI1-antibody patient cohort, despite treatment with B cell-directed immunotherapies. Moreover, the efficacy of PC-targeting immunotherapies such as Tocilizumab, an anti-interleukin-6 receptor (IL-6R) antibody, in patients with neuromyelitis optica (NMO) (Ayzenberg et al. 2013; Araki et al. 2013; Araki et al. 2014; Ringelstein et al. 2015) and Bortezomib, a PC-depleting proteasome inhibitor, in NMDAR-antibody encephalitis (Scheibe et al. 2017; Sveinsson et al. 2017; Schroeder et al. 2018; Keddie et al. 2018) in patients resistant to immunotherapies, including B cell-depleting anti-CD20 therapies, implicate PCs in disease perpetuation. These data implicate *BM-derived LLPCs* as a source of long-term autoantibody production in autoantibody-mediated neurological diseases.

Patient and public engagement (PPE) was also considered. For two annual meetings in succession at PPE days hosted by our group, patients and relatives afflicted by autoimmune

encephalitis identified that their singular most important unmet research need was '*disease causation'*. These findings warrant exploration of the BM compartment, and the role of BM-derived LLPCs in LGI1- and CASPR2-autoantibody syndromes.

5.1.2 Project Aims:

1. To determine the existence of bone marrow-derived long-lived plasma cells which secrete LGI1- and CASPR2-autoantibodies, in patients.
2. To develop a method for isolation of long-lived plasma cells from the human bone marrow, their maintenance in culture to allow functional antibody secretion, and detection of secreted antibody.

5.2 Preparatory Phases: Substantial Ethics Amendment

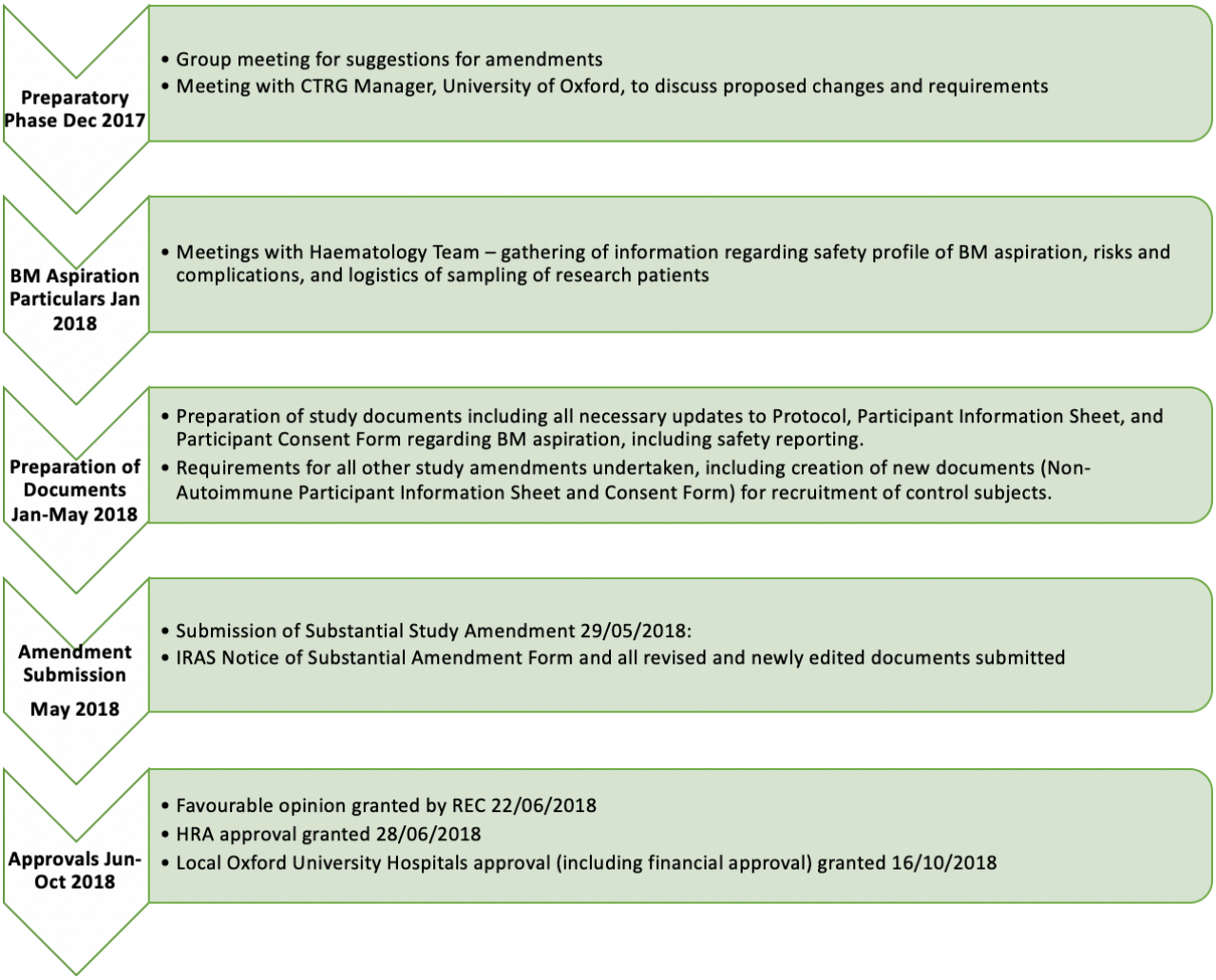
In order to sample BM from patients, the first necessary step was to obtain ethical permissions. Therefore, a substantial amendment to our group's ethics 'Immune Factors in Neurological Diseases', was prepared. The amendments and documents prepared are summarised in **Table 5.1** below, and a timeline of the process is outlined in **Figure 5.1**. A description of how the substantial ethics amendment was prepared is detailed within **Methods (Chapter 2, section 5.1)**. A comprehensive copy of documents relating to the ethics submission can be found within **Appendix (Appendix 5.1-5.8)**.

Table 5.1 Summary of Changes Made for Substantial Amendment to Study Ethics

Summary of Changes Made	Requirements
Addition of bone marrow aspiration	Several updates to Study Protocol, including listing of complications under 'Safety Reporting', and creation of 'Appendix D' to include bone marrow aspiration patient information sheet. Updates to Participant Information and Consent sheets.
Expansion of control groups – to allow recruitment of healthy controls and environmental controls.	New 'Non-Autoimmune Participant' Information and Consent sheets produced, and updates to Study Protocol
Inclusion of use of in vitro and in vivo animal models to study the effects of patient immune factors.	Updates to Study Protocol, Participant Information and Consent sheets, Consultee Information and Consent sheets, and Parental Information and Consent sheets
Addition of stool sampling	Updates to Study Protocol, Participant Information and Consent sheets, Consultee Information and Consent sheets, Non-Autoimmune Participant Information and Consent sheets, and Parental Information and Consent sheets
Adjustment of planned sample size to account for anticipated increased number of samples	Updates to Study Protocol
Updates to study funders, removal and addition of investigators, other logistical changes (e.g. addition of telephone numbers).	Updates to Study Protocol, Participant and Parental Information sheets

Summary of changes made for substantial amendment to 'Immune Factors in Neurological Diseases' study ethics, and details of requirements for each amendment, including documents updated, and new documents drafted.

Figure 5.1 Timeline of Workflow for Substantial Ethics Amendment



Flowsheet summarizing the workflow process for the substantial ethics amendment undertaken between December 2017-October 2018, which included addition of bone marrow aspiration. Study Title: 'Immune Factors in Neurological Diseases'; IRAS Project ID: 195152; Ethics Reference: 16/YH/0013.
CTRG, Clinical Trials and Research Governance; BM, bone marrow; IRAS, Integrated Research Approval System; REC, Research Ethics Committee; HRA, Health Research Authority.

5.3 Design of Flow Cytometry Panel for Isolation of BM LLPCs

An overview of PCs is given in the Introduction (**Chapter 1, Section 1.7**), including their morphology, development, and evidence for LLPC contribution to long-term antibody responses. Having gained a broad understanding of PCs, it was important to accurately define and isolate BM LLPCs. The change in cell surface markers during transition from B cells, to

PBs, PCs, and LLPCs is also summarised (**Introduction, Chapter 1, Section 1.7.5**), including differences in expression profiles between different tissue compartments.

5.3.1 Antibody Choice and Titration

Based upon the above analysis of PC cell surface markers, several markers were chosen for inclusion in a flow cytometry antibody panel. In general, a trend is clear: classical B-cell markers persist with intermediary expression on PBs, and gradually diminish to become absent on the surface of PCs with progression through development **(Figure 1.7, Introduction Chapter 1)**. A PC expression profile could range from the peripheral PB phenotype ($CD3^{-}CD56^{-}CD14^{-}DAPI^{-}CD20^{lo/-}CD19^{+}CD27^{+}CD38^{+}CD138^{lo/-}CD31^{+}$HLA-$DR^{+}CXCR4^{lo/+}$) to the BM-resident LLPC phenotype ($CD3^{-}CD56^{-}CD14^{-}DAPI^{-}CD20^{-}CD19^{-}CD27^{+}CD38^{+}CD138^{+}CD31^{++}$HLA-$DR^{-}CD45^{-}CXCR4^{++}$). Negative selection for CD3 and CD14 would allow exclusion of T cells and monocytes, and negative selection for DAPI aimed to select against dead cells. Selection for cells negative for CD56, or neural cell adhesion molecule (NCAM), would eliminate natural killer (NK) and dendritic cells. This is an important consideration for the gating strategy, as dendritic cells are large, and on forward scatter may appear in a similar position to LLPCs (also large, with abundant cytoplasm).

Inclusion of receptors in the antibody panel was generally avoided, due to the theoretical risk of receptor internalisation, although this would be unlikely with traditional 4°C flow cytometry protocols. CXCR4 however was deemed an important marker for positive selection of BM-LLPCs, and was included. Some likely unhelpful markers, such as BCMA, CD44, VLA-4, and CD24, as outlined earlier, were not included. Similarly, transcription factors were omitted since their staining for detection by flow cytometry would require cell fixation and permeabilization – this would not be feasible in our case, since our objective was to isolate *live* BM-derived LLPCs and maintain them extant in culture for several days with aim of functional antibody secretion. Since PCs have prominent RER, whereas other lymphocytes have very little and minimal cytoplasm, it was considered that detection of an ER^{+++} cell could be indicative of an antibody-secreting PC. However, incorporation of an ER marker within the panel was unsuccessful.

Once the cell surface markers were selected, the panel was designed with the assistance of Dr Jakob Theorell, a Postdoctoral Researcher with significant experience in flow cytometry. Careful consideration was given to place important markers such as CD138 on bright channels, with minimal spill-over from other channels where possible. Subsequently, antibodies were chosen (generally mouse-derived anti-human IgG1-κ) and allocated fluorochromes. Full antibody details, including clones, and methods for antibody titrations, can be found in **Methods (Chapter 2, Section 5.2)**. The antibody panel is summarised in **Table 5.2**.

Table 5.2 Antibody Panel for Bone Marrow-Derived Long-Lived Plasma Cells

Fusion II Laser and Filter Parameter	Fluorochrome	Antibody	Optimized Dilution
B 488-530/30	**AF488**	CD31	1:400
R 640-670/30	**APC**	CXCR4	1:50
R 640-730/45	**AF700**	CD20	1:200
R 640-780/60	**APC-Cy7**	CD45	1:50
V 405-450/40	**PacB**	DapiCD14	1:100
V 405-525/50	**BV510**	CD3/ CD56	1:50/ 1:200
V 405-610/20	**BV605**	CD38	1:50
V 405-660/20	**BV650**	HLA-DR	1:100
V 405-710/50	**BV711**	CD27	1:50
V 405-780/60	**BV785**	CD19	1:100
YG 560-780/60	**PE-Cy7**	CD138	1:50

Antibody panel developed for isolation of BM-derived LLPCs by flow cytometry using the BD FACSAria™ Fusion flow cytometer. Optimized dilution for each antibody, based on several titrations, is listed.
B, blue; R, red; V, violet; YG, yellow-green. Specified laser excitation wavelengths in nm.

5.4 Development of Culture Conditions for BM-Derived LLPCs

5.4.1 Choice of Cytokines: Combinations and Concentrations

In order to successfully culture BM-derived LLPCs, it is important to first understand the factors crucial for their maintenance ex-vivo – mainly cytokines. An overview of cytokines and the microenvironment which are key to PC survival is provided in the Introduction **(Chapter 1, Sections 1.7.6-1.7.7**). Several studies have attempted to culture PBs, typically one week following immunisation, or during disease relapses, in order to enrich for this rare population of circulating cells (Wilson et al. 2018; Chihara et al. 2011; Rodriguez-Bayona et al. 2013). Several factors limit the potential to culture directly isolated PBs however, including their low frequency and their typically poor survival capacity of only a few days. Thus, alternative platforms have been established, comprising in vitro differentiation of B cells into PBs, generating ASCs, and thus facilitating detection of antigen-specific antibodies (Wilson et al. 2018; Jourdan et al. 2014; Jego et al. 2001; Kitani et al. 1992; Klashman et al. 1991).

Maintenance of PBs and BM-derived PCs in culture are reported to necessitate dedicated cytokine conditions. PBs require a combination of both stimulatory and maintenance cytokines, including IL-6, and conditions mimicking infections such as R848 (Rodriguez-Bayona et al. 2013; Jego et al. 2001; Chihara et al. 2011; Wilson et al. 2018; Cocco et al. 2012; Klashman et al. 1991; Kitani et al. 1992; Dienz et al. 2009), which promote generation of PBs and antibody secretion, but not BAFF or APRIL (Chihara et al. 2011; Wilson et al. 2018). Co-culture with T cells (Dienz et al. 2009) or culture conditions mimicking aspects of T cell help such as IL-10, IL-21 or CD40L (Wilson et al. 2018; Rodriguez-Bayona et al. 2013; Cocco et al. 2012; Minges Wols 2005; Calame et al. 2003; Eto et al. 2011; Avery et al. 2003) may promote antibody secretion by PBs also, whilst mature BM PCs show poor responses to IL-10 and IL-21 (Rodriguez-Bayona et al. 2013). In contrast, BM PCs are reported to require combinations of chiefly IL-6, BAFF, APRIL, and CXCL12 for their maintenance and antibody secretion in culture (Rodriguez-Bayona et al. 2013; Minges Wols et al. 2007; Cassese et al. 2003; Jourdan et al. 2014; Ellyard et al. 2005; Alsayed et al. 2007; Tokoyoda et al. 2004). Some authors cite survival and continued immunoglobulin secretion by BM-derived PCs for at least 120 days in culture (Jourdan et al. 2014; Rodriguez-Bayona et al. 2013). Finally, co-culture with stromal feeder

cells, or their culture media, may aid both survival of and antibody production by BM PCs (Minges Wols et al. 2007; Cassese et al. 2003; Cocco et al. 2012; Jourdan et al. 2014; Ellyard et al. 2005), but not PBs (Wilson et al. 2018). There is no systematic study which directly compares culture conditions, however.

The differing culture condition requirements of both PBs and PCs based upon a literature review of several studies are summarised in **Table 5.3**. Based upon these general observations, the four key cytokines were selected for culture of our BM-derived LLPCs: IL-6, APRIL, BAFF, and CXCL12. For development and optimization of our culture conditions, these cytokines were used in various combinations, and at varying concentrations, both with and without supplementation with BM mesenchymal stromal cell supernatants. **Figure 5.2** summarises the 36 cytokine combinations developed for optimization of the BM-derived LLPC culture system.

Table 5.3 Effect of Various Culture Conditions on Plasma Cell Survival and Function

Cytokine and Concentration	Cell Cultured	Duration	Species	Finding(s)
IL-6 5 ng/ml **BAFF 100 ng/ml + Mega-APRIL 1 μg/ml + CXCL12 100 ng/ml (Rodriguez-Bayona et al. 2013)**	PBs and tonsillar PCs $1.25x10^5$cells/250 μl Purified PBs 10^4 cells/250 μl Purified BM PCs 10^4 cells/250 μl For total IgG production	7 d 7 d 14 d	Human HCs: BM and PBs 6 d post tetanus/ diptheria booster	IL-6 increased total IgG secretion by BM PCs and PBs. IL-6 5 ng/ml, BAFF 100 ng/ml, Mega-APRIL 1 μg/ml, CXCL12 100 ng/ml gave highest IgG secretion by BM PCs for up to 21 d.
IL-6 1 ng/ml **IL-6 1 ng/ml + CXCL12 10 ng/ml** **CXCL12 (5, 10, 20, 40, 80 ng/ml) (Minges Wols et al. 2007)**	5000 fresh BM PCs/well in 150ml + $7.5\text{-}10x10^3$ BM stromal cells 2500 fresh BM PCs/well For total and Ag-specific IgM and IgG production	7 d	Mice – early (4 d) and late (8-10 d) post secondary immunisation	IL-6 1 ng/ml + CXCL12 10 ng/ml gave highest IgM secretion by BM PCs at d 7. Specific IgG undetectable by d 7 when PCs cultured without BM stromal cells.
IL-6 0.01–50 ng/ml **CXCL12 10 ng/ml** **BM supernatant 10 ng/ml (Cassese et al. 2003)**	100 BM PCs/well/200 μl +/- BM stromal cells (10^5 cells/ml) To assess survival (by ELISpot)	3-7 d	IL-$5^{-/-}$ and IL-$6^{-/-}$ mice	BM feeder cells, or IL-6, or CXCL12 rescue PCs. IL-6 10 ng/ml optimum for BM PC survival.
IL-6 (1, 10, 100 ng/ml) (Urashima et al. 1996)	Mononuclear MM cells $2x10^5$/well	2 d	Human MM patients	IL-6 100 ng/ml significantly increased cell proliferation.

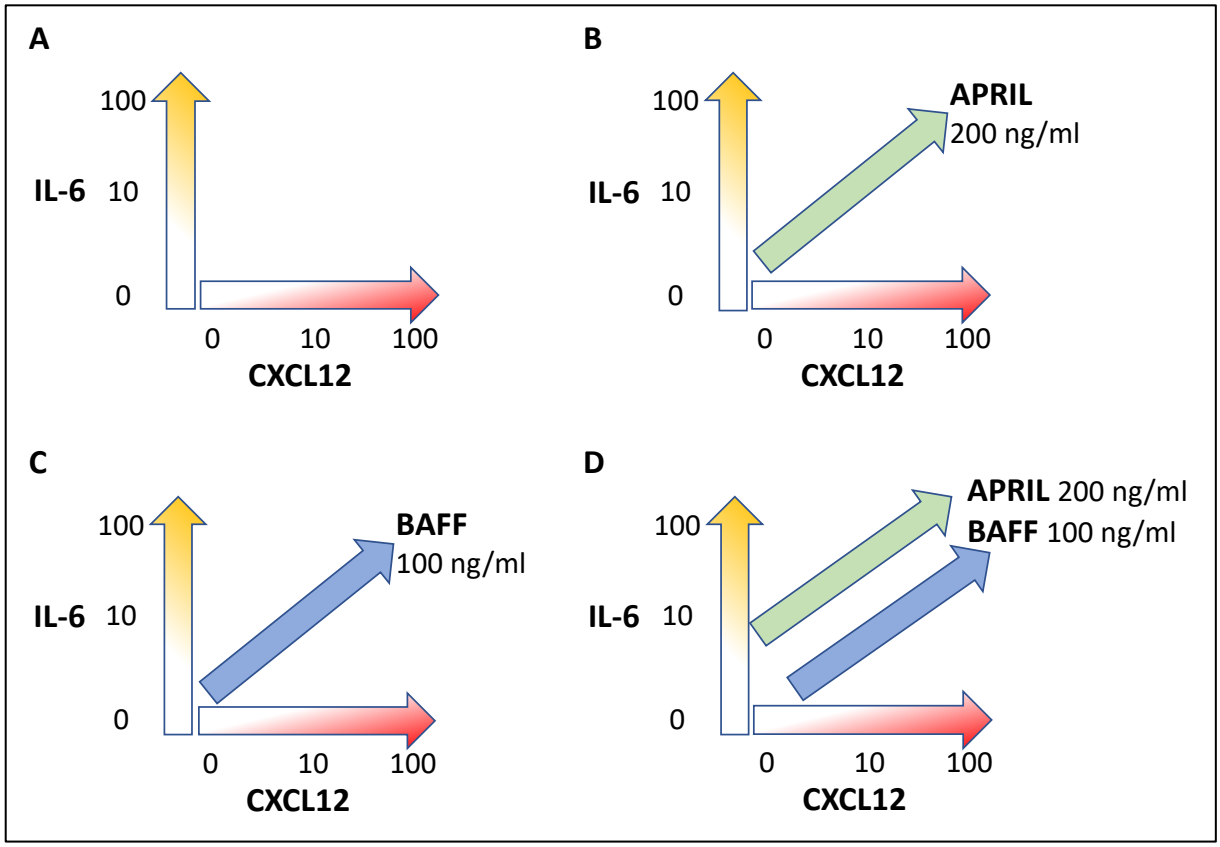
IL-6 10 ng/ml **BAFF 200 ng/ml + APRIL 300 ng/ml *or* BAFF 200 ng/ml + IL-6 10 ng/ml (Wilson et al. 2018)**	Fresh purified PBs 6000-12500/ well +/- M2-10B4 BM stromal cells - for total and specific AQP4-IgG production $2x10^5$ unfractionated PBMCS – for in vitro differentiation to PBs	13 d 6 d	Human NMO patients	Total IgG production not enhanced with BM stromal cells. No specific IgG production detected. Minimal differentiation of PBMCs to ASCs with BAFF+APRIL or BAFF+IL-6.
IL-6 1 ng/ml **BAFF 100 ng/ml** **APRIL 300 ng/ml (Chihara et al. 2011)**	PBs $1-2x10^4$/well – for specific AQP4-IgG production PBs $4×10^3$/well to assess survival	6 d 2 d	Human NMO patients and HCs	IL-6, but not BAFF or APRIL, increases PB survival and enhances specific IgG production.
IL-6 10 ng/ml + IL-21 50ng/ml + IFN-α 100 U/ml (Cocco et al. 2012)	In vitro generation of LLPCs from memory B cells (generation of PBs for 6 d with IL-2, IL-21, CD40L-cells), then PBs plated at $2.5-5x10^5$/ml with IL-6, IL-21, IFN-α, and M2-10B4 cells $4.16x10^4$/well. For cell division analysis, transwell assays, and total Ig secretion	62 d - **but averag e predict ed in vitro surviva l ~300 d**	Human HCs	IL-6 + IFN-α 100 promote PC generation and acquisition of CD138. Mesenchymal stromal cells support PC survival, increasing it up to 3-fold. Stromal cell support of PCs is contact-independent.
IL-6 50 ng/ml **IL-6 10 ng/ml + APRIL 200 ng/ml *or* BAFF 200 ng/ml *or* CXCL12 200 ng/ml (Jourdan et al. 2014)**	In vitro differentiation of PBMC memory B cells $1.5x10^5$/ml to PBs and PCs Generation of LLPCs from PCs Survival and total Ig secretion assessed by viability counts and ELISpot	4 d – **but LLPCs could survive for 120 days in these conditi ons**	Human HCs	PC survival is increased by culture with stromal cells *or* with IL-6 and either APRIL or BAFF. CXCL12 alone, or with IL-6 and/ or APRIL, had no effect on PC survival.
IL-6 10 ng/ml (Jego et al. 2001)	PCs generated from memory B cells isolated from HC tonsil	10 d	Human HCs	IL-6 is critical for PC growth and survival between days 0-7.
IL-6 0.05-1 units/ml (Klashman et al. 1991)	150 000 B cells/ 300 μl For specific tetanus toxoid IgG	4 d	Human HCs 5-7 d post tetanus immunisation	IL-6 is required for antibody production by B cells.
IL-6 20 U/ml (Kitani et al. 1992)	B cells $1x10^5$/well - for total and specific IgG production	7-12 d	Human active SLE patients and HCs	IL-6 drove differentiation of SLE B cells to antibody-secreting PCs and enhanced production of specific and total IgG.
IL-6 100 ng/ml (Dienz et al. 2009)	B cell-CD4+ T cell co-culture (1:3 T/B cell ratio); total splenocyte culture	7 d	$IL6^{-/-}$ mice	IL-6 promotes antibody production by B cells by inducing IL-21 production in $CD4^+$ T cells.
BAFF 2500 ng/ml **IL-6 (~10 ng/ml) secreted by stromal cells (Ellyard et al. 2005)**	Sorted PCs from BM+spleen $5x10^3$ cells/well +/- stromal cells For total Ig production	5 d	Human HCs	IL-6 secreted by splenic stromal cells enhances antibody secretion by splenic PCs. BAFF has no effect on antibody secretion by splenic or BM PCs.

BAFF 2500 ng/ml (Avery et al. 2003)	Memory B cells $2x10^5$/ml from spleens differentiated to PCs BM PCs $5x10^3$/well - for total Ig secretion	8 d 5 d	Human HCs	BAFF promotes survival but not proliferation of B cells. BAFF increases IgG secretion by BM PCs.
BAFF 200 ng/ml *or* IL-6 500 pg/ml *or* BAFF 200 ng/ml + IL-6 500 pg/ml (O'Connor et al. 2004)	Whole BM cells $8x10^6$ /well For total IgG-secreting BM PCs by ELISpot	4-10 d	Mice 51 d post immunisation	BAFF increases BM PC numbers 2-fold Culture combination BAFF+IL-6 for 10 d markedly increases BM PC numbers 4-fold.
APRIL, IL-6, BAFF (no concentrations specified) (Nguyen et al. 2019)	1000-2500 ASCs/ well co-cultured directly on irradiated mesenchymal stromal cells, or with irradiated mesenchymal stromal cells in transwells, or with mesenchymal stromal cell secretome 1000-1500 ASCs/ well co-cultured with irradiated mesenchymal stromal cells or in mesenchymal cell secretome only For survival and function of PCs by IgG ELISpot	6 d 7 – 56 d	Human HC ASCs 7 d post vaccination	Cell-cell contact not required for PC survival (no differences in number of surviving PCs). Co-culture with stromal cells only 20% ASCs survived to d 30, but when cultured in mesenchymal cell secretome, PCs survive and secrete IgG for 56 days – enhanced with APRIL and hypoxia. Addition of other single cytokines including BAFF and IL-6 had no effect.
CXCL12 0-100nM (Alsayed et al. 2007)	$2x10^5$ cells/ well for transwell migration assay: Human MM cell line with 0-100 nM CXCL12 $CD138^+$ PCs with 30 nM CXCL12	4 hours	Human MM cell line	Maximal migration of PCs seen at 20-30nM CXCL12. High levels of CXCL12 (75-100nM) inhibited PC migration.
CXCL12 100 ng/ml (Tokoyoda et al. 2004)	Fresh BM cells +/- CXCL12 (adhesion assay)	30 mins	Mice	More mature cells (pre- v. pro- B cells) were more responsive to CXCL12 and showed more chemotaxis.
CXCL12 *and* TGF-β secreted by BM mesenchymal stromal cells (Bonnaure et al. 2016)	Differentiation of memory B cells to PCs with BM mesenchymal stromal cells For Ig secretion; numbers of PCs measured by flow cytometry	20 d	Human HC	Mesenchymal stromal cells in serum-free medium allow generation of PCs through secreted CXCL12 and TGF-β.
IL-6 *and* APRIL secreted by eosinophils (Chu et al. 2011)	$1x10^2$ $CD138^+$ PCs/well: BM PC-eosinophil co-culture	1-2 d	Mice 6 d post immunisation	IL-6 and APRIL produced by BM eosinophils support long-term PC survival.

Summary of the differing culture condition requirements of both PBs and PCs, including BM-derived PCs, based upon several studies. Generation of peripheral PBs from B cells and their maintenance requires both stimulatory and maintenance cytokines including IL-6, R848, co-culture with T cells, or culture conditions mimicking T cell help such as IL-10, IL-21 or CD40L. In contrast, the optimal environment for maintenance of BM LLPCs and their continued antibody secretion includes combinations of IL-6, BAFF, APRIL, and CXCL12, and co-culture with BM mesenchymal stromal feeder cells or their supernatant.

PB, plasmablast; PC, plasma cell; BM, bone marrow; ASC, antibody secreting cell; d, days; PBMC, peripheral blood mononuclear cell; HC, healthy control; MM, multiple myeloma; NMO, neuromyelitis optica; SLE, systemic lupus erythematosus; M2-10B4, mouse fibroblastic mesenchymal stromal cell line.

Figure 5.2 Development of Optimum BM LLPC Culture Conditions



Based upon the studies summarised in Table 5.3, typical concentration ranges of cytokines used were: IL-6 1-100 ng/ml, BAFF 100-200 ng/ml, APRIL 200-300 ng/ml, and CXCL12 10-100 ng/ml. Therefore, for development of a cell culture system platform for BM-derived LLPCs, a 36-condition cytokine matrix was developed for optimization, consisting of 4 main groups of cytokine combinations:

A: IL-6 and CXCL12 group, with 9 combinations of increasing concentrations of both cytokines [e.g. bottom row: IL-6/ CXCL12 0 ng/ml (BCM only), IL-6 0 ng/ml + CXCL12 10 ng/ml, IL-6 0 ng/ml + CXCL12 100 ng/ml] etc. The same 9 combinations were used for **B-D**, with additions of APRIL **(B)**, BAFF **(C)**, or both **(D)** to each well.
B: IL-6, CXCL12 and APRIL group
C: IL-6, CXCL12 and BAFF group
D: IL-6, CXCL12, APRIL and BAFF group

NB The bottom left condition of each group represents a control condition: **(A)** BCM only; **(B)** APRIL 200 ng/ml only; **(C)** BAFF 100 ng/ml only; **(D)** APRIL 200 ng/ml and BAFF 100 ng/ml. BCM, B cell medium.

5.4.2 Sample Handling and Yield: Importance of Use of Fresh Plasma Cells

Other important experimental considerations include sample handling and yield, which is of particular relevance to a rare cell population. Percentage cell subsets of interest range from 1.8% for LLPCs from fresh BM ($CD138^+$ cells of the live, $CD3^-CD20low/^-$ gate) (Martinez-Murillo et al. 2016) to ~0.1-2% for PBs from PBMCs ($CD27^{hi}CD38^{hi}CD80^-$ of all $CD19^+$ cells) (Chihara et al. 2011; Chihara et al. 2013; Kyu et al. 2009). Several studies point to the importance of the use of fresh rather than frozen PC samples. Martinez-Murillo et al (Martinez-Murillo et al. 2016) showed that when staining fresh and frozen BM, the frequency of the functional, $CD138^+CD31^+$ PCs was 10 times higher in the fresh sample (4.73% v. 0.42%, respectively, of the live, $CD3^-CD20low/^-$ cells). Moreover, cryopreservation led to *selective* loss of the functional, antibody secreting $CD138^+CD31^+$ cells (Martinez-Murillo et al. 2016). Similarly, peripheral PBs also reduce following cryopreservation, with up to 70-80% falls reported (Kyu et al. 2009). Therefore, all experiments, including antibody titrations, optimization of culture conditions etc, employed use of fresh PCs, isolated from peripheral blood, thymus, or BM.

5.5 Results: Optimization Experiments for Isolation and Culture of BM-Derived LLPCs

These experiments utilized the new PC flow cytometry antibody panel to stain and isolate PCs from various sources. These included:

1. circulating PBs from healthy control peripheral blood
2. tissue-resident PCs from niches including:
    a. the thymus of a patient with MG
    b. the BM of a patient with multiple sclerosis, and a patient with LGI1-autoantibody encephalitis.

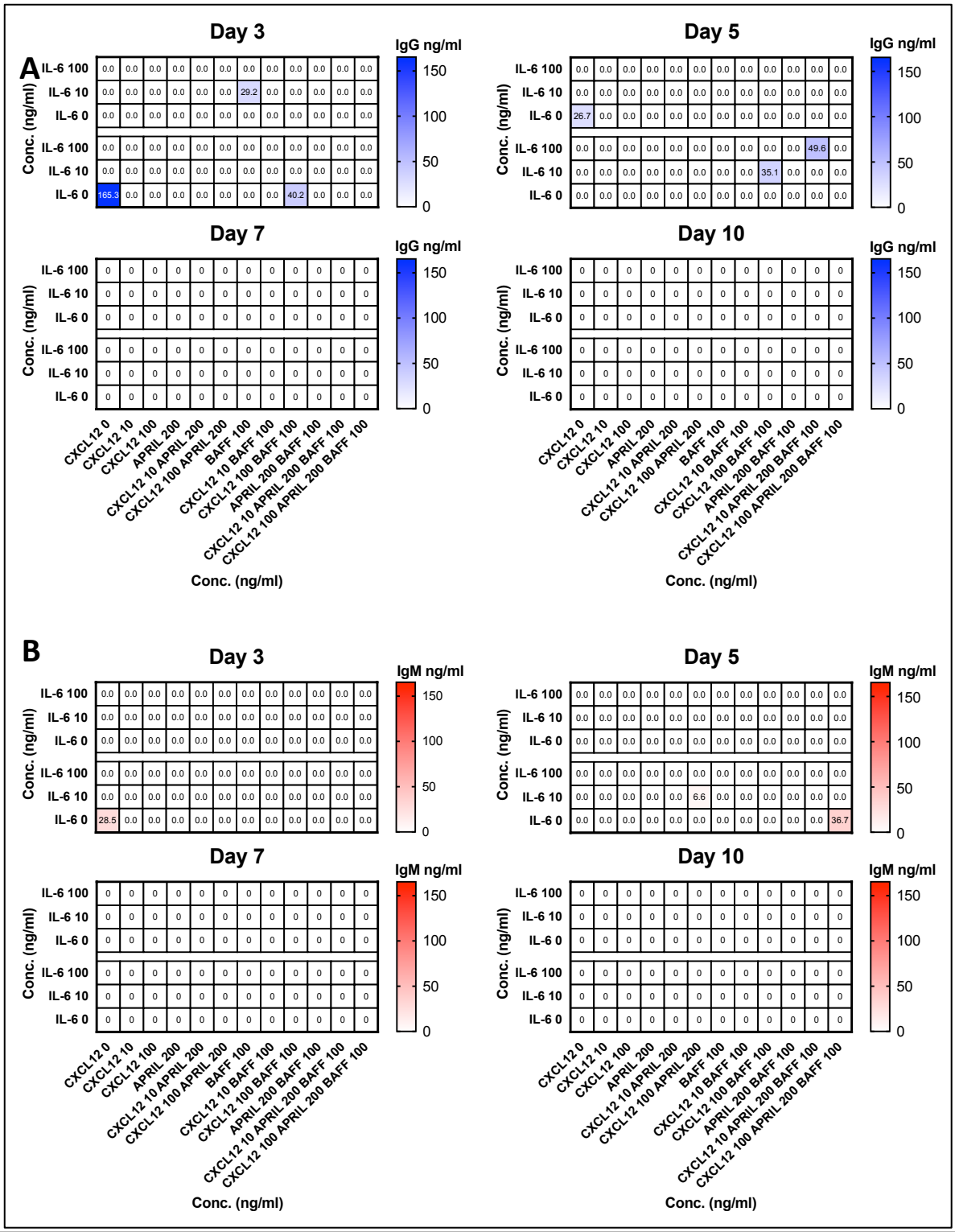
Next, the isolated PCs were plated at different cell densities (ranging from single cell to oligocultures up to 20 per well), in 36 various cytokine combinations plus or minus mesenchymal stromal cell supernatant **(Figure 5.2)**, for various durations (36 hours-10 days).

PC survival and functionality were subsequently determined by ELISpot and IgM/G ELISA after culture. Accordingly, these approaches permitted optimization of cell number, culture condition, and duration, for the novel LLPC culture system.

5.5.1 Cell Sort 1 – Healthy Control Peripheral Plasmablast Culture (5 cells/well): Peripheral plasmablasts secrete IgG and IgM antibodies in culture

For the first experiment, peripheral blood was taken from a 38-year-old male HC, 6 days after the onset of an upper respiratory tract infection, in order to enrich for peripheral circulating PBs. Fresh PBMCs were isolated on a Ficoll density gradient and stained with the newly developed PC antibody panel. 5 PBs (live, $CD45^{+}CD3^{-}CD56^{-}CD14^{-}CD20^{+}CD19^{+}CD27^{+}CD38^{+}$ cells) were sorted directly into each well of a plate containing the 36 cytokine combinations in 200 µL per well **(Methods, Chapter 2, Sections 5.4 and 5.5)**. Each condition was tested in duplicate. At timepoints day 3, 5, and 7, 100 µL of supernatant from each well was aspirated and frozen, and replenished with 100 µL of the respective cytokine mix (at double concentration). The culture was terminated at day 10, with supernatant stored from this timepoint also. Supernatants from each of the 4 timepoints were tested for total IgG and total IgM by ELISA at 1:3 dilution (**Figure 5.3**; and **Methods, Chapter 2, Section 5.7.1)**. Supernatants were initially tested by ELISA at 1:50 dilution, but no IgG was detected for any well from any of the timepoints (data not shown). Between days 3 and 5 of PB culture, IgG and IgM were detectable in 6/144 wells (4.2%; range 26.7-165.3 ng/ml) and 3/144 wells (2.1%; range 6.6-36.7 ng/ml), respectively (**Figure 5.3A and B**). Wells with detectable IgG appeared to more frequently derive from the IL-6, CXCL12, and BAFF cytokine group culture conditions (in 3/6), whilst no pattern was observed for IgM-positive wells. Intriguingly, and consistently, no secreted IgG or IgM were detected in day 7 or 10 PB culture supernatants.

Figure 5.3 Total IgG and IgM Levels from Healthy Control Peripheral Plasmablast Culture Supernatants Across Culture Conditions



Heat maps generated from data from 1 healthy control. Fresh PBs were purified from PBMCs and 5 sorted directly into each well with the 36-condition cytokine matrix; each condition was tested in duplicate on the same plate. Supernatant total IgG levels (ng/ml) in blue **(A)** and total IgM levels (ng/ml) in red **(B)** are shown for each culture condition for 4 timepoints: day 3, 5, 7 and 10. The repeat culture conditions are shown within each heat map (e.g. data for the IL-6 100 ng/ml + CXCL12 10 ng/ml condition is shown in row 1, column 2, and its duplicate is shown in row 4, column 2).

5.5.2 Cell Sort 2 – Myasthenia Gravis Thymic PC Culture (20 cells/well)

Thymic PCs survive and secrete IgG and IgM antibodies in culture

Next, fresh thymic tissue was obtained from a 29-year-old female with MG and positive AChR autoantibodies undergoing thymectomy for thymic hyperplasia. The thymic tissue was dispersed into a cell suspension and stained with the new PC antibody panel. PCs (live, $CD3^-$ $CD14^-CD45^+CD138^+$ cells) were purified from the thymic cell suspension using a cell sorter; CD19 was used to stain the cells but not as a selection marker. 20 PCs were sorted directly into each well of culture and ELISpot plates containing the 36 cytokine combinations in 100 µL per well **(Methods, Chapter 2, Sections 5.4 and 5.5)**. Each condition was tested in duplicate. For ELISpot, the number of IgG and IgM spots was assessed after 36 hours **(Methods, Chapter 2, Section 5.6)**. At day 3, the culture was terminated and the 100 µL of supernatant from each well was aspirated and frozen. Day 3 supernatants were tested for total IgG and total IgM by ELISA at 1:2 dilution **(Figure 5.4**; and **Methods, Chapter 2, Section 5.7.1)**. After 3 days *in vitro*, IgG and IgM from thymic PCs were detectable by ELISA in 5/72 wells (6.9%; range 8.3-30.4 ng/ml) and 2/72 wells (2.8%; range 6.7-8.9 ng/ml), respectively (**Figure 5.4A**). Detectable IgG appeared to correlate with PCs cultured in the IL-6, CXCL12, and BAFF cytokine group, whilst IgM appeared to correlate with wells of PCs cultured in the IL-6 and CXCL12 group.

ELISpot is more sensitive than ELISA for detection of PC survival and functionality

By contrast, IgG- and IgM-secreting PCs analysed by ELISpot were detectable after 36 hours of culture, in 100% of the 36 IgG wells, and 32/36 (88.9%) of IgM wells (**Figure 5.4B**). More IgG- and IgM-secreting PCs survived in the 'IL-6, CXCL12, and BAFF' group and 'IL-6, CXCL12, APRIL and BAFF' cytokine group conditions compared to the other two cytokine group conditions.

Ex-vivo PC survival and functionality is enhanced by IL-6, CXCL12, APRIL, and BAFF culture conditions

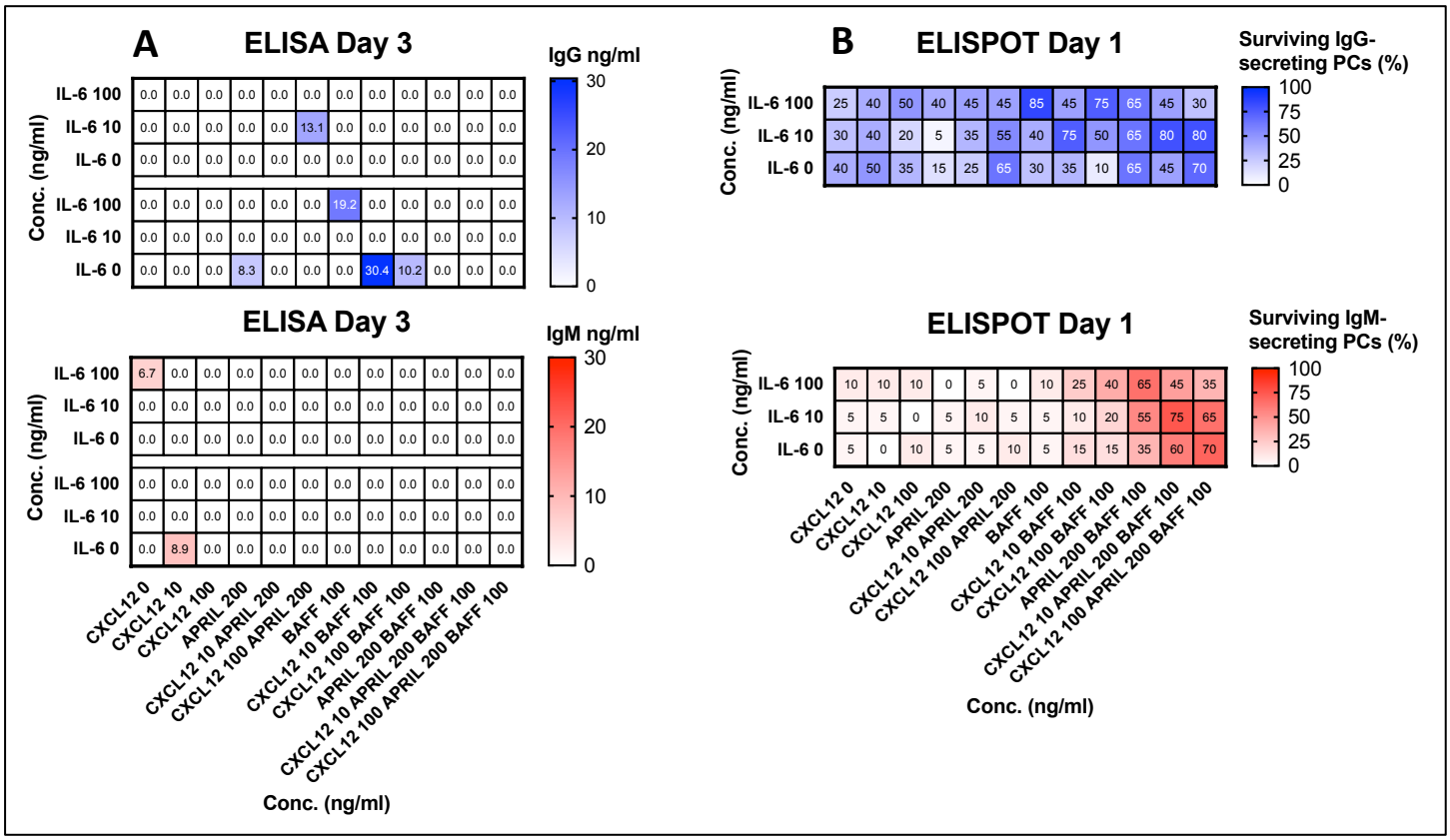
Within groups, survival of IgG-secreting PCs at 36 hours was significantly higher than IgM-secreting PCs for all cytokine groups except 'IL-6, CXCL12, APRIL, and BAFF', which produced similarly high survival rates; mean % PC survival for IgG versus IgM, respectively: 'IL-6 and CXCL12' 37% v. 6%, p<0.000001****; 'IL-6, CXCL12 and APRIL' 37% v. 5%, p=0.0002***; 'IL-6, CXCL12 and BAFF' 49% v. 16%, p=0.0019**; 'IL-6, CXCL12, APRIL and BAFF' 61% v. 56%, p=0.5623, ns; multiple unpaired t tests (**Figure 5.5A and C**). Survival of thymic PCs was significantly different *across* the four main cytokine groups for both IgG- and IgM-secreting PCs (5-85%, p=0.0264*, R squared 0.2474; 0-75%, p<0.0001****, R squared 0.8403, respectively; one-way ANOVA); **(Figure 5.5C)**. Whilst there were significant differences in *overall* survival of IgG- and IgM-secreting thymic PCs at 36 hours (46% versus 21%, respectively; p<0.0001****; two-way ANOVA; data not shown), maximal thymic PC survival was seen in the presence of IL-6, CXCL12, APRIL, and BAFF culture conditions, regardless of whether they produced IgG or IgM. For IgG, 'IL-6, CXCL12, APRIL, BAFF' versus 'IL-6, CXCL12' and 'IL-6, CXCL12, APRIL' p=0.0388*, and versus 'IL-6, CXCL12, BAFF' ns; for IgM, 'IL-6, CXCL12, APRIL, BAFF' versus all other groups p<0.0001****; Dunnett's multiple comparisons test (**Figure 5.5C)**.

When the number of IgG and IgM spots was analysed by reduction of the 36-condition matrix into 16 possible cytokine combination groups (irrespective of concentrations; **Figure 5.5B**), 8 IgG spots were detected in the BCM only control culture condition, and IL-6, CXCL12, APRIL, or BAFF cytokines alone yielded similar numbers of IgG. Except for 'BAFF plus CXCL12' combinations, addition of BAFF to any cytokine combination led to a profound increase in PC survival, almost doubling the number of IgG spots detected. For IgG-secreting PCs, culture in 'APRIL and BAFF', *or* 'IL-6, APRIL and BAFF' increased survival compared to IL-6 alone (p=0.046* and p=0.046*, respectively; Tukey's multiple comparisons test). Additionally, any of 'IL-6 and BAFF', *or* 'IL-6, CXCL12, and BAFF', *or* 'APRIL and BAFF', *or* 'IL-6, APRIL and BAFF', *or* 'CXCL12, APRIL and BAFF', *or* 'IL-6, CXCL12, APRIL and BAFF' significantly increased survival compared to APRIL alone (p=0.0028**, p=0.0041**, p=0.0013**, p=0.0013**, p=0.0121*,

and p=0.0085**, respectively; Tukey's multiple comparisons); **(Figure 5.5B)**. Similarly, 'IL-6 and BAFF', *or* 'IL-6, CXCL12, and BAFF', *or* 'APRIL and BAFF', *or* 'IL-6, APRIL and BAFF' increased survival compared to 'IL-6 and APRIL' (p=0.024*, p=0.0334*, p=0.0121*, and p=0.0121*, respectively). 'IL-6, CXCL12, plus APRIL' combinations produced a mean of 9 IgG spots, comparable to control (p>0.9999), whilst 'IL-6, CXCL12, APRIL, and BAFF' combinations showed enhanced PC survival (mean of 12 IgG spots). Addition of CXCL12 increased PC survival when added to IL-6 (IL-6 plus CXCL12 mean IgG spots 8 versus IL-6 alone 6; p=0.9997) or APRIL (APRIL plus CXCL12 mean IgG spots 9 versus APRIL alone 3; p=0.3727), or to IL-6 plus APRIL (IL-6, APRIL, plus CXCL12 mean IgG spots 9 versus IL-6 plus APRIL 5; p=0.6960), although not significantly, and not to the same extent as BAFF. APRIL did not augment the effects of IL-6 or CXCL12 alone (p>0.9999) or together (p>0.9999). Similarly, IL-6 did not augment the effects of APRIL or CXCL12 alone (p>0.9999) or together (p>0.9999). IgM spots were detected beyond control levels almost exclusively in culture conditions containing APRIL plus BAFF with either IL-6 or CXCL12, or both. The individual combinations yielded significantly different survival rates for both IgG and IgM-secreting thymic PCs (p <0.0001**** and <0.0001****, respectively; two-way ANOVA; data not shown).

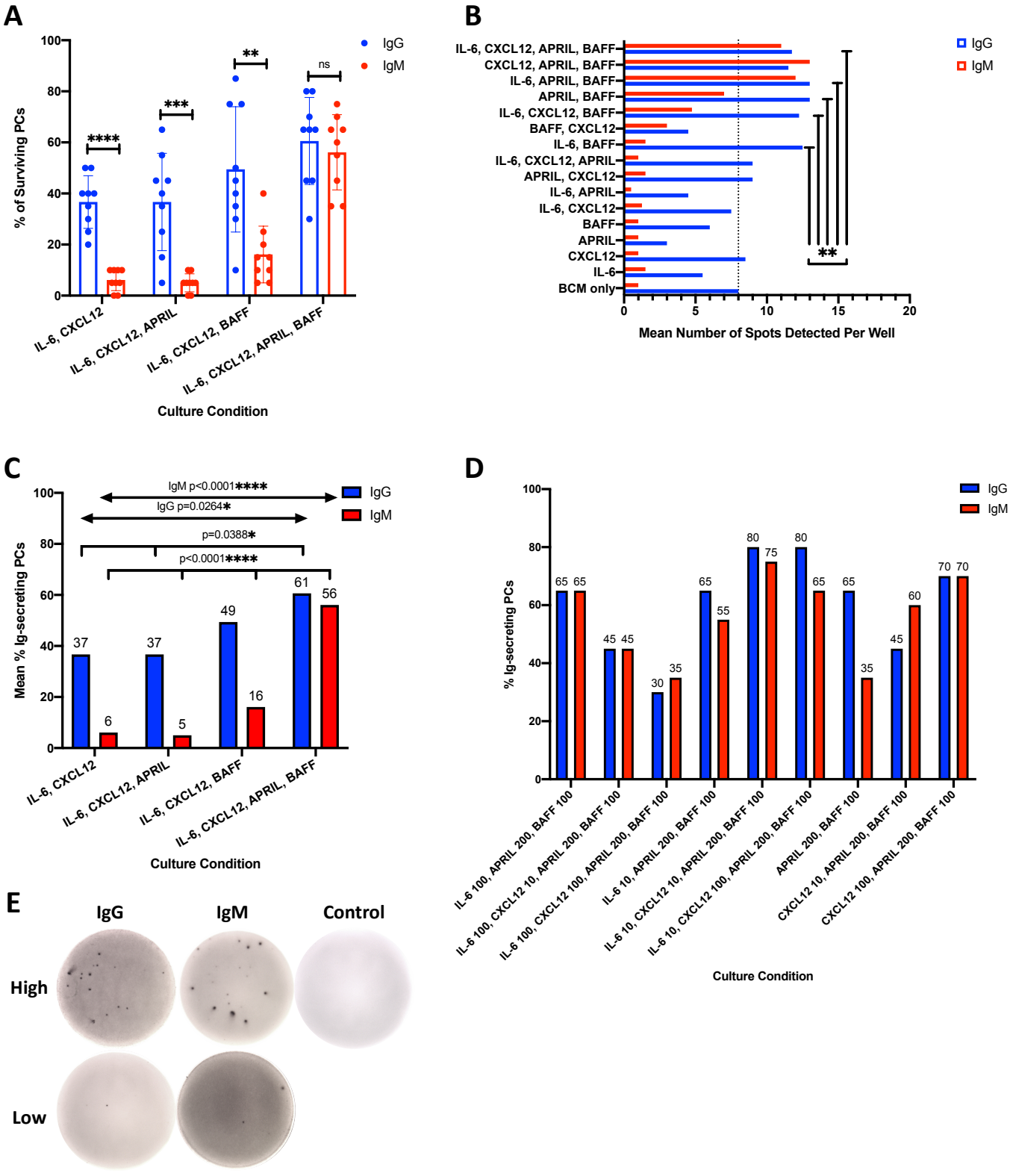
The 'IL-6, CXCL12, APRIL and BAFF' group associated with the highest PC survival rate, yielding 61% and 56% mean survival for IgG- and IgM-secreting PCs, respectively (**Figure 5.5C**). Of the 9 individual conditions within the 'IL-6, CXCL12, APRIL and BAFF group', highest PC survival was seen in the 'IL-6 10 ng/ml, CXCL12 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml' condition (80% for IgG and 75% IgM), and the lowest PC survival was observed in wells cultured in the 'IL-6 100 ng/ml, CXCL12 100 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml' condition (30% for IgG and 35% IgM); (**Figure 5.5D**). *The IL-6 10 ng/ml, CXCL12 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml culture condition was selected as the optimized condition for ex-vivo culture of BM-derived LLPCs.* ELISPOTS are shown in **Figure 5.5E**, with representative spots from select culture conditions; IgM spots tended to be larger than IgG spots.

Figure 5.4 Myasthenia gravis thymic plasma cell survival and functionality and total IgG and IgM levels from plasma cell culture supernatants across culture conditions



Heat maps generated from data from 1 patient. PCs (live, $CD3^{-}CD14^{-}CD45^{+}CD138^{+}$ cells) were purified from fresh thymic tissue obtained from a patient with myasthenia gravis. 20 PCs were sorted directly into each well of culture and ELISpot plates containing the 36-condition cytokine matrix. **(A)** Supernatant total IgG levels (ng/ml) in blue **(top panel)** and total IgM levels (ng/ml) in red **(bottom panel)** at day 3 are shown for each culture condition. Repeat culture conditions are shown within each heat map (e.g. data for the IL-6 100 ng/ml + CXCL12 10 ng/ml condition is shown in row 1, column 2, and its duplicate is shown in row 4, column 2). **(B)** The percentage of IgG-secreting PCs in blue **(top panel)** and IgM-secreting PCs in red **(bottom panel)** assessed by ELISpot at 36 hours is shown for each culture condition.

Figure 5.5 Thymic Plasma Cell Survival and Functionality by ELISpot at Day 1 Across Culture Conditions



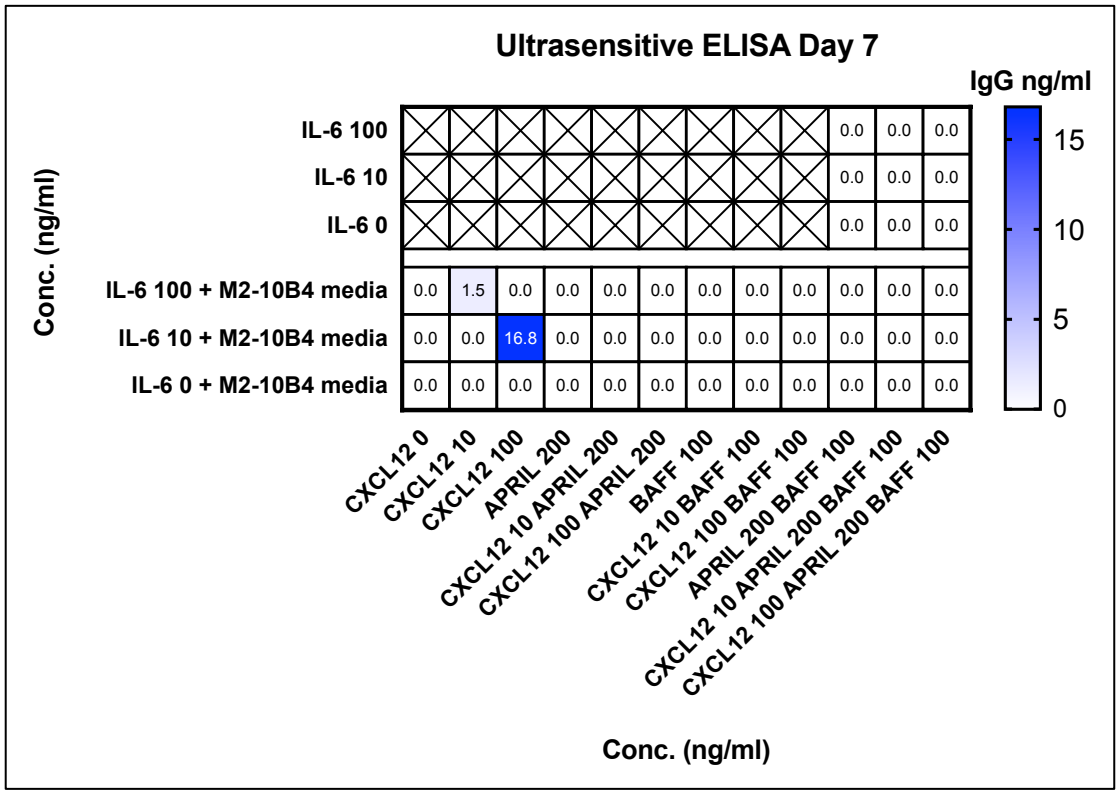
A: Percentage of surviving IgG-secreting (blue) and IgM-secreting (red) thymic PCs at 36 hours for each of the 9 conditions within the 4 main cytokine matrix groups (for each well, number of Ig spots detected out of the 20 plated PCs is plotted as a percentage; mean +/- SD). For each group, IgG- versus IgM-secreting PC survival compared by multiple t tests.
B: 36-condition matrix subdivided into 16 possible cytokine combination groups (irrespective of concentration). Mean number of IgG (blue) and IgM (red) spots detected after 36 hours plotted; dotted line set at 'BCM only' (control condition). IgG-secreting PC survival is significantly higher for several cytokine combinations compared to APRIL alone (Tukey's multiple comparisons test); only highest p values plotted.
C: Mean percentage of surviving IgG-secreting (blue) and IgM-secreting (red) thymic PCs at 36 hours. For each of the 4 main cytokine matrix groups, the mean percentage of the 9 conditions is plotted. IgG- and IgM-secreting PC survival compared across 4 main cytokine groups (ordinary one-way ANOVA). For IgG- and IgM-secreting PCs, % survival compared for 'IL-6, CXCL12, APRIL, and BAFF' conditions versus all other groups (Dunnett's multiple comparisons test).
D: Percentage of surviving IgG-secreting (blue) and IgM-secreting (red) thymic PCs at 36 hours for each of the individual 9 conditions (with concentrations) within the 'IL-6, CXCL12, APRIL, and BAFF' cytokine matrix group, which produced the highest mean % PC survival as shown in **(C)**.
E: representative spots from ELISpot wells. Left: IgG (top panel – high spots 16/20 detected; IL-6, CXCL12, APRIL and BAFF cytokine group; bottom panel – low spots 1/20 detected; IL-6, CXCL12, and APRIL group). Middle: IgM (top panel – high spots 13/20 detected; IL-6, CXCL12, APRIL, and BAFF cytokine group; bottom panel – low spots 2/20 detected; IL-6 and CXCL12 group). Right: control well with BCM only and no plated PCs (no IgG spots detected).
PC, plasma cell; BCM, B cell medium.

5.5.3 Cell Sort 3 – MS Bone Marrow PC Culture (10 cells/well): BM PCs may survive and secrete IgG antibodies in culture for up to 7 days

A BM aspirate was obtained from the femur of a 74-year-old female with multiple sclerosis (MS) undergoing total knee replacement. PCs (live, $CD3^-CD56^-CD14^-CD20^-CD19^{-/low}CD138^+$ cells) were purified from the BM aspirate, which was prepared by Ficoll density gradient. 10 PCs were sorted directly into each well of a plate containing the 36-condition cytokine matrix in 100 µL per well; each condition was tested in duplicate on the same plate, with the addition of enriched mesenchymal stromal cell media ('M2-10B4 media') to repeat wells **(Methods, Chapter 2, Sections 5.4 and 5.5)**. At day 7, the culture was terminated and the 100 µL of supernatant from each well was aspirated and frozen. Given low levels of IgG found in previous experiments (26.7-165.3 ng/ml from peripheral blood PB cultures, and 8.3-30.4 ng/ml from thymic PC cultures), day 7 supernatants were tested for total IgG using a high

sensitivity IgG ELISA detection kit **(Figure 5.6**; **Methods, Chapter 2, Section 5.7.2)**. Due to insufficient numbers, only 450 PCs were sorted into wells. Only 9/36 (25%) of the non-enriched cytokine matrix wells contained plated PCs (**Figure 5.6**; top panel; far right, corresponding to 'IL-6, CXCL12, APRIL and BAFF' group); of these, IgG was detected in 0/9 wells (0%). IgG was detected in 2/36 (5.6%; range 1.5-16.8 ng/ml) of wells which contained enriched mesenchymal stromal cell media ('M2-10B4 media'; **Figure 5.6**; bottom panel). Both wells with detectable IgG were of PCs cultured in the 'IL-6 and CXCL12' group.

Figure 5.6 Total IgG levels from multiple sclerosis patient bone marrow plasma cell culture supernatants across culture conditions



Heat maps generated from data from 1 patient. PCs (live, $CD3^-CD56^-CD14^-CD20^-CD19^{-/low}CD138^+$ cells) were purified from fresh BM aspirate obtained from the femur of a patient with MS undergoing total knee replacement. 10 PCs were sorted directly into each well of a plate containing the 36-condition cytokine matrix (top panel); each condition was tested in duplicate on the same plate, with the addition of enriched mesenchymal stromal cell media ('M2-10B4 media'; bottom panel). Supernatant total IgG levels (ng/ml) at day 7 are shown. Wells marked 'X' indicate empty wells, due to insufficient numbers of PCs.

5.5.4 Cell Sort 4 - LGI1-Autoantibody Encephalitis Bone Marrow LLPC Culture (20 cells/well): BM LLPCs show similar ex-vivo survival and functionality across all culture conditions

For research purposes, LLPCs (live, $CD3^{-}CD14^{-}CD20^{-}CD19^{-/low}CD138^{+}$ cells) were purified from fresh BM aspirate obtained from the posterior iliac crest a 56-year-old male patient with LGI1-autoantibody encephalitis. 20 PCs were sorted directly into each well of ELISpot plates containing the 36-condition cytokine matrix in a total volume of 100 µL per well; each condition was tested in duplicate on the same plate **(Methods, Chapter 2, Sections 5.4 and 5.5)**. Two plates were prepared: one using cytokines in normal BCM, and the other using enriched mesenchymal stromal cell media ('M2-10B4 media'). After 1 day in culture, the number of IgG spots was assessed by ELISpot **(Methods, Chapter 2, Section 5.6)**. Owing to both the rarity of a BM sample, and to the lower yield of IgM- compared to IgG-production observed in previous experiments **(Figures 5.3, 5.4, and 5.5)**, particularly when assessed by ELISpot in the thymic optimization experiments **(Figure 5.5)**, we elected to assess only IgG secretion as our output measure. Accordingly, the 36 conditions were tested in duplicate with IgG repeats rather than singular IgM and IgG; moreover, use of both non-enriched and enriched plates allowed 4 biological repeat results for each cytokine condition, facilitating generation of more robust data.

After 1 day *in vitro*, IgG-secreting LLPCs were detectable by ELISpot in 100% of wells in both non-enriched and enriched plates **(Figure 5.7A)**. The overall mean proportion of surviving BM LLPCs was 52% (mean across all non-enriched conditions 57%, and 47% for enriched). The mean proportion of surviving BM LLPCs cultured in non-enriched and enriched conditions ranged from 1-20 (5-100%; data not shown); mean range 7-18 (35-90%), and 1-18 (5-90%; data not shown); mean range 2-15 (10-75%), respectively (**Figure 5.7A**). The mean proportion of surviving BM LLPCs cultured across each of the four main cytokine groups was similar for both those cultured in non-enriched and enriched conditions (range 55-60%, p=0.9107, R squared 0.0078; range 41-51%, p=0.2668, R squared 0.0561, respectively; one-way ANOVA); **(Figure 5.7B)**. Although overall there appeared to be decreased trends for survival in the presence of enriched media, use of enriched media did not significantly affect LLPC survival

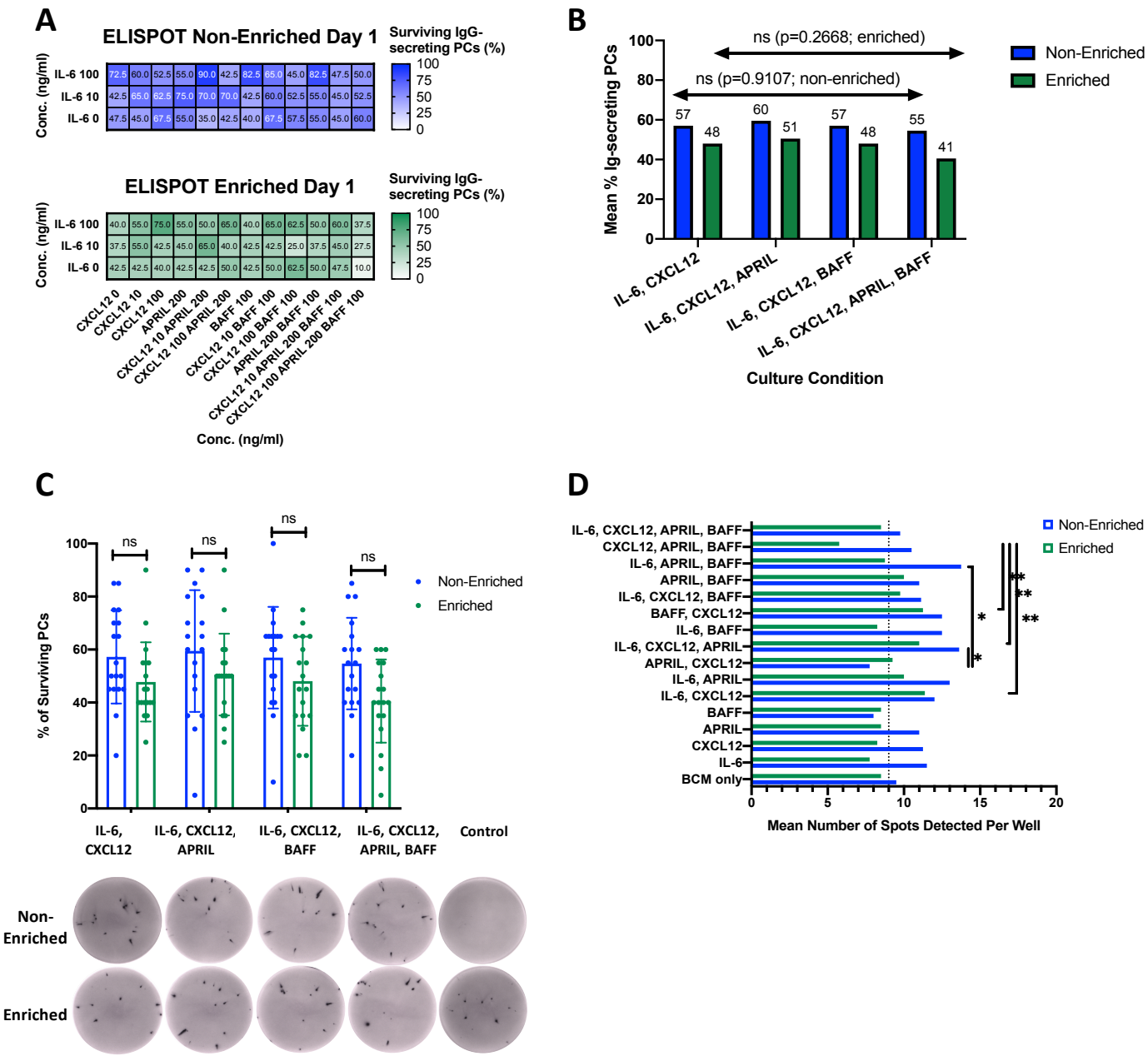
(mean survival for non-enriched versus enriched conditions, respectively: IL-6 and CXCL12 57% v. 48%, p=0.0919; IL-6, CXCL12, and APRIL 59% v. 51%, p=0.1822; IL-6, CXCL12 and BAFF 57% v. 48%, p=0.1485; IL-6, CXCL12, APRIL, and BAFF 55% v. 41%; p=0.0146; multiple unpaired t tests); **(Figure 5.7C)**.

When the 36-condition matrix was subdivided into 16 possible cytokine combinations (irrespective of concentration), the individual combinations yielded significantly different BM LLPC survival rates for both non-enriched and enriched conditions (p=0.0083** and p=0.0023**, respectively; two-way ANOVA; data not shown). For BM LLPCs in non-enriched wells, culture in 'IL-6, CXCL12, and APRIL', *or* 'IL-6, APRIL, and BAFF' both significantly increased survival compared to 'APRIL and CXCL12' (p=0.0494* and p=0.0397*, respectively; Tukey's multiple comparisons test; mean number of spots/20 per well: 'IL-6, CXCl12 and APRIL' 13.63; 'IL-6, APRIL and BAFF' 13.75; 'APRIL and CXCL12' 7.75); **(Figure 5.7D)**. For BM LLPCs in enriched wells, culture in 'IL-6 and CXCL12', *or* 'IL-6, CXCL12, and APRIL', *or* 'BAFF and CXCL12' significantly increased survival compared to 'CXCL12, APRIL and BAFF' (p=0.0023**, p=0.0068**, and p=0.0034**, respectively; Tukey's multiple comparisons; mean number of spots/20 per well: 'IL-6 and CXCL12' 11.38; 'IL-6, CXCl12 and APRIL' 11; 'BAFF and CXCL12' 11.25; 'CXCL12, APRIL and BAFF' 5.75); **(Figure 5.7D**). Consistently, as shown in **Figure 5.7C**, no significant differences were observed in BM LLPC survival for any of the individual 16 combinations when cultured in non-enriched versus enriched conditions (p value range 0.0662-0.7582; multiple t tests; data not shown).

As with thymic PCs **(Figure 5.5B)**, BM LLPC culture in 'IL-6, APRIL, and BAFF' conditions increased survival **(Figure 5.7D)**, and whilst the presence of BAFF did appear to enhance survival, its effect on BM PCs did not appear as profound as that on thymic PCs. For example, BM LLPC survival in 'IL-6, APRIL, and BAFF' combinations was similar to 'IL-6 and APRIL' combinations, and survival in 'APRIL and BAFF' combinations was similar to APRIL alone **(Figure 5.7D)**. Rather, BM LLPCs appeared to survive well in most conditions, although some particularly prominent effects of 'IL-6 and APRIL', 'IL-6, APRIL, and BAFF', and 'IL-6, CXCL12, and APRIL' mixes were clear.

IgG spots secreted by BM-derived PCs **(Figure 5.7C)** appeared larger than those secreted by thymic PCs **(Figure 5.5E)**. Of note, BCM alone was sufficient to sustain survival and function of almost 50% of BM LLPCs for 24 hours; as expected, no IgG spots were detected in the presence of BCM only without any plated PCs **(Figure 5.7C)**. Finally, a characteristic 'snail-track', or trail line was observed in many of the ELISpot wells **(Figure 5.7C)**. This may be due to natural movement of PCs within the culture wells, leading to a 'trail' of secreted Ig. Alternatively, it may be due to repeated opening and closing of the door of the incubator directly above the one in which the BM culture plates were stored.

Figure 5.7 Bone Marrow Long-Lived Plasma Cell Survival and Functionality by ELISpot at Day 1 Across Culture Conditions



Data from 1 patient. LLPCs (live, $CD3^{-}CD14^{-}CD20^{-}CD19^{-/low}CD138^{+}$ cells) were purified from fresh BM aspirate obtained from a patient with LGI1-antibody encephalitis. 20 LLPCs were sorted directly into each well of ELISpot plates containing the 36-condition cytokine matrix.

A: Heat maps of surviving IgG-secreting BM LLPCs assessed by ELISpot at day 1 using non-enriched media (blue; top panel) and enriched media (green; bottom panel); for each condition, mean percentage of 2 repeat wells is shown.

B: Mean percentage of surviving IgG-secreting BM LLPCs for each of the 4 main cytokine matrix groups when cultured in non-enriched media (blue) and enriched media (green). BM LLPC survival across the 4 main cytokine groups analysed by ordinary one-way ANOVA.

C: Percentage of surviving BM LLPCs plotted individually with 2 repeat wells for each of the 9 conditions within the 4 main cytokine groups; mean +/- SD. No significant differences were observed in BM LLPC survival when cultured in non-enriched versus enriched conditions (multiple t tests). Representative IgG spots from ELISpot wells. Non-enriched wells (top panel), left to right: 15/20 spots detected (IL-6-100 ng/ml); 11/20; 10/20; 17/20 (IL-6 100 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml); control well with BCM only and no plated PCs (no spots detected). Enriched wells (bottom panel), left to right: 10/20 spots detected; 11/20; 10/20; 9/20 (IL-6 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml); control BCM only condition with 20 plated PCs (8/20 spots detected).

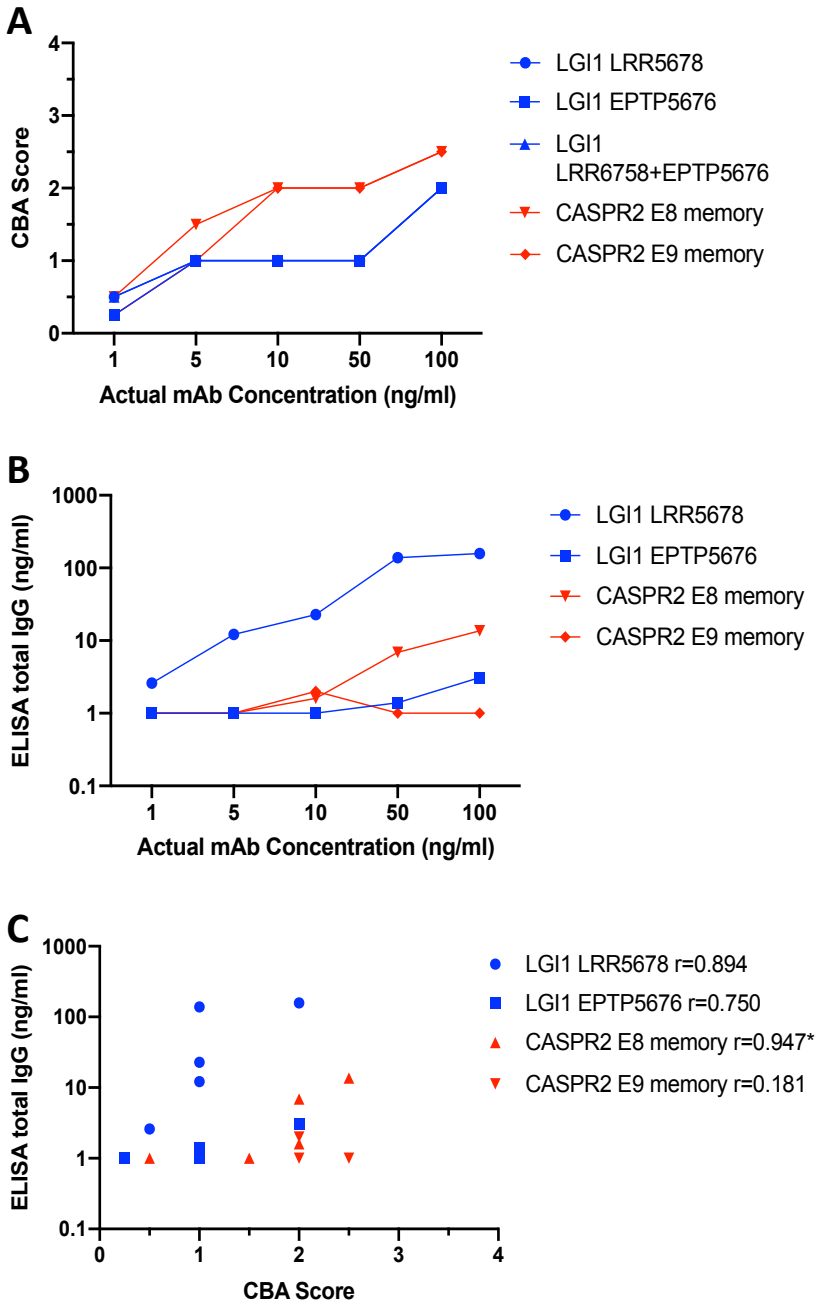
D: Mean number of BM LLPC IgG spots detected after 1 day of culture in non-enriched media (blue) and enriched media (green) for 16 cytokine combination groups; dotted line set at 'BCM only' (control). Conditions with significant differences in BM LLPC survival plotted (Tukey's multiple comparisons test). LLPC, long-lived plasma cell; BM, bone marrow; BCM, B cell medium.

5.5.5 LGI1- and CASPR2-Antibodies are detected at concentrations of 1 ng/ml by live 96-well CBA, but not ELISA

Since we had now established methods for PC isolation and ex-vivo culture, ascertained survival and functionality of the PCs, and gauged their approximate total immunoglobulin secretion levels, we proceeded to focus on *specific* antibody detection. This was important since our ultimate aim was to ascertain whether *LGI1-specific* BM-derived LLPCs can be detected. The low *total* levels of IgG (1.5-165 ng/ml) observed in our preliminary PC cultures (with several PCs per well) would suggest that our expected secreted *specific* LGI1 antibody would be even lower. Thus, in order to ensure that a BM aspirate which would be performed on a patient purely for research purposes would have the potential to generate clear results, it was crucial for us to first ensure that it would be possible for us to detect low levels of specific antibody using our detection systems.

To this end, two LGI1 and two CASPR2 patient-derived monoclonal antibodies were titrated to end point by live CBA, and for measurement of total IgG using ultrasensitive ELISA **(Methods, Chapter 2, Sections 5.7.2 and 5.8)**. All monoclonal LGI1- and CASPR2- antibodies were detected at the lowest concentration of 1 ng/ml by live CBA **(Figure 5.8A)**. In contrast, only one monoclonal antibody (LGI1 LRR5678) was detected accurately by ELISA **(Figure 5.8B)**. The remaining antibodies were detected at levels discrepant to their known concentration (for example CASPR2 E8 at 100 ng/ml concentration was detected as 13.6 ng/ml, and at 50 ng/ml was detected as 6.9 ng/ml; **Figure 5.8B)**. Moreover, except for CASPR2 E8 ($r=0.947$; $p=0.0333$*; Spearman's correlation) the ELISA results did not correlate with those obtained by live CBA **(Figure 5.8C)**, confirming that live-CBA is a relatively more sensitive and accurate detection tool for low concentrations of specific antibody. Thus, our 96-well CBA system has the capacity to detect specific antibody levels as low as 1 ng/ml. This justified both viability and pursuit of research BM aspirate sampling to detect antigen-specific antibodies in our patients.

Figure 5.8 Validation of LGI1 and CASPR2 Antibody Concentration Detection Thresholds by Live 96-well CBA and ELISA



LGI1 (n=2) and CASPR2 (n=2) patient-derived monoclonal antibodies were titrated to specific concentrations (100, 50, 10, 5, and 1 ng/ml). Additionally, a mix of 1 monoclonal LGI1 LRR and 1 monoclonal LGI1 EPTP antibody (LRR6758+EPTP5676) was tested (by CBA only). To assess whether the antibodies could be detected at given concentrations, they were tested in their varying concentrations by live 96-well CBA for specific LGI1- or CASPR2-antibody **(A)**, and total IgG was measured by ultrasensitive ELISA **(B)**. For each monoclonal antibody, ELISA IgG results (ng/ml) were correlated with the CBA score **(C)**: LGI1 LRR5678 r=0.894, ns (p=0.1000); LGI1 EPTP5676 r=0.750, ns (p=0.3000); CASPR2 E8 memory r=0.947, p=0.0333*; CASPR2 E9 memory r=0.181, ns (p>0.9999); Spearman's correlation. EPTP, epitempin; LRR, leucine-rich repeat

5.6 Results: Isolation and Culture of BM-Derived LLPCs from LGI1-Autoantibody Encephalitis Patients

Having obtained clear evidence from the PC optimization experiments that we were able to: 1. maintain ex-vivo survival and functionality of BM LLPCs, 2. detect even minimal levels of specific antibody, and hence 3. justify BM sampling in our research patients, we began recruitment. We approached patients already under our clinical care, and who had previously participated in research. In order to better delineate the potential role of BM-derived LLPCs in disease, we first considered patients who were in clinical remission, and/or no longer taking any immunotherapies. Second, by the same rationale, we considered those with refractory disease, despite high-dose, second-line immunotherapies, such as Rituximab. Third, we approached those in either the acute onset or relapse phase. Finally, we included patients with double-positive antibodies, to determine whether both LGI1- and CASPR2-specific BM-derived LLPCs can be detected from a single timepoint from the same patient. Ten patients were approached for participation in donation of a research BM aspirate. 8/10 (80%) consented, and BM aspirate was obtained from 2/8 (25%); 1/8 (12.5%) attended but the procedure was abandoned. In 2/8 (25%), BM aspiration was cancelled due to significant changes in clinical circumstances, including thymoma relapse requiring chemotherapy, and initiation of anticoagulants, a contraindication to BM aspiration. For the remaining 3/8 (37.5%), appointments were pending allocation in February-March 2020 during unprecedented halting of clinical research due to the COVID-19 pandemic. Recruitment of participants, including an additional booking protocol devised for BM aspiration, is detailed within **Methods (Chapter 2, Section 5.3)**. A copy of the BM referral form can be found within **Appendix 5.9**.

5.6.1 LGI1-Specific BM-Derived LLPCs Can be Detected in LGI1-Autoantibody Encephalitis Patients

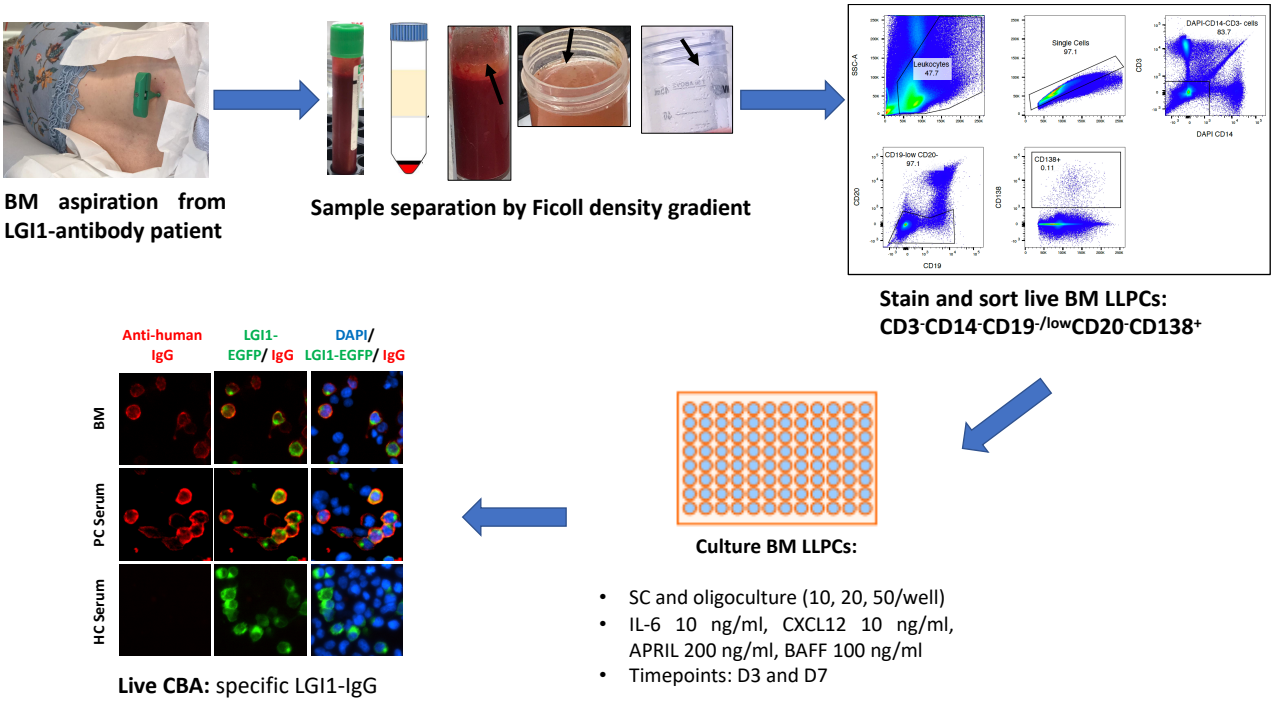
A schematic detailing an overview of the process of BM LLPC sampling, preparation, staining, isolation, and culture is provided **(Figure 5.9)**. For the two patients who underwent BM aspiration, 8ml of fresh BM aspirate was sampled from the left posterior iliac crest. It was

noted that patient JR051 reported a two-day history of viral illness, consisting upper-respiratory tract symptoms, headache, myalgia, and fatigue. Despite these symptoms, he was keen to proceed with the research procedure. Approximately 80 million BM mononuclear cells were counted from each sample following separation by Ficoll density gradient (83 million from participant JR879; 81 million from JR051). BM LLPCs, defined as live, $CD3^-CD14^-CD20^-CD19^{-/low}CD138^+$ cells, represented 0.11% of the $CD19^{-/low}/CD20^-$ cells (1204/ 1,053,425). BM LLPCs were purified and cultured in the optimized culture conditions of IL-6 10ng/ml, CXCL12 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml in a total volume of 100 µL per well for up to 7 days **(Figure 5.9; Methods, Chapter 2, Sections 5.4 and 5.5)**. Additionally, to assess whether PCs may escape through the Ficoll layer due to their large size, we additionally stained the BM pellet, and analysed the cells by flow cytometry. We confirmed that no PCs were lost through preparation of the sample by Ficoll density gradient, since the cells contained within the BM pellet did not express any PC characteristics.

For patient JR879, two plates of 50 BM LLPCs per well and one plate of single BM LLPCs per well were cultured; on each plate, PCs were sorted into 60 wells. After 7 days, 0/6060 culture supernatants contained LGI1-specific IgG antibodies by live CBA **(Methods, Chapter 2, Section 5.8)**. For patient JR051, one plate of 50 BM LLPCs per well, one plate of 20 BM LLPCs per well, one of 10 BM LLPCs per well, and one plate of single BM LLPCs were cultured for 7 days. An additional single cell plate was cultured for 3 days. For reference, **Table 5.4** summarises all PC experiments, including the clinical characteristics of the donors, the PC sources, differences in experimental design, and results of each experiment.

LGI1-IgG autoantibodies were detected with strong signal within the culture supernatant from one well of patient JR051 (specifically, from the culture plate containing 10 BM LLPCs per well; **Figure 5.9)**. *Thus, LGI1-specific autoantibodies were detected at a frequency of 1:4920 of total cultured BM LLPCs.* Intriguingly, four weeks following the BM aspiration, patient JR051 developed a relapse of LGI1-autoantibody encephalitis with FBDS, necessitating inpatient admission for prompt steroid therapy.

Figure 5.9 Schematic for Isolation and Culture of BM-Derived LLPCs



A research participant with LGI1-antibody encephalitis depicted undergoing BM aspiration from the left posterior iliac crest (full written consent for photography obtained). 8ml of BM aspirate is collected into a sodium heparin tube and prepared with Ficoll density gradient (middle top panel). Difficulties during processing of BM samples shown (middle top panel; 3x far right images). BM aspirate is highly susceptible to coagulation, despite containment within heparin, and must be agitated frequently. High lipid content renders the sample difficult to aspirate and a larger bore pipette must be used; separation of fat layer at the surface of BM aspirate sample is indicated by arrows; despite several washes, lipid droplets can be seen along the container walls (arrows, far right image). Following sample preparation, the BM pellet is stained with the full PC antibody panel but sorted upon only 6 selection markers: $DAPI^{-}CD3^{-}CD14^{-}CD19^{-/low}CD20^{-}CD138^{+}$. BM LLPCs are cultured in IL-6 10ng/ml, CXCL12 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml for up to 7 days. Culture supernatants are tested for specific antibody by live-CBA. Images from a culture supernatant containing positive LGI1-IgG antibodies (top panel; BM), with co-localization of anti-human IgG antibody (red), to the surface-expressed eGFP-tagged LGI1 (green); control sera, positive (middle panel, PC), and negative (bottom panel, HC) for LGI1-antibodies; x20 magnification.

BM, bone marrow; LLPCs, long-lived plasma cells; SC, single cell; D3, day 3; CBA, cell-based assay; PC, positive control; HC, healthy control.

Table 5.4 Summary of PC Experiments and Clinical Characteristics of Donors

PC Source	Disease/ Antibody	Age/ Sex at Sampling	Time from Onset at Sampling	IT/ Relevant Medications at Sampling	Experiment Details/ Cells Cultured	Result
HC peripheral blood	HC	38 M	N/A	Nil	Live, $CD45^+CD3^-CD56^-CD14^-CD20^+CD19^+CD27^+CD38^+$ cells 5/ well cultured in 200 µL cytokine matrix for 3, 5, 7, and 10 days Total IgG and IgM measured by ELISA	Peripheral PBs survive and secrete Ig in culture for up to 5 days (26.7-165.3 ng/ml IgG; 6.6-36.7 ng/ml IgM)
DC thymic tissue from thymectomy	Generalised MG/ AChR antibody positive	29 F	5 months	Nil IT; Pyridostigmine	Live, $CD3^-CD14^-CD45^+CD138^+$ cells 20/ well cultured in 100 µL cytokine matrix for: Survival and function by ELISpot at day 1 Total IgG at 3 days measured by ELISA	Thymic PCs survive and secrete Ig in culture for ≥3 days (8.3-30.4 ng/ml IgG; 6.7-8.9 ng/ml IgM) Ex-vivo PC survival enhanced by IL-6, CXCL12, APRIL and BAFF (highest survival in IL-6 10 ng/ml, CXCL12 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml) ELISpot more sensitive than ELISA for PC survival and functionality
DC BM aspirate from femur; JR1084	Secondary progressive MS	74 F	35 years	Nil; previous IVMP >30 years ago	Live, $CD3^-CD56^-CD14^-CD20^-CD19^{-/low}CD138^+$ cells 10/ well cultured in 100 µL cytokine matrix for 7 days for total IgG by ELISA	BM PCs may survive and secrete Ig in culture for up to 7 days (1.5-16.8 ng/ml IgG)
Patient BM aspirate; JR879	LGI1 antibody positive; latest serum titre 1:100; in clinical remission (latest seizure 6 months prior)	73 F	2 years	2mg oral prednisolone OM only	Live, $CD3^-CD14^-CD20^-CD19^{-/low}CD138^+$ cells 7-day culture in 100 µL IL-6 10 ng/ml, CXCL12 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml: 2 plates 50/well 1 SC plate for specific LGI1-antibody by live CBA	No positive LGI1 IgG antibodies detected (frequency: 0/6060 LLPCs)
Patient BM aspirate; JR051	LGI1 antibody positive;	56 M	3 years, 1 month	Nil (steroid cessation	Live, $CD3^-CD14^-CD20^-CD19^{-/low}CD138^+$ cells	BM LLPCs show similar ex-vivo survival and functionality across all

	latest serum titre >1:1000; in clinical remission (latest seizure 1 year prior)			2 months prior)	20/well cultured 100 µL cytokine matrix for survival and function by ELISpot at day 1 Culture in 100 µL IL-6 10 ng/ml, CXCL12 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml: SC (n=1 plate for 3 days) 50/well 20/well 10/well SC (all n=1 plate for 7 days) for specific LGI1-antibody by live CBA	culture conditions (41-60% survive at day 1) LGI1-specific BM-derived LLPCs exist in patients with a frequency of ~1/5000 LLPCs

Summary of n=5 PC experiments (clinical details, differing PC sources, experimental design, and findings). All PCs were collected from fresh tissue/ samples. For culture, 60 wells of each plate were used.

PC, plasma cell; IT, immunotherapy; HC, healthy control; PB, plasmablast; DC, disease control; MG, myasthenia gravis; AChR, acetylcholine receptor; BM, bone marrow; MS, multiple sclerosis; IVMP, intravenous methylprednisolone; OM, omni mane (every morning); SC, single cell; CBA, cell-based assay; LLPC, long-lived plasma cell.

5.6.2 CD138$^+$ Plasma Cells Isolated from Different Compartments Show Differential Cell Surface Marker Expression Profiles

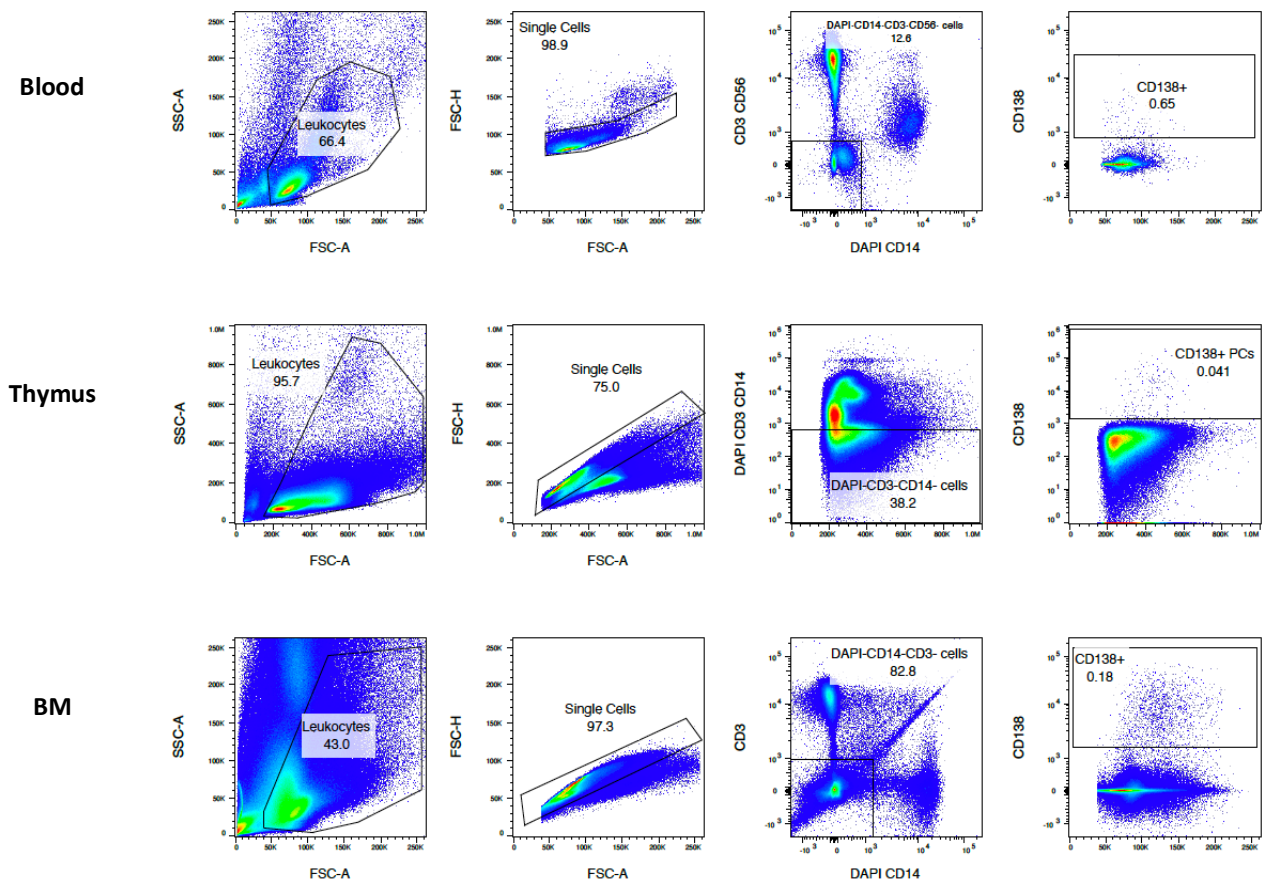
Comparison of cell surface marker expression profiles of CD138$^+$ cells across the three PC compartments sampled revealed interesting trends (**Figure 5.10)**. Since the cell sorting strategy differed for the isolation of PCs from the blood, thymus and BM (namely in relation to selection of CD19 and CD20), the CD138$^+$ cells compared were gated directly from live, CD3-CD14- cell populations (**Figure 5.10A)**. For peripheral blood, CD56$^-$ cells were inevitably selected also, since this marker was included in the original PC panel, prior to removal for BM experiment; this could not be removed from the gating in this case, since it was on same channel as CD3, so that they may be used together as 'dump channel markers'. Similarly, for logistical reasons, and to coincide with other experiments, thymic cells were not stained with the full PC panel, therefore comparison of only some of the cell surface markers was possible for CD138$^+$ cells from the thymus.

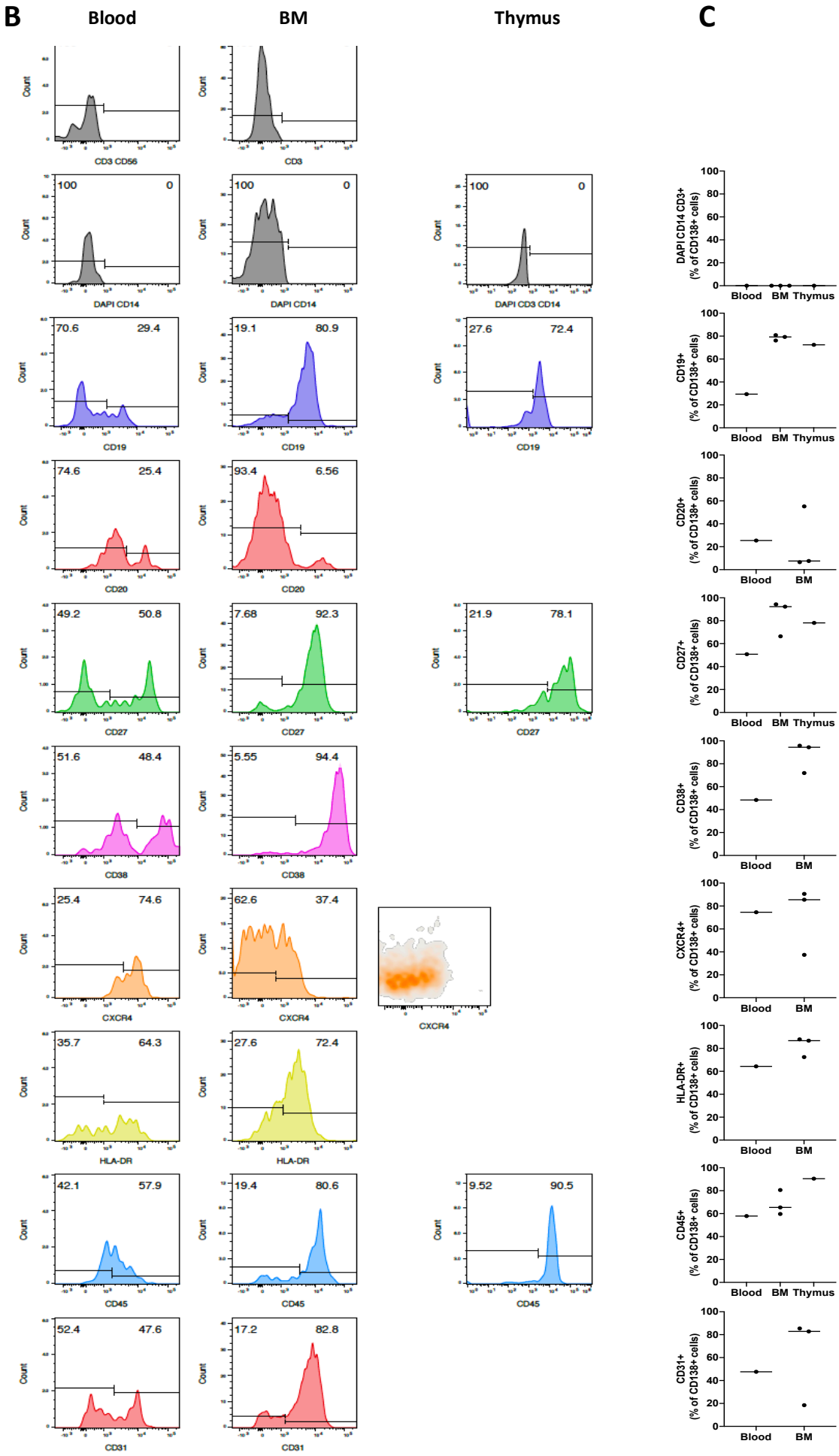
For blood (n=1), BM (n=3, with median % and range), and thymus (n=1), respectively, the percentage of expression for each marker on CD138+ cells was as follows: DAPI/CD14/CD3 (0; 0; 0), CD19 (29.4; 79.3, range 76.2-80.9; 72.4), CD20 (25.4; 7.56, range 6.56-55.2; N/A), CD27 (50.8; 92.3, range 66.4-94.3; 78.1), CD38 (48.4; 94.4, range 71.9-95.8; N/A), CXCR4 (74.6; 85.5, range 37.4-90.6; N/A), HLA-DR (64.3; 86.7, range 72.4-87.9; N/A), CD45 (57.9; 65.4, range 59.7-80.6; 90.5), CD31 (47.6; 82.8, range 18.5-85.4; N/A); **(Figure 5.10B** and **C)**. Whilst no significant differences were identified in expression of any cell surface marker across the PC compartments (not shown; one sample t and Wilcoxon test p=0.25; Sidak's multiple comparison test p>0.9999), clear trends can be seen **(Figure 5.10B)**. Much higher expression of CD19 is seen on CD138$^+$ cells in the BM and thymus compared to the blood. Contrastingly, and as expected, almost none (7.6%) of the BM CD138$^+$ cells express CD20, whereas one quarter of those in the blood maintain this on their surface. Corroborating their definitive PC phenotype, almost all (~95%) of BM CD138$^+$ PCs express CD27 and CD38, compared to only half of blood PBs. Interestingly, CD27 expression by thymic PCs is around 75%, suggesting an intermediate phenotype, and thus increasing gradient of CD27 expression by PCs from blood, to thymus, to BM. As expected, we found CXCR4 expression to be high on blood PBs (75%),

consistent with its role in facilitating their homing to the BM by CXCL12. By comparison, expression by BM PCs was wide ranging (37-91% from n=3 subjects). This may reflect the different PC subsets within BM, with those highly expressing CXCR4 likely to be more immature PCs, having recently entered the BM and hence still retaining CXCR4 on their surface, and those with low expression likely to be more mature, LLPCs, residing for some duration within the BM, suggesting CXCR4 downregulation once PCs no longer require responsiveness to CXCL12. Perhaps surprisingly, HLA-DR expression was similar in blood and BM PCs. CD45 was marginally higher in BM PCs than blood PBs (65.4% versus 57.9%), but markedly high in thymic PCs (>90%). Finally, CD31 was expressed on almost all $CD138^{+}$ BM PCs, and less than half of blood PBs.

Figure 5.10 Comparison of cell surface marker expression by CD138+ PCs from blood, thymus, and bone marrow compartments

A




B
Blood
BM
Thymus
C
CD3 CD56
CD3
DAPI CD14
DAPI CD3 CD14
CD19
CD20
CD27
CD38
CXCR4
HLA-DR
CD45
CD31
DAPI CD14 CD3+ (% of CD138+ cells)
CD19+ (% of CD138+ cells)
CD20+ (% of CD138+ cells)
CD27+ (% of CD138+ cells)
CD38+ (% of CD138+ cells)
CXCR4+ (% of CD138+ cells)
HLA-DR+ (% of CD138+ cells)
CD45+ (% of CD138+ cells)
CD31+ (% of CD138+ cells)
Blood
BM
Thymus

(A) Gating for live, CD3-CD56-CD14-CD138$^+$ cells from the peripheral blood, and live, CD3-CD14-CD138$^+$ cells from the thymus and BM; phenotyping data, not representative of sorting strategies. **(B)** Expression profiles of CD138+ PCs compared for the 3 tissue compartments; histograms for 8 cell surface markers; DAPI, CD14, CD3, and CD56 (dump channels) plotted, confirming their absence in the gated CD138$^+$ cells. For logistical reasons, thymic cells were stained with only 4 of the 8 new PC panel markers, in order to coordinate other experiments. Additional density plot provided for CXCR4 within the BM compartment, to demonstrate how delineation of the positive and negative populations was assessed. **(C)** for each marker, % expression on CD138+ cells plotted for the 3 PC compartments (for BM, median % of n=3 experiments plotted). No significant differences identified in expression of any cell surface marker across the PC compartments (not shown).

5.7 Discussion

Herein, we have demonstrated a method for isolation of LLPCs from the human BM, a platform for their ex-vivo maintenance with preservation of their functionality, and detection of secreted antibody at low concentrations. We have shown, to our knowledge for the first time, the presence of LGI1-specific BM-derived LLPCs in a patient with autoimmune encephalitis by direct sampling of their BM. One other study showed GAD autoantibody production by ex-vivo cultured BM mononuclear cells in a patient with GAD-antibody encephalitis (Thaler et al. 2019), although the antibody is not a neuronal surface antibody, and the BM cells were cultured in bulk directly from the BM sample, rather than isolated as a defined PC phenotype. In this case, peripheral blood contamination is plausible, with antibody production by B cells from the periphery (as already demonstrated within their study).

Antibody Secretion by PCs in Culture

The daily immunoglobulin production rate of PCs is influenced by many factors, including the tissue niche, and level of PC maturity, with LLPCs thought to have lower secretion rates than short-lived PCs (Bonnaure et al. 2016). Reported immunoglobulin secretion rates by PCs vary widely in the literature. From a molecular context, exceptional rates of antibody release by PCs have been determined as 10^8 Ig molecules/ cell/ hour, which are comparable for IgG, IgM, and IgA isotypes (Hibi & Dosch 1986). In PCs generated by *in vitro* differentiation from human

HC B cells, Cocco et al observed Ig secretion rates of ~120 pg IgG/cell/day, and ~20 pg IgM/cell/day (Cocco et al. 2012), whilst others found 10-18 pg/cell/day IgG and 1pg/cell/day of IgM (Jourdan et al. 2014; Bonnaure et al. 2016). Others report secretory values as high as 1.8 ng Ig/cell/day from PCs of all isotypes (Brinkmann & Heusser 1993). In direct *ex-vivo* cultures of BM-derived human PCs, rates are typically 0.7 ng IgG/cell/24 hours (Hibi & Dosch 1986), which matches that of murine BM PCs (~0.4 ng IgG/cell/day) (Manz et al. 1997). In malignant PCs, Salmon et al reported IgG synthetic rates of only 5-14 pg/myeloma PC/day (Salmon & Smith 1970). Based on the above studies, we shall infer *an approximate daily IgG production of 500 pg from a single PC*.

One of the most striking findings was the antibody concentration detected in supernatants of cultured PCs. IgG levels were 26.7-165.3 ng/ml in day 3-5 peripheral PB cultures, 8.3-30.4 ng/ml from day 3 thymic PCs, and 1.5-16.8 ng/ml in day 7 BM PC supernatants. IgM levels were 6.6-36.7 ng/ml and 6.7-8.9 ng/ml in day 3-5 peripheral PB and day 3 thymic PC supernatants, respectively. Whilst at first glance, these overall immunoglobin levels of 1.5-165 ng/ml may appear to be in stark contrast to those reported by others, after normalization of experimental parameters between studies, they appear largely consistent. In fact, the levels generated from our PC cultures may even be more copious than expected.

Firstly, using the assumption of *daily IgG production of ~500 pg from a single PC*, the BM PCs plated at 10 per well in our experiments would produce a minimum of 500 pg per day if only one were to survive, and a maximum of 5000 pg IgG per day if all survived. Since they were cultured in a total volume of 100 µL, the concentration range per day would be equivalent to 5-50 ng/ml. For the 7-day MS BM PC culture, one would expect a range of 35-350 ng/ml by day 7. Thus, this would suggest that for the well found to have an IgG concentration of 1.5 ng/ml, only one of the ten PCs survived for a maximum of 24 hours, and the well with 16.8 ng/ml IgG is likely to be a result of either a single PC surviving for ~3 days, or due to survival of 3 PCs for 24 hours. By the same approach, the expected concentration in our peripheral PB supernatants for a minimum of 1 and maximum of 5 surviving, functional PCs would be 7.5-37.5 ng/ml IgG at 3 days, and 12.5-62.5 ng/ml IgG at 5 days. Similarly, from our thymic PC cultures we would expect 15-300 ng/ml IgG production from survival of 1-20 PCs after 3 days. These extrapolations are compatible with our findings.

Now, by comparison, others report total Ig secretion by BM PCs from human HCs of ~170 ng/ml IgG and ~12 ng/ml IgM at 5 days (Avery et al. 2003; Ellyard et al. 2005), and ~1500 ng/ml at 7 days (Rodriguez-Bayona et al. 2013). However these are from cultures generated from $5x10^3$ cells per well (Avery et al. 2003; Ellyard et al. 2005) and 10^4 cells per well in 250 µL (Rodriguez-Bayona et al. 2013), equating to 500- and 400-fold, respectively, more cells than ours, which used 10 per well in 100 µL. Thus, – if one assumes that secreted antibody levels correlate with the cell number – then the reported IgG levels of 170 /500 would equate to 0.34 ng/ml at day 5, and 1500/ 400 would equate to 3.75 ng/ml at day 7; these are comparable to the total IgG levels of 1.5-16.8 ng/ml detected at day 7 in our MS BM PC culture. Whilst we did not assess total IgM secretion from BM PCs, secretory rates as low as 35 ng/ml IgM are reported from murine BM PC cultures (5000 per well) after 7 days (Minges Wols et al. 2007).

Peripheral PB culture supernatant IgG levels range from ~100 ng/ml at day 13 (or roughly 25 ng/ml at day 3; 6000-12500 per well from NMO patients) (Wilson et al. 2018) to ~300 ng/ml at day 3 (100,000 per well from HCs) (Rodriguez-Bayona et al. 2013), and 800 ng/ml at day 6 (which we may equate to ~400 ng/ml at day 3; 10, 000 per well from NMO patients) (Chihara et al. 2011). Thus, considering that our IgG values of 26.7-165.3 ng/ml in day 3-5 peripheral PB cultures were generated from only 5 PBs per well, these are in comparison much higher than expected. One likely explanation is that our results are based only upon concentrations from wells with IgG *detectable* by ELISA, which represented merely 4.2% of wells. If the PBs in other wells secreted IgG at much lower rates, comparable to those reported by others, it is likely that such concentrations would be below the ELISA detection threshold, and thus the levels observed in the positive wells may portray a skewed, overrepresentation of 'abnormally high IgG-secreting PBs'. Other factors could include sample handling, such as time from isolation to culture, and temperature, use of fresh versus thawed cells, study design, and donor differences, including age. We used fresh PBs, isolated from a HC 7 days post onset of upper respiratory tract infection, replicating methods employed by others to enrich for antibody-secreting PCs by selecting donors shortly (~7-10 days) following antigen-stimulation by vaccination or infection. Medina et al (Medina et al. 2002) observed that the frequency of $CD38^{hi}$ cells in the peripheral blood of HCs increased at least 20-fold at 6 days following tetanus booster – also the time at which detection of tetanus-specific IgG-secreting ASCs was

maximal. It is not known however, whether their individual antibody-secreting capacity also increases. Indeed, minimal levels of secreted antibody in culture supernatants from patient PBs prompted use of an *in vitro* differentiation platform of B cells to PBs by Wilson et al (Wilson et al. 2018). Total IgG levels from cultured PBs generated using this platform were up to 80, 000 ng/ml, compared to merely ~100 ng/ml by direct ex-vivo culture of PBs, using the same culture conditions. This highlights not only the fragility and limited ex-vivo survival of isolated PBs, but also, antithetically, the antibody-secreting potential of these cells. Hence, the concentration of 80, 000 ng/ml at day 13, which estimates ~30, 750 ng/ml by day 5, is in fact much more comparable to the IgG concentrations detected in our PB cultures; since this used 1850 times more cells than used in our PB culture, 30, 750/ 1850 equates to 16 ng/ml total IgG at day 5 – similar to ours. Therefore, it is possible that the PBs isolated for our experiments were more viable, and/ or had higher secretory capacity.

Whilst secreted Ig rates from thymic PC cultures are not widely reported, cultures of human HC PCs from other compartments range from 180 ng/ml IgG after 7 days from $1.25x10^5$ tonsillar PCs (Rodriguez-Bayona et al. 2013), and 15.6-18.7 IgG and ~220 ng/ml IgM from day 5 cultured splenic PCs at $5x10^3$ per well (Ellyard et al. 2005). Based on our culture parameters of 20 thymic PCs per well, these reported rates assume ~0.03 ng/ml and 0.5 ng/ml of expected IgG and IgM secretion, respectively, by day 3. Thus, as with the aforementioned extrapolated PB culture levels, our thymic PC culture supernatant concentrations of 8.3-30.4 ng/ml IgG and 6.7-8.9 ng/ml IgM appear to be at least 10-fold higher than expected; however, HC tonsillar and splenic PC secretion rates are not a direct comparison to patient thymic PCs. The same factors discussed above could account for these differences, as could higher secretory rate by PCs from patients with autoimmune disease versus controls; Kitani et al showed that B cell cultures from SLE patients contained 4-5 times higher levels of antibody than those from HCs (Kitani et al. 1992). Another explanation may relate to differences in the starting cell density of PC cultures reported in the literature (in some cases 10, 000 - 100, 000) compared to our oligocultures of only 5-20 PCs per well. It is conceivable that PCs contained within low density cultures may fare better than those cultured in higher density, where many may undergo apoptosis. Apoptosis of even few cells with release of toxic materials may change the culture environment significantly, and detrimentally to other PCs. Even in culture conditions consisting of equivalent combinations and concentrations of cytokines, cell numbers as

discrepant as 20, 000-fold may promote better survival of few (~5-20) PCs distributed in abundant, rich cytokine media, compared to those cultured at higher density.

Overall, the Ig rates detected within our cultures appear feasible based on the above calculations. Moreover, the consistent ranges of total IgG and IgM from experiments from both peripheral blood and thymus are supportive for legitimate antibody production by the cultured PCs. Secondly, the frequency of positive wells across the blood day 3-5, thymus day 3, and BM day 7 (4.2% versus 6.9% versus 5.6%, respectively for IgG; 2.1% wells versus 2.8% wells respectively for IgM; BM IgM not tested) was similarly consistent. Of note, no IgG or IgM was detected in *any* of the wells from the day 7 and day 10 PB cultures when measured by ELISA. This could be due to replenishment of cytokines at days 3, 5, and 7 leading to further dilution of a low concentration of antibody contained within the well, falling below the ELISA detection threshold. Alternatively, the PCs may have been aspirated during the media changes, although this is unlikely since this was performed with extremely low-pressure graduated aspiration, with avoidance of sudden suction, and with tilting of the culture plates; moreover, it would be unlikely that PCs from all wells of all plates were aspirated. Finally, the lack of antibody could signify PC death, or abnormal function, leading to cessation of antibody secretion; without survival studies, we are unable to confirm this. The ELISpot data suggest otherwise however, since 100% of IgG wells were positive from cultured thymic and BM PCs, and 89% IgM positivity was found in thymic PCs tested by ELISpot. Thus, ELISpot appears to be much more sensitive than ELISA at detecting secreted antibody from PCs, and suggests that the PCs are indeed surviving and producing antibody, but this is likely to be below ELISA detection limits. ELISA plates are usually coated with 0.1-1 μg antibody (1 μg in our methodologies), whereas ELISpot plates are coated with ~1 μg antibody (1.5 μg in our system), and the porous structure of the PVDF membrane on ELISpot plates permits high capture antibody binding, and high detection sensitivity of secreted antibodies. Finally, one study showed that only 24% of plated BM LLPCs secrete Ig antibodies (IgG 60%, IgA 33%, IgM 7%) (Martinez-Murillo et al. 2016), and typically several thousands of cells are plated in ELISpot wells (up to 100,000 PCs/ well by Hallilley et al (Hallilley et al. 2015)). Our findings of 5-61% and 41-60% Ig secretion from only 20 plated thymic and BM PCs, respectively, may indicate superior viability and/ or functionality of PCs, possibly due to handling, culture conditions, or donor factors (autoimmunity).

Specific LGI1 Autoantibody Secretion by BM-Derived LLPCs in Ex-Vivo Cultures

The culture well which produced specific LGI1-IgG antibodies was deemed to be a strong positive result, with a CBA score of 3. Based on the CBA validation experiments which tested monoclonal antibodies across several concentrations, a CBA score of 3 would equate to an LGI1-IgG antibody concentration of ~100 ng/ml **(Figure 5.8A)**. Considering that this was from a well containing 10 BM LLPCs in a total volume of 100 µL cultured for 7 days, and we assume that a single PC produces ~500 pg IgG per day, if only a single BM LLPC survived, we would expect a concentration of 35 ng/ml IgG after 7 days. In the event of survival of all ten BM LLPCs, the maximum detectable IgG concentration would be ~350 ng/ml. Whilst it is possible that the LGI1-antibody detected in this well was a result of specific antibody production by more than one LLPC (for instance two or three), it is unlikely that they would all coincide in this one well, rather than be dispersed amongst the remaining 4919; additionally, culture conditions across plates were the same. It is much more likely that the LGI1-antibody detected was produced by a *single* surviving, functional LGI1-specific BM-derived LLPC, with exceptionally good Ig secretion capacity, since we assume that it secreted three times the expected amount of antibody for the given time in culture. Moreover, not only can we infer that the cell bore exceptionally effective Ig production, but also that the PC was likely viable upon elective termination of the culture at day 7, and that it may have survived for many more days or even weeks in these ex-vivo culture conditions. Jourdan et al demonstrated a powerful cell culture platform in which they could maintain LLPCs in vitro for at least 120 days (Jourdan et al. 2014). Similarly, Cocco et al (Cocco et al. 2012) generated in vitro differentiated LLPCs, which showed equivalent CD138 expression profiles to normal human BM PCs, and common strong expression of secretory genes shared with BM PCs. They developed a cell culture system in which mature PCs could survive until at least the point of elective culture termination at 62 days, although, strikingly, their mathematical predictions concluded that the average and maximum lifespans of these LLPCs were ~300 days, and 700 days, respectively. Whilst these studies indicate remarkable ex-vivo survival capabilities, in contrast to our oligocultures, the methods employed bulk culture of several thousands of cells per well. Additionally, IgG secretion is higher in cultures which initiate from B cells differentiating

into PCs, and numbers of IgM-, IgG-, or IgA-secreting cells can exceed the number of functional precursors by 15-fold (Brinkmann & Heusser 1993).

Frequency of LGI1-Specific BM-Derived LLPCs in Ex-Vivo Cultures

The frequency of LGI1-specific BM-derived LLPCs detected in our culture system was 1:4920 (0.02%) from patient JR051, and 0:6060 from patient JR879. Whilst this may represent patient factors, since patient JR879 was in clinical remission, whereas JR051 was sampled 4 weeks prior to a disease relapse, the extremely low frequency of ~1:5000 likely reflects the low in vivo frequency of individual antigen-specific PCs amongst a rare cell subpopulation (*long-lived* PCs). Thus, isolation of antigen-specific PCs in good quantities is inherently difficult, and this is further exacerbated by the difficulties in their ex-vivo maintenance. In NMO, for example, no specific AQP4-IgG was detected in culture supernatants of PBs isolated directly from patients, however when an in vitro differentiation system of patient B cells to PBs was adopted, 1 of 12 patients produced specific AQP4 IgG in culture (Wilson et al. 2018). The reported frequency of cultured antigen-specific PCs is ~0.5%, since Hallilely et al found a mean frequency of 0.6% (range 0-1.8%) measles- and mumps-specific IgG-secreting ASCs of total IgG ASC by ELISpot (Hallilely et al. 2015).

We sought to compare our determined frequency of antigen-specific PCs to those estimated from the literature. Data from human HC BM aspirates indicates that filling 80% of a 10ml syringe (as in the case of the 8ml BM aspirates obtained from our patients within a 10ml tube), should yield ~20 x10^6 BM mononuclear cells/ml (Hernigou et al. 2013). Thus, we would expect 160 x10^6 BM mononuclear cells in the 8ml aspirate. Since we counted ~80 x10^6 BM mononuclear cells from both research aspirates, we shall use this figure. Next, analysis of BM aspirates from rhesus macaques obtained from the iliac crest, matching the sampling site of our patients, revealed that the frequency of all IgG-secreting ASCs was 4000/10^6 BM cells, and that the frequency of specific IgG-secreting ASCs was 5/10^7 BM cells (Hammarlund et al. 2017). Therefore, the estimated total number of IgG-secreting PCs within our 8ml aspirate is 4000 x 80 (320, 000), and the estimated number of LGI1-specific IgG-secreting PCs within our sample is 0.5 x 80, equating to only 40 (0.01%). To have successfully cultured and detected one of ~40 antigen-specific cells is noteworthy.

It should be considered that the frequency of antigen-specific antibodies may have been enhanced within our patient, since the BM sample was obtained four weeks prior to a clinical relapse, and he had completed steroid immunotherapy three months prior. Thus, LGI1 autoantibodies may have been quiescently expanding within the BM compartment. Additionally, the coinciding viral illness at the time of sampling may have triggered polyclonal immune activation, including within the BM compartment, promoting antibody-secretion by LGI1-specific LLPCs. A combination of these factors is also possible.

Finally, there are several possible reasons for non-production of LGI1-autoantibodies by patient JR879. Firstly, as already outlined, it is likely that there was low frequency or absence of LGI1-specific LLPCs in the acquired BM sample, which may have been further influenced by her increased age (73 years). Secondly, reduced viability and functionality of BM LLPCs is possible due to ex-vivo delays from time of sampling to introduction of isolated PCs into culture. Further, staining and sorting is known to impact PC survival and functionality. Nguyen et al demonstrate almost 3-fold decreased PC functionality after cell sorting due to shear stress (Nguyen et al. 2019). Other factors, such as the environmental niche and culture conditions, may influence Ig secretion by PCs in vitro.

Influence of Culture Conditions on Ex-Vivo PC Survival and Function

Since BM is a rare sample to obtain, and our study ethics do not permit sampling of BM from HCs, access to BM-derived PCs was limited. Thus, the initial optimization experiments utilised opportunistic availability of PCs from various tissue niches and sources in order to optimize culture conditions for [BM] LLPCs prior to embarking on BM sampling from patients with autoantibody-mediated encephalitis for research purposes. Whilst the experiments using peripheral PBs and thymic PCs offered no *direct* comparison to BM, they enabled testing of culture conditions across various PC compartments (peripheral blood, versus thymus, versus BM). It should be borne in mind that the cells cultured from each compartment had varying cell surface phenotypes, since the cell sorting strategy for PCs from each compartment differed slightly, owing to their different maturity and phenotype. The optimization experiment conducted with BM from a patient with MS provided insufficient PC numbers to

enable comparison across all culture conditions to include ELISpot, although nonetheless generated useful data in other respects.

Another limitation to be considered is the differing numbers of plated PCs between experiments (HC peripheral PBs 5/well, MG thymic PCs 20/well, MS BM PCs 10/well). The initial choice of 5 per well reflected initial attempts to – ultimately – optimize single PC culture conditions, however owing to paucity of Ig levels detected in the first PB sort, this prompted increases to 20 cells per well for the subsequent thymic PC sort. In turn, the sufficient data generated from 20 PCs per well, combined with the fact that the next sort would be of BM-derived PCs – which were anticipated to produce higher levels of Ig per cell – led to the reduction to 10 per well for the MS BM PC sort. Whilst one could normalise the percentage survival of Ig-secreting PCs across the three experiments, this does not necessarily take into account any supportive effects individual PCs may have on each other, and how this may influence their survival and/ or function. Despite differences in numbers of plated PCs, from the peripheral PB and thymic PC experiments, we were able to infer both optimum duration of culture (at least 3, and up to 5 days), and optimum culture conditions (IL-6, CXCL12, and BAFF cytokine group conditions for PBs, and IL-6, CXCL12, APRIL and BAFF conditions for thymic PCs). Moreover, we established that ELISpot sensitivity is superior to ELISA for determination of PC survival and function; ELISA demonstrated only 6.9% of wells positive for any IgG, and 2.8% for IgM after 3 days' culture, compared to IgG and IgM spots determined by ELISpot in 100% and 89% of wells, respectively, after only half of the duration (36 hours) in culture. Although data were limited, BM PCs appeared to survive longer in culture (potentially up to 7 days).

We used the secretome of mesenchymal cells within our cultures rather than the cells directly acting as feeder cells, since there was temporarily no access to the radiation facility. Cocco et al (Cocco et al. 2012) demonstrated that direct cell contact is unnecessary, and using mass spectrometry, identified that the M2-10B4 secretome includes proteins appropriate for a BM environment, including LGALS1 (Galectin-1), linked to PC generation and survival, and SPP1 (osteopontin). Interestingly, osteopontin permits myeloma cell growth, and an analogous mechanism may occur within the physiological state to maintain human LLPCs within the BM. Paradoxically, we observed overall decreases in survival in BM PCs cultured in conditions

supplemented with BM mesenchymal stromal cell media compared to those without, although the trends were not significant. It is possible that culture conditions were altered by cytokine production by the mesenchymal stromal cells. For example, mesenchymal stromal cells can secrete up to 335 ng/mL of CXCL12 (Bonnaure et al. 2016), and BM stromal cells secrete ~2 ng/ml of IL-6 (Minges Wols et al. 2007). One possibility is therefore that the cytokines in wells supplemented with enriched media became overly concentrated, causing a potentially toxic effect on the PCs.

BM LLPCs showed no trend towards any particular cytokine condition(s), and exhibited up to 90% survival in enriched and 100% survival in non-enriched conditions when assessed by ELISpot. Moreover, BCM alone was sufficient to sustain survival and function of almost 50% of BM LLPCs for 24 hours. One murine in vivo study found that *either* BAFF or APRIL can support the survival of BM-derived LLPCs (Benson et al. 2008), thus use of both of these TNF ligands may be superfluous. Whilst there were no significant differences in BM LLPC survival between 'IL-6, CXCL12 and APRIL' and 'IL-6, CXCL12 and BAFF' group conditions, indicating that this may be true, no reductions in PC survival were observed in only 'IL-6 and CXCL12' conditions.

Whilst BM PCs appeared to survive well irrespective of culture condition, one of the most striking observations was the effect of BAFF on thymic PC survival and/or function. Except for 'BAFF plus CXCL12' combinations, addition of BAFF to any cytokine combination led to a profound increase in PC survival, almost doubling the number of IgG spots detected compared to the control 'BCM only' condition (**Figure 5.5B**). Whilst 'IL-6 plus APRIL' combinations yielded lower IgG spots than control, both 'IL-6 plus BAFF' and 'APRIL plus BAFF' combinations led to dramatic increases – almost trebling – in IgG spot numbers, or PC survival. Moreover, 'IL-6, CXCL12, plus APRIL' combinations produced a mean of 9 IgG spots (little different to the control), whilst 'IL-6, CXCL12, APRIL, and BAFF' combinations showed increased IgG secretion (mean of 12 IgG spots). Finally, APRIL did not appear to augment the PC survival effects of either IL-6 or CXCL12 used alone or in combination; similarly, IL-6 did not enhance the effects of either CXCL12 or APRIL used alone or in combination. Together, these findings suggest that the observed dramatic increases in thymic PC survival (and/or function) are due to the effects of BAFF. Interestingly, and perhaps unexpectedly, CXCL12 also increased PC survival, although

not to the same extent as BAFF. Whilst the survival effects of CXCL12 on BM PCs are well established (Rodriguez-Bayona et al. 2013; Minges Wols et al. 2007; Cassese et al. 2003), its effect on thymic PCs has not been reported.

Synergistic effects of cytokine combinations appear to be important – for instance, APRIL enhances IL-6-induced PC survival by almost 7-fold (Jourdan et al. 2014). Our findings indicate that concentration may be equally as important. Of interest, of the 9 individual conditions within the 'IL-6, CXCL12, APRIL and BAFF' group, the condition which led to the highest thymic PC survival was 'IL-6 10 ng/ml, CXCL12 10 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml' (80% for IgG and 75% IgM), yet the lowest PC survival was found in the 'IL-6 100 ng/ml, CXCL12 100 ng/ml, APRIL 200 ng/ml, BAFF 100 ng/ml' condition (30% for IgG and 35% IgM) (**Figure 5.5D**). These dramatic 63% and 53% drops in survival (for IgG- and IgM-secreting PCs, respectively) correlate with 10-fold increases in IL-6 and CXCL12 concentrations, which may be considered negligible. Whilst this may be considered a coincidental finding, each of the 36 conditions was tested in duplicate (one well each for IgG and IgM), and this observation was consistent for both IgG and IgM (**Figure 5.5D**); moreover, clear trends can be seen across the 4 main cytokine groups (**Figure 5.4B, Figure 5.5A and C**), supportive of validity of these data. These observations highlight both the sensitivity of PCs to subtle changes in cytokine milieu for their ex-vivo maintenance, and the importance of testing culture conditions in various cytokine combinations and across a range of concentrations.

In the thymic PC culture, IgM-secreting PCs were detected far less frequently than IgG-secreting PCs, including for matched culture conditions. IgM-secreting PCs were detected beyond control levels almost exclusively in conditions containing APRIL plus BAFF with either IL-6 or CXCL12, or both. This may be explained by the fact that adult thymocytes consist of predominantly IgG^+ B cells. One study showed that whilst human thymic B cells in infants as young as 5 days are largely IgM^+ (70%), this population decreases in children as it is replaced by class-switched IgG^+ cells. An adult of 29 years of age had almost no IgM^+ B cells within thymocytes (~7%) and the functional antibody secreting PCs secreted predominantly IgG (Nuñez et al. 2016), akin to our findings. Since our subject was of the same age, the infrequent detection of IgM, yet copious IgG secretion by the PCs, is likely explained by accumulation of memory B cells within the thymus.

Bone Marrow Staining and Lymphocyte Subsets

Due to the nature of our study ethics, BM sample was available only for final research experiments. Thus, antibody titration and optimization were performed on peripheral blood. On the day of BM sorts, FMOs were prepared using fresh PBMCs from HCs in the morning, in advance of acquisition of the BM sample. One reason was to minimize time from BM sampling to PC culture, thus maximizing PC viability. Additionally, our research participants travelled from various areas of the UK, and were offered lunchtime appointments (final slots following NHS Haematology patients), thus the BM aspirate was often only acquired at 13:30-14:00. Since staining at the relevant dilutions optimized for blood would likely be insufficient for BM, since the expression of certain markers (e.g. CD19, CD20, CD38, CD138) was much higher on BM, antibody dilutions were upscaled for CD19 and CD20 **(Methods, Chapter 2, Table 5.5.2)**. Without these adjustments, too little antibody would mean that the BM cells expressing the markers of interest would not stain brightly, and as a result prevent adequate separation from the negative populations.

Few human BM studies are published detailing proportions of lymphocyte subsets, and any such studies pertaining to PCs are particularly rare. Most published human BM data referencing PCs are based on frequencies of malignant PCs, typically in the context of multiple myeloma (MM). We compared cell yield from our BM aspirates to other studies of human BM. One study examined BM aspirates from 10 HCs (24-42 years), and stained the aspirates with monoclonal antibodies against lymphocyte, monocyte, and myeloid antigens (Clark et al. 1986). Using flow cytometry, they found that T cells (defined as CD3 positivity), are found in high numbers in BM samples, comprising 46% of lymphocytes from BM aspirates. Our flow cytometry data suggest that T cells (based on CD3 and CD14 positivity) comprise a mean 26% of BM lymphocytes (range 17.2–39.4%). Reasons accounting for these discrepancies could include the age differences between the two cohorts, with average age of subjects included by Clark et al of 33 years (range 24-42), compared to ours (66 years; range 53-74). Moreover, our samples were taken from patients with autoimmune diseases, two of whom were taking long-term immunosuppressive treatments either at the time of or shortly before sampling, which may have altered proportions of lymphocyte subsets within the marrow. Finally, whilst the same sampling site was used for both studies (posterior iliac crest), Clark et al sampled

only 1-1.5ml volume, and this has been shown to affect both the concentration of the sample, and degree of peripheral contamination, thus influencing differential cell counts. In fact, the group importantly identified that BM aspirates, but not BM biopsies, can be substantially contaminated by peripheral blood. B cells (defined by the pan B cell marker, CD19), were mean 12% of BM lymphocytes. Since no PC markers were used, we are unable to compare the proportion of BM samples represented by PCs.

Distinctive Expression Profiles of CD138$^+$ Plasma Cells Isolated from Different Plasma Cell Compartments

Our finding of much higher CD19 expression on CD138$^+$ cells in the BM and thymus compared to the blood (79.3% and 72.4% versus 29.4%, respectively) is consistent with that of others; the majority (~80%) of CD38^{hi}CD138^{hi} antigen-specific LLPCs in the human BM are CD19$^+$ (Brynjolfsson et al. 2017), and only 40% of PBs express CD138 (Chihara et al. 2011). Our data showing 25.4% CD20 expression on blood PCs versus only 7.6% on BM PCs corroborate successive PC maturation with downregulation of CD20 from blood to BM, consistent with evidence that BM PCs almost never (~2-3%) express CD20 (Halliley et al. 2015; Medina et al. 2002). The almost universal (~95%) expression of CD27 and CD38 on BM CD138$^+$ PCs affirms their definitive PC phenotype, whilst only half of the blood CD138+ cells from our experiments expressed these markers. This is likely to reflect increasing PC maturity between the two compartments and hence more robust PC phenotype within the BM, although it is in contrast with other studies, which report >90% expression of CD27 on blood CD138$^+$ cells (Halliley et al. 2015).

One intriguing marker we explored is CXCR4. CXCR4 expression increases with increasing PC maturity, from 12-21% expression by blood PCs (although these studies examined only CD38$^+$ and not CD138$^+$ cells (Chihara et al. 2013; Medina et al. 2002)), to 70% in human BM LLPCs (Halliley et al. 2015). Whilst our experiments were in keeping with this trend, revealing 75% expression by blood CD138$^+$ cells and a median of 86% by BM PCs, the expression on BM PCs was wide-ranging (37-91%). This may reflect a variety of PC subsets within the BM, with more mature LLPCs upregulating CXCR4 to maintain their retention within the BM. It is also plausible that over time, the most mature LLPCs downregulate CXCR4 as they no longer

require responsiveness to CXCL12 once they become established within their niche. Indeed, these cells may be the hardest to access or disturb from their niche through BM sampling. Alternatively, low expression levels could represent peripheral blood contamination of the BM sample. Additionally, the apparent low expression of 37% could be an underrepresentation of the true expression due to receptor internalisation; whilst the sample was prepared and maintained at 4°C, it is possible that the sample temperature increased during the protracted sorting time, allowing some degree of internalisation. Furthermore, in MM, 60% of blood PCs express CXCR4 compared to only 26% of BM PCs, shown to be due to active internalisation of CXCR4 induced by CXCL12, and BM CXCL12 levels are markedly elevated compared to HCs (6571 pg/ml versus 2632 pg/ml, respectively) (Alsayed et al. 2007). These findings highlight that in PC disorders such as MM, conventional PC expression profiles may be altered. This may be true for the autoimmune encephalitides, which include abnormal production of autoantibodies by aberrant, autoreactive PCs, which may be similarly over-responsive to high levels of CXCL12. Of interest, the lowest CXCR4 expression by BM PCs (37%) was seen in patient JR051, from whom the LGI1-specific BM LLPC was isolated; the two other patients showed CXCR4 BM expression levels of 85.5% and 90.6%. Moreover, patient JR051 experienced a relapse of LGI1-autoantibody encephalitis only four weeks following the BM aspirate, suggesting that the CXCR4/CXCL12 pathway may be of pathophysiological relevance to autoimmune encephalitis. All of these considerations support the initial decision of non-inclusion of CXCR4 as a selection marker.

We found HLA-DR expression to be similar amongst blood and BM PCs (64.3% and median 86.7%, respectively). Degree of peripheral expression is comparable to others (>80-90%) (Medina et al. 2002; Halliley et al. 2015), whilst HLA-DR expression by BM PCs ranges from <10% (Halliley et al. 2015), to >70% (Medina et al. 2002). The most conflicting aspect of our finding compared to the literature is the higher HLA-DR expression within the BM compartment, which should typically be lost as PCs mature. Our BM data are drawn from n=3 subjects, and peripheral from n=1, which may explain this discrepancy.

CD45 was initially included within our PC panel to potentially facilitate removal of RBCs from BM aspirates. As it is expressed on almost all nucleated haematopoietic cells except for erythrocytes (Nakano et al. 1990), CD45 has been utilized in other flow cytometry panels

within our research group as a means of removing RBCs from heavily blood-contaminated lymph node aspirates. Whether we would be able to include this as a negative selection marker to reliably exclude only RBCs, however, was unclear, since reports suggest that some BM LLPCs may also lack CD45 expression. Indeed, approximately one third of $CD138^{+}$ BM LLPCs isolated in our experiments lacked CD45, thus when this became apparent during the first BM sort, we avoided inclusion of CD45 within the cell sorting strategy, although we opted to keep this marker within the PC panel to obtain phenotypic data. Whilst CD45 expression typically decreases with increasing PC maturity, with clear reductions in BM PCs (Medina et al. 2002), we found that CD45 expression was marginally *higher* in BM PCs than blood PCs (65.4% versus 57.9%), and markedly high in thymic PCs (>90%). The absence of clear downward trend between blood and BM is likely to be due to our data comprising only one blood sample; nonetheless, the percentage expression by BM PCs is comparable to others (~62%) (Medina et al. 2002). CD45 expression is heterogeneous in both abnormal and normal BM PCs (Pellat-Deceunynck 2004). To our knowledge, the expression of CD45 by $CD19^{+/-}$ BM PCs has not been addressed, but as it is known that BM contains $CD45^{+/-}$ PCs, and since both $CD19^{-}$ and $CD45^{-}$ BM PCs express more CD56 and proliferate less than their positive counterparts (Pellat-Deceunynck 2004; Mei et al. 2015), it seems likely that a more terminally differentiated, long-lived $CD45^{-}CD19^{-}$ PC subset exists within the BM. Taken together, these data corroborate that CD45 should not be incorporated into cell selection panels for BM-derived PCs.

Finally, we show that CD31 was expressed highly on $CD138^{+}$ BM PCs (median 82.8%), and on less than half of blood PCs. Some authors report that human BM PCs show 4-fold higher expression of CD31 than non-BM PCs (Medina et al. 2002). Whilst not included in our sorting strategy, CD31 selection may be prudent for effective isolation of functional BM LLPCs, since *double-positive* $CD138^{+}CD31^{+}$ $CD20^{-}$ cells have been defined as the *functional* antibody secreting PCs of the human BM (Martinez-Murillo et al. 2016).

5.8 Conclusions and Clinical Implications

We show, for the first time, the existence of LGI1-specific BM-derived LLPCs in patients with LGI1-autoantibody encephalitis. These proof-of-concept data suggest a role for BM-derived

LLPCs in patients with autoimmune encephalitis and similar autoantibody-mediated diseases. The coincidental temporal relationship of the BM aspirate 4 weeks prior to onset of relapse in patient JR051 perhaps more convincingly substantiates their direct contribution to pathogenesis. These data have allowed us to better characterize the cell types which produce the autoantibodies and the underlying immunobiology of autoantibody-mediated encephalitis. Furthermore, the findings provide novel insights regarding therapeutic responses of patients, with direct clinical implications. The detection of antigen-specific CD20-negative BM-derived LLPCs could explain the reasons for relapse in around one third of LGI1-autoantibody patients, despite adequate immunotherapies. Whilst traditional immunotherapies including plasma exchange, intravenous immunoglobulin, and steroids may be efficacious in reducing peripheral circulating autoantibodies, they are unlikely to eliminate autoantibodies which are continuously contributed to by BM-resident LLPCs. Moreover, some patients relapse despite potent second-line immunotherapies such as Rituximab. Rituximab, an anti-CD20 antibody which targets B cells at most stages of development, but does not target PCs, particularly mature LLPCs, since they lack CD20 (Mei et al. 2015). Indeed, $CD19^-$ BM LLPCs are robust and resistant to in vivo depletion with Rituximab (Mei et al. 2015). It is possible, therefore, that the group of patients who relapse despite Rituximab represent those in whom BM-derived LLPCs are likely a dominant source of autoantibody. This study implicates the BM as a potentially major site for long-term autoantibody production in patients with autoantibody-mediated encephalitis. Further studies of BM-derived LLPCs are warranted in this group of patients, to determine the extent of their contribution to the peripheral antibody pool, and to disease. Overall, these findings could lead to profound changes in the management of patients with autoimmune encephalitis, and other similar autoantibody-mediated neurological diseases.

CHAPTER 6. DISCUSSION

6.1 Introduction: Overview of Key Concepts

This thesis explored patients with LGI1-autoantibodies across two main strands: their clinical characteristics and their immunological phenotypes, using one of the largest international cohorts. We gained new knowledge regarding aspects of both their clinical features, and mechanisms by which autoantibodies are produced and maintained over time. Through these findings, we gleaned insights into how these immunobiological processes may contribute to disease perpetuation, and influence patient response to therapies.

6.2 Clinical Features of LGI1-Autoantibody Patients

6.2.1 Novel Clinical Characteristics

We described the clinical phenotypes and outcomes of 107 LGI1-autoantibody patients, the third largest known international cohort (Gadoth et al. 2017; Muñiz-Castrillo 2021). Whilst many of our findings replicated those from other studies, including the original descriptions (Irani et al. 2010; Irani et al. 2011; Gadoth et al. 2017; Finke et al. 2017; Thompson et al. 2017; Binks et al. 2018), we reported several novel findings. We described seizure semiologies, classifying events into 11 overall distinct seizure types, and showed that individual patients manifest up to 5 different seizure semiologies, and notably, significantly more seizure types compared to a large pharmacoresistant epilepsy cohort. This is a potentially useful distinguishing diagnostic feature, particularly in the ~40% patients without FBDS. We highlight potentially life-threatening ictal neurocardiac syndromes in 2%, and significant psychiatric manifestations of suicidal ideation and attempts in 6% – both are important associations of LGI1-autoantibody disease of which clinicians should be aware. Additionally, clinicians should be vigilant that patients with FBDS show significantly higher rates of falls compared to those without FBDS, particularly since ~25% of patients suffered falls, and more than one third sustained significant injuries. We found that sleep disorders were present in a high proportion (70%), and that 11% suffered parasomnias. Through initial observations of select patients, we were able to validate and establish the frequencies of novel clinical features on a large scale across the cohort. These included ‘complex motor behaviours’, obsessive compulsive-like behaviours, hoarding, punding, and overspending. The complex motor behaviours are likely

to represent the first report of the sleep disorder agrypnia excitata in patients with LGI1-autoantibodies, although epileptic aetiology is an alternative.

The above findings are important since firstly, they expand the clinical phenotypes of LGI1-autoantibody patients, increasing recognition and facilitating earlier diagnosis and treatment. Recent similar descriptions which expand the phenotypes include novel descriptions of paroxysmal dizzy spells (PDS) as a seizure semiology (Gadoth et al. 2017) and the association of parasomnias in patients with LGI1-autoantibodies (Blattner 2019). Secondly, the sleep disorders, obsessive compulsive, and punding behaviours provide insights into novel pathophysiological pathways and brain regions implicated in these patients. Thirdly, these data may directly influence the management of LGI1-autoantibody patients, since they suggest that antidepressants or melatonin may be important pharmacological adjuncts to immunotherapies and anti-seizure medications, and that multidisciplinary care comprising Neuropsychiatrists and Sleep Specialists is likely to be beneficial. Finally, we demonstrated that presence of either hyponatraemia or abnormal EEG at initial presentation (found in 71% and 66%, respectively) significantly associated with worse clinical severity at nadir. This highlights the utility of routine investigations, which could aid decisions for initiation of aggressive immunotherapy regimes in select patients.

6.2.2 Relapse Patterns and Potential Triggers: Immunobiological Insights

We showed that relapses were common, occurring in 36%, most often precipitated by prednisolone weaning or cessation (79%). This is important for clinician awareness and highlights the need for standardised treatment protocols for this group of patients. The apparent steroid-dependency is unlikely to fully explain relapses, since 90% of the cohort received oral prednisolone, and were subjected to weaning and/ or cessation, yet only one third relapsed. Moreover, 3% of relapses occurred in immunotherapy-naïve patients. Rather, other patient factors are likely to predispose to relapse, as suggested by the finding that 21% of relapsing patients showed *multiple* relapses (in some cases up to 3), an observation also reported by others (Gadoth et al. 2017). The relapsing patients may provide insights to a subgroup of patients with underlying disease pathogenesis distinct to that of non-relapsing patients. One plausible explanation could be a more sustained GC B cell-driven

immunobiology in relapsing versus non-relapsing patients. Or a role for LLPCs in more refractory patients. These hypotheses indicate that differing mechanisms of underlying immunopathogenesis and autoantibody production may exist even within the same autoantibody disease, hence providing one possible explanation for a subset of patients who relapse (often multiply), and/ or are more dependent on certain B cell-targeting therapies.

We showed evidence for a potential role for several triggers to LGI1-autoantibody encephalitis, in particular prodromal infections and vaccinations. Whilst one major limitation of our study was a lack of control group, the close temporal relationships of the infections and vaccinations with disease onset was highly suggestive of their relevance to disease pathogenesis. We propose a mechanism of polyclonal immune activation in response to any form of immune challenge (infection, vaccination, or significant stress) in susceptible patients with genetic risk factors. Whilst the role of specific organisms remains unknown in LGI1-autoantibody encephalitis, we highlighted potential roles of herpes viruses including HSV1 and VZV, possibly triggering neuronal surface antibody production secondary to viral-induced neuronal damage. Additionally, the simultaneous onset of LGI1-autoantibody encephalitis alongside other autoimmune disorders in a small proportion suggests a widespread loss of immune tolerance, particularly given the shared HLA associations amongst the conditions (e.g. IgG4-related disease; (Huijbers et al. 2015). LGI1-autoantibody syndromes are not considered to be paraneoplastic (Irani et al. 2010). Whilst 13% of our cohort had tumours, they did not classically associate with certain tumours such as ovarian teratomata in NMDAR-autoantibody encephalitis (Dalmau et al. 2007) and thymomas in CASPR2-autoantibody syndromes (Irani et al. 2012). Finally, we propose a role for altered LGI1 expression in the immunopathogenesis of LGI1-autoantibody encephalitis. Since there is evidence for LGI1 as a tumour suppressor gene due to its downregulation or lack of expression in brain tumours (Chernova et al. 1998), we hypothesise that its *overexpression* results in manifestation of autoimmunity with LGI1-autoantibody encephalitis as a protective mechanism against primary brain tumours in a group of typically elderly males who would have otherwise developed glioblastoma multiforme. Overall, we demonstrate evidence for several potential triggers of LGI1-autoantibody encephalitis, which, together with relapse patterns, inform some aspects of the immunobiology of these patients.

6.2.3 Patient Outcomes

Overall, patients showed good outcomes, with significant improvements in both cognitive and functional disability scores at latest follow-up compared to nadir, and median self-reported 'percentage back to normal' ratings of 85%. Median MRS scores were 1 at latest follow-up in our study and another (Muñiz-Castrillo 2021). Additionally, 85% of patients maintained seizure freedom at the time of latest follow-up. However, despite these improvements, many patients had persistent cognitive impairment and fatigue which impacted their quality of life. Correspondingly, several studies report severe, persistent memory deficits with marked hippocampal atrophy or mesial temporal sclerosis in up to half of patients (Finke et al. 2017; Miller et al. 2017; van Sonderen et al. 2016). These findings confirm that physical disability is typically minimal in this group of patients, yet they continue to be affected by significant cognitive deficits. Thus, whilst MRS is widely used as an outcome measure for many neurological conditions, it does not reflect the residual impairments seen in LGI1-autoantibody encephalitis, which are chiefly related to cognition and fatigue. There is paucity of studies which compare equivalent outcome measures across centres, partly due to the rarity of the condition. We highlight the need for harmonised outcome measures in LGI1-autoantibody encephalitis, which should focus on standardised cognitive, mood and fatigue scores, with less emphasis on functional disability scales such as MRS.

Additionally, several studies evidence that earlier diagnosis and treatment lead to improved outcomes in LGI1-autoantibody encephalitis (Thompson et al. 2017; Miller et al. 2017; Finke et al. 2017). However, no clear consensus treatment guidelines exist for this condition. Importantly, we found a 5% fatality rate over a follow-up period of 0-15 years (median 41 months); none known to be directly related to disease or immunotherapy. Others however report fatality rates of 19%, with a proportion of deaths directly related to steroid treatment (van Sonderen et al. 2016). This further underscores the need for standardised treatment guidelines for this vulnerable group of patients.

6.3 Evidence for a Dominant Role of GC Contribution to Autoantibody Production

We used IgMs, the LGI1 epitopes LRR and EPTP, and CXCL13 as surrogate markers for GC reactions. We found evidence both for and against a dominant role for GC contribution to autoantibody production, summarized below and in **Table 6.1**.

Evidence supporting a dominant role of GCs:

1. **Patient LGI1 autoantibodies are polyclonal, directed towards both EPTP- and LRR-domains in comparable proportions throughout disease duration.** In almost all patients, autoantibodies with reactivity to both EPTP- and LRR-LGI1 domains were detected in equal proportions throughout disease. This autoantibody polyclonality and possible epitope spread suggests ongoing dendritic cell presentation of both LGI1 antigens (EPTP and LRR) to B cells within GCs throughout the course of the disease, leading to the sustained production of both EPTP- and LRR-directed LGI1 autoantibodies.
2. **LGI1-autoantibody patients show markedly elevated CXCL13 levels.** Serum CXCL13 levels were significantly higher in patients with LGI1 autoantibodies compared to controls and CASPR2-autoantibody patients. LGI1-autoantibody patients had elevated serum CXCL13 levels throughout the course of disease, and very high serum CXCL13 levels were detected at disease onset, with sharp rises during relapses, suggesting ongoing GC activity throughout disease in LGI1-autoantibody syndromes.
3. **LGI1 IgMs are present in some patients.** LGI1-IgMs were detected in 17% of patients. Almost all (88%) IgMs were from relapsing patients, and the remainder from acute patients, within one month of disease onset. This suggests that IgM production from GC reactions is enhanced during heightened disease activity such as acute illness onset or relapse. LGI1 IgMs significantly associated with relapsing patients, suggesting that detection of LGI1-IgM may be a marker of relapse.
4. **CASPR2 IgMs are detectable for long disease durations.** In the subgroup of patients with CASPR2 IgMs (16%), IgMs were detectable for as long as 2551 days (7 years) post disease onset, with a median time of 321 days (10.5 months). Akin to NMDAR-antibody encephalitis (Makuch et al 2018), this suggests that ongoing GC reactions

may be responsible for persistent autoantibody production in some patients with CASPR2-autoantibody syndromes.

Evidence against a dominant role of GCs:

1. **CXCL13 levels are not elevated in CASPR2-autoantibody patients.** Only 27% of samples showed elevated serum CXCL13 levels. No dynamic change was observed longitudinally, even in the presence of relapse.
2. **LGI1- and CASPR2-IgMs are found in only few patients.** LGI1-IgMs were present in only 17% of LGI1-autoantibody patients, and CASPR2-IgMs in only 16% of patients with CASPR2-autoantibodies.
3. **Identification of bone marrow-derived LGI1-specific LLPCs.** The detection of LGI1-autoantibodies in BM-derived LLPC cultures from a patient with LGI1-autoantibody encephalitis implicates the BM as a major alternative source of long-term autoantibody in these patients.

Table 6.1 Evidence for Germinal Centre B Cell Contribution to Autoantibody Production in LGI1- and CASPR2-Autoantibody Diseases

Study	LGI1-Autoantibody Patients	CASPR2-Autoantibody Patients
LGI1 Epitopes	**Strong:** 37/38 (97%) patients had LGI1-autoantibodies to both EPTP- and LRR- epitopes. 97/100 (97%) sera bound both EPTP and LRR, with tight correlation in end-titres (r 0.9257; p<0.0001****) across disease course.	N/A
IgM	**Some:** LGI1-IgMs detected in 5/30 (17%) patients (8/70; 11% samples). LGI1 IgMs significantly associated with relapsing patients.	**Some:** CASPR2-IgMs detected in 4/25 (16%) patients (7/51; 14% samples) with median time from onset 321 days.
CXCL13	**Strong:** Significantly elevated serum CXCL13 levels throughout disease, with rises during relapses.	**Weak:** Serum CXCL13 levels positive in only 17/63 (27%) samples with

	Serum CXCL13 levels positive in 88/161 (55%) samples.	no dynamic longitudinal changes.
BM-Derived LLPC Cultures	**Weak:** LGI1 autoantibodies detected in culture supernatants of direct ex-vivo BM-derived LLPCs from a patient with LGI1-autoantibody encephalitis (frequency ~1:5000).	N/A

Thesis project results summarised and categorised as either 'strong', 'weak' or 'some' evidence supporting a significant contribution of GC activity to autoantibody production. LGI1 IgM positivity rate: by CBA with IgG-depletion. CASPR2 IgM rate: positivity by both live optimized CBA and FACS.

6.4 Evidence for Bone Marrow-Derived Long-Lived Plasma Cell Contribution to LGI1-Autoantibody Production

6.4.1 Detection of LGI1-Specific Bone Marrow-Derived Long-Lived Plasma Cells: Potential Significance

Overall, the above findings suggest that sustained, ongoing GC-reactions may act as a dominant mechanism of persistent autoantibody production in some patients with LGI1- and CASPR2-autoantibodies. However, most CASPR2-autoantibody patients lacked elevated serum CXCL13 levels, and LGI1- or CASPR2-IgMs were infrequent (<20%), suggesting that in the majority, other mechanisms of autoantibody production operate and predominate. This warranted the exploration of the BM compartment and the role of LLPCs.

We showed, for the first time, the presence of LGI1-autoantibodies from ex-vivo cultures of BM-derived LLPCs from a patient with LGI1-autoantibody encephalitis. LGI1-specific LLPCs were detected in one of two patients sampled, although their rarity (determined frequency ~1:5000 of plated BM-LLPCs) suggests that the possibility of their existence in the second patient is not excluded. To our knowledge, this is the first study to directly isolate and culture antigen-specific LLPCs from the BM compartment of patients with neurological autoantibody-mediated conditions. Whilst the BM has been sampled in MG (Fujii et al. 1986) and GAD-autoantibody encephalitis (Thaler et al. 2019) previously, both studies employed bulk culture of BM mononuclear cells cultures rather than isolated PCs. In both cases, autoantibody

secretion could have arisen either from B cells which differentiated into antibody-secreting PCs in culture, or from peripheral B cell contamination of the BM sample. As outlined in previous chapters, BM PC studies are rare, and human studies are few, typically in the context of haematological malignancies, or immune responses against vaccine or infectious antigens, with little data on autoimmune conditions and examination of autoreactive BM PCs. Whilst the current COVID-19 pandemic has drawn some attraction to the BM compartment (Turner et al. 2021), ex-vivo cultures of BM-derived LLPCs remain rare.

Whilst our study is limited by small patient numbers, it provides proof-of-concept data for the existence of LGI1-specific BM-derived LLPCs, and substantiates the likely contribution of the BM compartment and LLPCs to the peripheral autoantibody pool and disease perpetuation in LGI1-autoantibody mediated syndromes. The onset of relapse in patient JR051 four weeks following BM aspiration perhaps more convincingly substantiates their direct contribution to pathogenesis. The findings likely provide a paradigm for mechanisms of long-term autoantibody production in other similar autoantibody diseases.

6.4.2 Clinical Implications and PC-Targeted Therapies

Our findings are important for several reasons. Knowledge gleaned regarding which immune cells are responsible for autoantibody production, their cell surface marker characteristics, and the immune compartments within which they reside will enhance our understanding of several aspects of these autoantibody-mediated syndromes. These include not only the underlying immunopathogenesis of the condition, but also prediction of treatment response, including specific immunotherapies to which patients may respond, and the optimal method of delivery.

The detection of antigen-specific CD20-negative BM-derived LLPCs could partially explain the relapses seen in around one third of LGI1-autoantibody patients despite seemingly adequate immunotherapies. Traditional management of these patients includes rapidly effective treatments such as plasma exchange, intravenous immunoglobulin, and high-dose intravenous steroids, followed by a long-term oral steroid regime. Whilst potent steroids and plasma exchange provide temporary reduction of circulating autoantibodies, these agents

would likely fail to eliminate the peripheral pool of autoantibodies if they are continuously contributed to by BM-resident LLPCs. This is a plausible explanation for the relapses observed during prednisolone weaning or shortly following discontinuation – factors which associated with ~80% of relapses in our cohort. Moreover, some patients relapse despite potent second-line immunotherapies such as Rituximab. Rituximab is an anti-CD20 antibody which targets B cells at most stages of development, but does not target PCs, particularly mature LLPCs, since they lack CD20 surface expression (Mei et al. 2015). Indeed, $CD19^-$ BM LLPCs are robust and resistant to in vivo depletion with Rituximab (Mei et al. 2015), and tetanus-specific IgG-secreting LLPCs have been shown to persist in primate BM for 10 years despite memory B-cell depletion with Rituximab (Hammarlund et al. 2017). Correspondingly, Thaler et al report a patient with GAD-antibody encephalitis who showed high serum GAD-antibody levels despite B-cell depletion with Rituximab and azathioprine (Thaler et al. 2019). Thus, whilst Rituximab has been shown to be beneficial for select LGI1-autoantibody encephalitis patients (Brown et al. 2014), it may not be efficacious for all. In fact, Rituximab and other B-cell targeting therapies may even promote PC populations and further drive disease. In immune thrombocytopenia, splenic LLPCs were detected in Rituximab-treated patients, indicating that like BM LLPCs, splenic LLPCs are resistant to Rituximab (Mahévas et al. 2013). Intriguingly, splenic LLPCs were not found in untreated patients. This suggests that B cell depletion may paradoxically *promote* the repopulation of pathogenic antibody-secreting LLPCs within various tissue niches – including the BM. Some postulate that increased serum BAFF levels observed post Rituximab drive repopulation of autoreactive PCs (Pranzatelli et al. 2013). Further, LLPCs are resistant to apoptosis by cyclophosphamide since they are non-proliferative (Cupps et al. 1982). These observations could underpin potential mechanisms for disease perpetuation and the clinical relapses observed in our subgroup of patients who relapsed despite potent immunotherapies including cyclophosphamide, mycophenolate, and azathioprine.

The potential contribution of BM-LLPCs to autoantibody production supports a need for both PC-targeting and BM-penetrating immunotherapies in our patients. Many PC therapies are available, with widespread use in the PC malignancy multiple myeloma. Bortezomib, a proteasome inhibitor, is efficacious in reducing both $CD19^+$ and $CD19^-$ BM-derived human

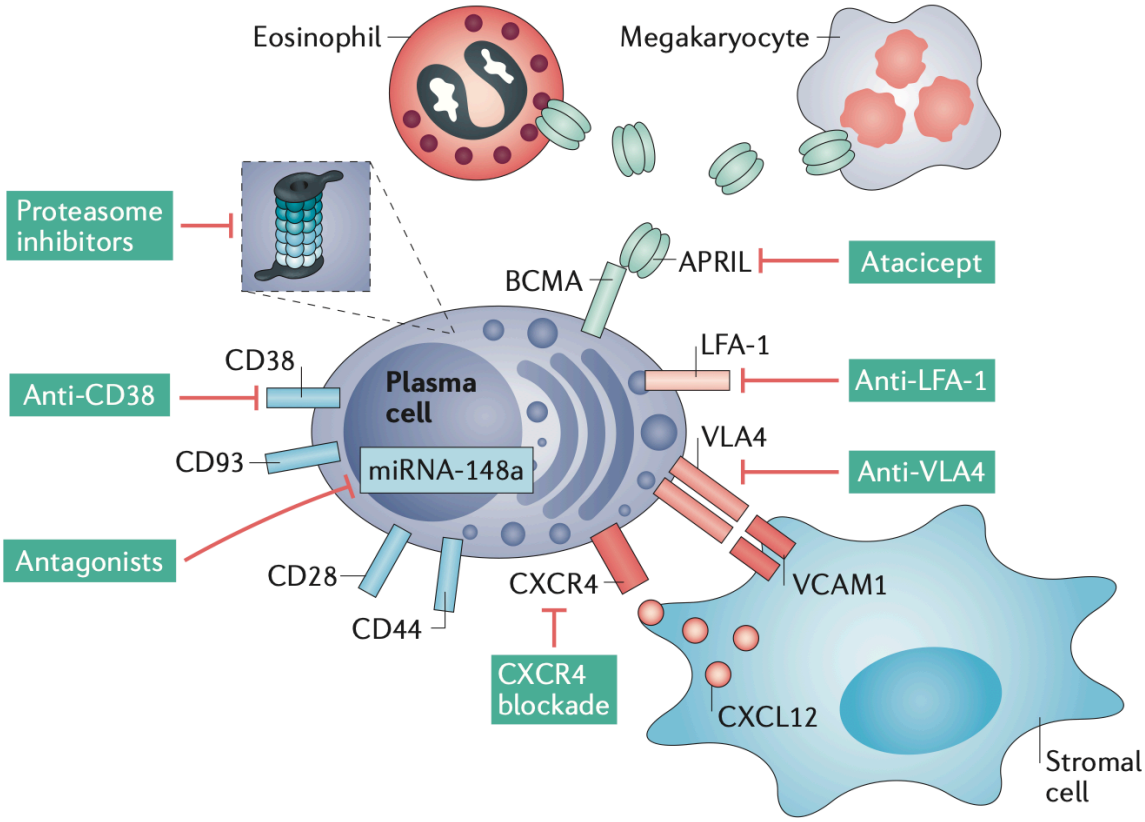
LLPCs both in vivo and in vitro (Mei et al. 2015). Additionally, Bortezomib has been shown to be beneficial in NMDA-receptor antibody encephalitis, including achieving disease remission (Scheibe et al. 2017; Sveinsson et al. 2017; Schroeder et al. 2018; Keddie et al. 2018). However, other small series show limited effect of Bortezomib (Shin et al. 2018), which may be attributable to its dominant peripheral response and poor CNS penetrance highlighted by some authors (Rüegg & Irani 2017). Therefore, whether PC depletion in the blood and BM is sufficient to reduce secondary CNS effects remains to be elucidated. Tocilizumab, a humanized IL-6R antagonist which acts directly on PCs, has shown benefit in NMO with striking reductions in annual relapse rates and reduction in PBs and circulating antibody titres in otherwise treatment-refractory patients, including B cell-depleting anti-C20 therapies (Ayzenberg et al. 2013; Araki et al. 2013; Araki et al. 2014; Ringelstein et al. 2015). The clinical utility of Tocilizumab has also been reported in new-onset refractory status epilepticus (NORSE), an explosive autoimmune disorder where serum and CSF IL-6 levels are markedly elevated compared to controls (Jun et al. 2018). These studies suggest that PC-directed therapies may be useful in LGI1-autoantibody encephalitis and other autoantibody-mediated diseases.

6.4.3 Novel PC-Therapeutic Targets

Many therapeutic targets of BM PCs exist **(Figure 6.1)**, which have led to the development of several novel drugs, such as antibodies against CXCR4 and CD38 (Hiepe & Radbruch 2016). In mice, CXCR4 blockade prevents migration of PCs to the BM (Hiepe & Radbruch 2016). If such therapies were administered early, they could theoretically aid prevention of the establishment of antigen-specific LLPCs within the BM, which are difficult to target and eliminate once established within their niche. Daratumumab, a monoclonal antibody targeting CD38, is currently used for relapsed, refractory MM, with substantial reductions of BM PCs (Hiepe & Radbruch 2016; Cho et al. 2018). Use of Daratumumab however is limited by the expression of CD38 on many normal tissues and cells. BCMA-directed therapies may minimise non-targeted effects, since BCMA is expressed almost exclusively by healthy and malignant PCs (Cho et al. 2018). However, this may not eliminate risk of reduced immunity and infection, or even death, which are major reported adverse effects of global PC-depletion with Bortezomib and Tocilizumab (Hiepe & Radbruch 2016; Jun et al. 2018; Shin et al. 2018). BAFF and APRIL blockade with newer agents such as Atacicept is an attractive therapeutic

strategy (Hiepe & Radbruch 2016). Interestingly, dual BAFF and APRIL blockade in mice depletes BM-derived PCs, but not memory B cells, suggesting that this is a safer way to deplete specific cell subsets such as LLPCs without removing an individual's immunological memory to previous pathogens (Benson et al. 2008). More drastic options to eliminate LLPCs and PC memory include immunoablation with cyclophosphamide and anti-thymocyte globulin (ATG) followed by stem cell transplantation, currently used for refractory autoimmune disorders including MS (Hoyer et al. 2008; Hiepe & Radbruch 2016). T cells may also be important therapeutic targets for PC-mediated disorders, since CD4+ 'helper' T cells may promote differentiation of PCs via IL-21 (Rao et al. 2017). Various therapeutic targets of PCs are summarised in **Figure 6.1**.

Figure 6.1 Potential Therapeutic Targets of PCs Within the BM



Schematic taken from Hiepe et al (Hiepe & Radbruch 2016). Proteasome inhibitors such as Bortezomib cause PC apoptosis and are widely used in haematological malignancies and autoimmune disorders such as SLE. Anti-CD38 monoclonal antibodies such as Daratumumab are effective agents in MM. CXCR4 blockade may inhibit PC homing to the BM via CXCR4-CXCL12 interactions. Atacicept blocks both BAFF and APRIL, inhibiting their interaction with BCMA on the PC surface, which is essential for PC survival. Silencing miRNA-148a, or targeting adhesion molecules such as VLA4 and CD44 which aid PC survival within the BM, may be effective therapeutic strategies to deplete PCs. Other cells in the BM microenvironment such as eosinophils play key roles BM PC survival, and may be alternative therapeutic targets for depletion of BM PCs.

6.5 Conclusions and Future Studies

Herein, we have expanded the clinical and immunological characteristics of patients with LGI1-autoantibody syndromes. We have described novel clinical features, several potential triggers, relapse patterns, and clinical outcomes. These descriptions should increase recognition of these rare syndromes, with ultimate aim of earlier diagnosis and treatment, and thus improved outcomes. Awareness of the novel clinical features, for example, may be particularly important for clinicians to aid diagnosis in the 43% of patients who lack the characteristic FBDS. From an immunological perspective, we have shown evidence for roles of both GC reactions and BM-derived LLPCs in the pathogenesis of LGI1-autoantibody encephalitis. A unique finding of this thesis was the detection of LGI1-specific BM-derived LLPCs in patients, implicating the BM as a potentially major site for long-term autoantibody production in autoimmune encephalitis. We conclude that BM-derived LLPCs likely directly contribute to the peripheral autoantibody pool and to ongoing disease perpetuation in LGI1-autoantibody syndromes. The production of LGI1-IgG by BM LLPCs indicates that targeting GC B cells alone would not be a sufficient therapeutic target, and that future treatments in LGI1-autoantibody encephalitis may need to address both peripheral, BM, and even CNS B cells and PCs. Future work should include clinical trials of PC-directed therapies such as Bortezomib, which may be beneficial in some patients. Clinical efficacy, cost-effectiveness, and safety profiles should be determined.

Further studies of BM-derived LLPCs are warranted in this group of patients, to determine the extent of their contribution to the peripheral antibody pool, and to disease. Additionally, the antigenic epitope targets of BM-derived LLPCs remain to be explored. We have developed a novel culture platform for the isolation of BM LLPCs, their ex-vivo maintenance in culture, and detection of secreted LGI1-IgG using our live CBA system. These techniques should be applied to future work. Sequencing of the antigen-specific LLPCs to produce monoclonal antibodies, with direct comparison of their effects between peripheral, CSF, BM, and lymph node compartments would be important. Development of monoclonal antibodies could yield key targeted therapies, and should be a chief future research focus, since it is desirable to selectively eliminate autoreactive PCs, whilst preserving protective PCs – this is a major unmet research need. Comparison of the characteristics of BM LLPCs from a wider group of patients

should include antibody status (LGI1- versus CASPR2-autoantibody or both), disease phase (acute versus chronic or relapsing disease), tumour (those with and without underlying tumour), and treatment status (treatment-naïve versus those treated with various long-term immunotherapies). Investigation of these factors and their influence on BM-LLPCs would allow better understanding of the immunobiology, which has yet to be fully defined, although this is likely to be patient-specific. It is possible that even within this one condition, the immunological phenotypes may show heterogeneity. For instance, patients with LGI1-autoantibodies may be subdivided into those with a predominant GC B-cell driven disease, those with a predominant LLPC-driven phenotype, and potentially a third subtype of patients who possess a 'combination phenotype'. Our data support the concept that dual mechanisms of autoantibody production exist within LGI1-autoantibody encephalitis, with ongoing short-lived GC reactions, and parallel, long-term autoantibody production by BM-derived LLPCs, which lie 'hidden' within their BM niche and are resistant to conventional immunotherapies. Future studies should aim to elucidate methods of stratifying patients into 'immunological subcategories' as early as possible following presentation, to define whether an individual is likely to respond superiorly to B-cell or PC-targeting treatments, and thus better tailor immunotherapies. A tailored future approach could include patient stratification based upon measurement of serum LGI1-IgM, LGI1-IgG, CXCL13 and sodium levels, radiological and EEG features, and even BM analysis for presence of antigen-specific BM-derived LLPCs.

These data advance our understanding of LGI1-autoantibody syndromes, and have allowed us to better characterize the cell types which produce the autoantibodies, and delineate mechanisms behind long-term autoantibody production and maintenance in autoantibody-mediated encephalitis. The validations shown herein provide insights into therapeutic responses, support a need to incorporate PC-targeting treatments for this patient group, and will help to inform novel future therapeutic strategies. Our study may have therapeutic implications, with alterations to the traditional management of patients with autoimmune encephalitis, and other similar autoantibody-mediated neurological diseases. Finally, by expanding the clinical phenotypes of this relatively new disease entity, we anticipate increased clinical recognition of these rare disorders, consequential expedited immunotherapies, and ultimately improved outcomes in these patients.

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Wutte, N. et al., 2011. CXCL13 chemokine in pediatric and adult neuroborreliosis. *Acta neurologica Scandinavica*, 124(5), pp.321–328.

Xiang, Z. et al., 2007. FcgammaRIIb controls bone marrow plasma cell persistence and apoptosis. *Nature Publishing Group*, 8(4), pp.419–429.

Yamagata, A. et al., 2018. Structural basis of epilepsy-related ligand-receptor complex LGI1-ADAM22. *Nature Communications*, 9(1), pp.1546–13.

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Zhong, X. et al., 2011. Cerebrospinal fluid levels of CXCL13 are elevated in neuromyelitis optica. *Journal of Neuroimmunology*, 240-241(C), pp.104–108.

APPENDIX

Appendix 5.1 IRAS Notice of Substantial Amendment Form (Submitted 29/05/2018)

Notice of Amendment IRAS Version 5.8.0

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please complete the questions in order. If you change the response to a question, please select 'Save' and review all the questions as your change may have affected subsequent questions.

Please enter a short title for this project (maximum 70 characters)
Immune Factors in Neurological Diseases v1

1. Is your project research?

◉ Yes ○ No

2. Select one category from the list below:

○ Clinical trial of an investigational medicinal product
○ Clinical investigation or other study of a medical device
○ Combined trial of an investigational medicinal product and an investigational medical device
○ Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
◉ Basic science study involving procedures with human participants
○ Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
○ Study involving qualitative methods only
○ Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
○ Study limited to working with data (specific project only)
○ Research tissue bank
○ Research database

If your work does not fit any of these categories, select the option below:

○ Other study

2a. Will the study involve the use of any medical device without a CE Mark, or a CE marked device which has been modified or will be used outside its intended purposes?

○ Yes ◉ No

2b. Please answer the following question(s):

a) Does the study involve the use of any ionising radiation?	○ Yes	◉ No
b) Will you be taking new human tissue samples (or other human biological samples)?	◉ Yes	○ No
c) Will you be using existing human tissue samples (or other human biological samples)?	◉ Yes	○ No

1 195152/1214475/13/44/77751

d) Will the study involve any other clinical procedures with participants (e.g. MRI, ultrasound, physical examination)? (●) Yes ( ) No

3. In which countries of the UK will the research sites be located? *(Tick all that apply)*

- [x] England
- [ ] Scotland
- [ ] Wales
- [ ] Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

- (●) England
- ( ) Scotland
- ( ) Wales
- ( ) Northern Ireland
- ( ) This study does not involve the NHS

4. Which applications do you require?

IMPORTANT: If your project is taking place in the NHS and is led from England select 'IRAS Form'. If your project is led from Northern Ireland, Scotland or Wales select 'NHS/HSC Research and Development Offices' and/or relevant Research Ethics Committee applications, as appropriate.

- [ ] IRAS Form
- [x] NHS/HSC Research and Development offices
- [ ] Social Care Research Ethics Committee
- [x] Research Ethics Committee
- [ ] Confidentiality Advisory Group (CAG)
- [ ] Her Majesty's Prison and Probation Service (HMPPS)

For NHS/HSC R&D Offices in Northern Ireland, Scotland and Wales the CI must create NHS/HSC Site Specific Information forms, for each site, in addition to the study wide forms, and transfer them to the PIs or local collaborators.

For participating NHS organisations in England different arrangements apply for the provision of site specific information. Refer to IRAS Help for more information.

5. Will any research sites in this study be NHS organisations?

(●) Yes ( ) No

5a. Are all the research costs and infrastructure costs (funding for the support and facilities needed to carry out research e.g. NHS Support costs) for this study provided by a NIHR Biomedical Research Centre, NIHR Biomedical Research Unit, NIHR Collaboration for Leadership in Health Research and Care (CLAHRC), NIHR Patient Safety Translational Research Centre or a Diagnostic Evidence Co-operative in all study sites?

Please see information button for further details.

( ) Yes (●) No

Please see information button for further details.

5b. Do you wish to make an application for the study to be considered for NIHR Clinical Research Network (CRN)

Support and inclusion in the NIHR Clinical Research Network Portfolio?

Please see information button for further details.

◉ Yes ○ No

The NIHR Clinical Research Network provides researchers with the practical support they need to make clinical studies happen in the NHS e.g. by providing access to the people and facilities needed to carry out research "on the ground".

If you select yes to this question, you must complete a NIHR Clinical Research Network (CRN) Portfolio Application Form (PAF) immediately after completing this project filter question and before submitting other applications. Failing to complete the PAF ahead of other applications e.g. HRA Approval, may mean that you will be unable to access NIHR CRN Support for your study.

6. Do you plan to include any participants who are children?

◉ Yes ○ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

◉ Yes ○ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the Confidentiality Advisory Group to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

◉ Yes ○ No

Please describe briefly the involvement of the student(s):
Medically qualified DPhil students will be involved in the assessments for some of the patients and in the laboratory assays as part of their degree.

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

◉ Yes ○ No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

○ Yes ◉ No

11. Will identifiable patient data be accessed outside the care team without prior consent at any stage of the project (including identification of potential participants)?

○ Yes ◉ No

NOTICE OF SUBSTANTIAL AMENDMENT

Please use this form to notify the main REC of substantial amendments to all research other than clinical trials of investigational medicinal products (CTIMPs).
The form should be completed by the Chief Investigator using language comprehensible to a lay person.

Details of Chief Investigator:

Title	Forename/Initials	Surname
Dr	Sarosh	Irani

Work Address	Level 6, West Wing, Nuffield Department of Clinical Neurosciences, John Radcliffe Hospital, Oxford
PostCode	OX3 9DS
Email	sarosh.irani@ndcn.ox.ac.uk
Telephone	01865234531
Fax	

For guidance on this section of the form refer to the guidance

Full title of study:	Immune Factors in Neurological Diseases
Lead sponsor:	
Name of REC:	YORKSHIRE AND THE HUMBER / LEEDS EAST
REC reference number:	16/YH/0013

International Standard Randomised Controlled Trial Number (ISRCTN):	
ClinicalTrials.gov Identifier (NCT number):	

Additional reference number(s):

Ref.Number Description	Reference Number

Name of lead R&D office:	Oxford University Hospitals NHS Foundation Trust
Date study commenced:	13/04/2016
Protocol reference (if applicable), current version and date:	3 19/01/2017

Amendment number and date:	2 29/05/2018

Type of amendment

(a) Amendment to information previously given in IRAS

◉ Yes ○ No

If yes, please refer to relevant sections of IRAS in the "summary of changes" below.

(b) Amendment to the protocol

◉ Yes ○ No

If yes, please submit either the revised protocol with a new version number and date, highlighting changes in bold, or a document listing the changes and giving both the previous and revised text.

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

◉ Yes ○ No

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.

Is this a modified version of an amendment previously notified and not approved?

○ Yes ◉ No

Summary of changes

Briefly summarise the main changes proposed in this amendment. Explain the purpose of the changes and their significance for the study.
If this is a modified amendment, please explain how the modifications address the concerns raised previously by the ethics committee.
If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.

1. Removal and addition of investigators

- A number of additional individuals have been added to the project as investigators, since they are new group members:
Drs Isabel Leite, Sophia Michael, Bettina Balint, Valentina Damato, and Adam Handel; Miss Archana Ramesh; Ms Rhea Zambellas

- Prof Bethan Lang and Dr Jenny Taylor have been removed from the project investigator list, since they are no longer group members.

2. Amendment of current investigators' titles, updated CTRG telephone number, and corrected page number

- Titles for Drs Irani and Sen have been updated to 'AProf' within the Protocol investigator list.
- The CTRG telephone number has been updated within the Participant Information Sheet, so that participants may have accurate contact information.
- The page number within the footer of the Consultee Information Sheet has been corrected from '1 of 4' to read '1'.

3. Inclusion of use of in vitro and in vivo models to study the effects of patient immune factors.
- We propose that patient samples may be used for in vitro and in vivo studies, including cell culture and in vivo experimental animal models, which will allow characterization of the immunological factors to better understand disease biology.
- These proposals have been outlined within the Protocol 19/01/2017 ('Synopsis', 'Objectives' and 'Amendment History, Appendix C'). The Participant Information Sheet 'what will happen to the samples I give?' section has been updated, and an additional box has been added to the Patient, Consultee, and Parental Consent Forms for sample

options 'to be used to study the effects on cellular models and in live organisms'.

4. Adjustment of planned sample size to account for anticipated increased number of samples
- Larger sample sizes are anticipated, considering the emerging availability and uptake of antibody tests, leading to increased diagnosis and therefore identification of relevant selected patients. The ability to study cohorts would be enhanced by larger sample sizes.
- Amendments have been made to the Protocol under 'Synopsis' 1., 'Study Participants' 6.1, 'Recruitment' 7.1 and 'Number of Participants' 10.2, to further outline a breakdown of our proposed sample numbers.

5. Amended specificity of lymph node sample site from 'submental' to 'cervical'
- Based upon our increasing experience with this procedure along with our radiology colleagues, it is thought that other cervical nodes are more easily accessible and without increased risk.
- This proposed sampling site change has been updated within the Protocol 'Samples' section, including Table 1., Participant Information Sheet 'Ultrasound-guided FNA' section, and Participant Consent Form 'Optional' section.

6. Addition of bone marrow aspiration and stool collection
- 6.1 Bone marrow aspiration – we would like to include the option for participants to donate cells from the bone marrow for research purposes. Sampling of the bone marrow compartment would be a natural extension of our work to date, which has studied immune cells contained within the blood and lymph nodes, and improved our understanding of cells in the circulation and lymph nodes which make autoantibodies. Bone marrow studies will determine whether these cells are present in the bone marrow, a potential niche for cells which make antibodies, and will have implications on identifying the cell types and their naïve precursors which produce antibodies, and future targeted therapies. Indeed, our recent Public and Patient Engagement day in November 2017 (Corpus Christi College, Oxford), provided patient and relative feedback which suggested that the main unmet research need is disease causality, which has guided the development of our next research questions.
- An experienced Haematologist would perform the procedure, which is done routinely in Haematology practice, and has few major risks. The bone marrow aspirate would be taken from the hip bone, under local anaesthetic. The most notable risk is of major haemorrhage, which is extremely rare, occurring at a rate of 1 in 1000 cases. Patients will be advised to be familiar with their aftercare leaflet, and in the instance of any noted bleeding through their dressing site, to apply pressure and seek medical attention immediately. This procedure would be offered to patients only (not to healthy volunteers, a proposed new control group – see below), and only to patients adjudged to have capacity to consent.
- A new 'Bone Marrow Aspiration' section has been created within the Participant Information Sheet, including disadvantages and risks, and the box 'I agree to potential bone marrow aspiration' has been added to the Participant Consent Form under the 'Optional' section. The Protocol has been updated to include complications under 'Safety Reporting' 9., and the procedure is included within 'Analysis of Outcome Measures' 10.3. An Appendix D has been created within the Protocol detailing the proposed patient information for the procedure.

- 6.2 Stool collection would allow study of the gut microbiome and its effect on both T and B cells on disease causality. The gut microbiome has been shown to influence many major diseases.
- Amendments have been made to the Participant, Non-Autoimmune, and Parental Information Sheets, to include stool sampling. The option for stool sample donation has been included within the Patient Consent Form, Consultee Consent Form, Parental Consent form, and Non-Autoimmune Participant Consent Form (new document, see below).

- 6.3 - For proposed bone marrow and stool sampling additions, amendments have been made to the Protocol ('Synopsis' 1. Primary and Secondary Outcome Measures, 'Objectives and Outcome Measures' 4., Study Procedures – 'Study Assessment' 7.3, and 'Sample Handling' 7.5, including Table 1.).

7. Recruitment of healthy controls
- Expansion of our control groups to allow recruitment of healthy volunteers would allow acquisition of a large number of appropriately age- and sex-matched healthy volunteers and relatives to allow comparative data with patients. This would be particularly important, considering our anticipated increasing patient numbers. Our proposed healthy volunteer groups would include healthy patient spouses, relatives (e.g. siblings), or friends who may have lived in close contact with patients. Such volunteers would serve to act not only as healthy controls, but potentially additionally as 'environmental controls', and 'age and sex-matched controls', thus facilitating more powerful comparative data such as potential influence of environmental factors on these diseases. Control participants would be eligible for blood, stool, saliva, urine and lymph node aspiration sampling, but not CSF or bone marrow aspiration sampling.
- New information sheets and consent forms for 'Non-autoimmune participants' have been produced.
- Amendments have been made to the Protocol, under 'Synopsis', where 'study participants' has been updated to include controls, and 'planned sample size' and 'Inclusion Criteria' further define healthy volunteers to include environmental controls. 'Recruitment' 7.1 has been updated, and within 'Study Assessment' 7.3, the interview/ questionnaire times have been increased to maximum 90 minutes, where longer interviews may allow our more accurate study and understanding of certain domains, such as environmental factors.

8. Re-consent of selected participants already recruited for new changes
- We will approach selected patients, currently participating in the study, that may be suitable to help with the new additions (animal models and bone marrow aspirates). They will be re-consented as per the current informed consent pathways. The new consent forms will be stored alongside the original consent forms.

9. Updates to child and parental documents
- We thought it was suitable to add the following statement to the parental and older child information sheets: "You may also choose to make your samples available for transfer into cell and animal models." We have reflected this in the parental consent form also. We do not intend to obtain lymph node or bone marrow aspirates from children.
- Within the Parental Information Sheet, we have additionally updated the CTRG telephone number, detailed the ethics committee, and added Arjune Sen's contact details.

Any other relevant information

Applicants may indicate any specific issues relating to the amendment, on which the opinion of a reviewing body is sought.

nil

List of enclosed documents

Document	*Version*	*Date*
Protocol - Immune Factors in Neurological Diseases	4	29/05/2018
Participant Information Sheet	4	29/05/2018
Participant Consent Form	4	29/05/2018
Consultee Information Sheet	2	29/05/2018
Consultee Declaration	3	29/05/2018
Non-Autoimmune Participant Information Sheet	1	29/05/2018
Non-Autoimmune Participant Consent Form	1	29/05/2018
Child information sheet ages 12-16	2	29/05/2018
Parental information sheet	2	29/05/2018
Parental consent form	3	29/05/2018

Declaration by Chief Investigator

1. *I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.*
2. *I consider that it would be reasonable for the proposed amendment to be implemented.*

This section was signed electronically by Dr Sarosh Irani on 29/05/2018 11:16.

Job Title/Post:	CI; Associate professor; Honorary Consultant neurologist
Organisation:	University of Oxford
Email:	sarosh.irani@ndcn.ox.ac.uk

Declaration by the sponsor's representative

I confirm the sponsor's support for this substantial amendment.

This section was signed electronically by Dr Ronja Bahadori on 29/05/2018 11:21.

Job Title/Post:	Senior Research Support Specialist
Organisation:	University of Oxford
Email:	ronja.bahadori@admin.ox.ac.uk

Appendix 5.2 List of Submitted Documents

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Notice of Substantial Amendment (non-CTIMP)	Substantial Amendment 2, 29/05/18	29 May 2018
Participant consent form [Parental consent form Immune Factors]	3 - clean	29 May 2018
Participant consent form [Participant consent form Immune Factors]	4 - clean	29 May 2018
Participant consent form [Participant consent form Immune Factors]	4 - tracked	29 May 2018
Participant consent form [Consultee declaration Immune Factors]	3 - clean	22 June 2018
Participant consent form [Consultee declaration Immune Factors]	3 - tracked	22 June 2018
Participant consent form [Non-Autoimmune Participant consent form Immune Factors]	1	22 June 2018
Participant consent form [Parental consent form Immune Factors]	3 - clean	22 June 2018
Participant consent form [Parental consent form Immune Factors]	3 - tracked	22 June 2018
Participant consent form [Participant consent form Immune Factors]	4 - clean	22 June 2018
Participant consent form [Participant consent form Immune Factors]	4 - tracked	22 June 2018
Participant information sheet (PIS) [Consultee Information Sheet Immune Factors in Neurological Diseases]	2 - clean	29 May 2018
Participant information sheet (PIS) [Consultee Information Sheet Immune Factors in Neurological Diseases]	2 - tracked	29 May 2018
Participant information sheet (PIS) [Parental Participant Information Sheet Immune Factors in Neurological Diseases]	2 - clean	29 May 2018
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Participant information sheet (PIS) [Participant Information Sheet Immune Factors in Neurological Diseases]	4 - clean	29 May 2018
Participant information sheet (PIS) [Participant Information Sheet Immune Factors in Neurological Diseases]	4 - tracked	29 May 2018
Participant information sheet (PIS) [Child information sheet ages 12-16]	2 - clean	22 June 2018
Participant information sheet (PIS) [Child information sheet ages 12-16]	2 - tracked	22 June 2018
Participant information sheet (PIS) [Non-Autoimmune Participant Information Sheet Immune Factors in Neurological Diseases]	1	22 June 2018
Participant information sheet (PIS) [Parental Participant Information Sheet Immune Factors in Neurological Diseases]	2 - clean	22 June 2018
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Participant information sheet (PIS) [Participant Information Sheet Immune Factors in Neurological Diseases]	4 - clean	22 June 2018
Participant information sheet (PIS) [Participant Information Sheet Immune Factors in Neurological Diseases]	4 - tracked	22 June 2018
Research protocol or project proposal [Protocol - Immune Factors in Neurological Diseases]	4 - clean	29 May 2018
Research protocol or project proposal [Protocol - Immune Factors in Neurological Diseases]	4 - tracked	29 May 2018
Research protocol or project proposal [Protocol - Immune Factors in Neurological Diseases]	4 - clean	22 June 2018
Research protocol or project proposal [Protocol - Immune Factors in Neurological Diseases]	4 - tracked	22 June 2018

Appendix 5.3 Research Ethics Committee (REC) Favourable Opinion Approval Letter 22/06/2018

NHS
Health Research Authority

Yorkshire & The Humber - Leeds East Research Ethics Committee

NHSBT Newcastle Blood Donor Centre
Holland Drive
Newcastle upon Tyne
NE2 4NQ

Tel: 0207 1048 088

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

22 June 2018

Dr
Level 6, West Wing
Nuffield Department of Clinical Neurosciences
John Radcliffe Hospital
Oxford
OX3 9DS

Dear Dr

Study title:	**Immune Factors in Neurological Diseases**
REC reference:	**16/YH/0013**
Amendment number:	**Substantial Amendment 2, 29/05/18**
Amendment date:	**29 May 2018**
IRAS project ID:	**195152**

The above amendment was reviewed at the meeting of the Sub-Committee held by correspondence.

Summary of amendment

This amendment was submitted in order to make various changes to the study protocol, including: the use of in vitro and in vivo models to study the effects of patient immune factors, adjust the planned sample size to account for anticipated increased number of samples, amended specificity of lymph node sample site from 'submental' to 'cervical', and include the addition of bone marrow aspiration and stool collection.

Ethical opinion

Following the review of this amendment, it was noted that the sentence 'You may also choose to make your samples available for transfer into cell and animal models.' was added to all information sheets; however the Sub-Committee considered that this would not be understandable to the majority of the general public, and definitely not 6-12 year old children. Therefore, it was requested that this sentence was re-written for clarity.

A revised sentence was included in the information sheets as requested.

The Sub-Committee requested a simple summary for each group, detailing the numbers of participants who were included, what the proposed numbers were, and why the numbers were being changes by so much.

It was explained that there had been a number of important research observations made over the last year or so; one way to extend and confirm these, and explore some subgroups with the same baseline hypotheses, was to use more samples and more controls. T

Therefore, the adjustment to the sample sizes would account for such increased numbers of samples. It was confirmed that the total sample size would be 5000 participants, comprising of 1500 patients with autoimmune neurological disease (such as autoimmune encephalitis and autoimmune peripheral nerve diseases), 500 samples from healthy volunteers (including environmental controls) – recruitment from clinic and biobanks, and 3000 samples from research participants who consented to the use of their samples in further research from cohorts recruited by collaborators.

With regards to the recruitment of healthy volunteers, it was queried whether this would be all relative/friends of participants who go to the clinic. The Sub-Committee noted that any publicity for recruitment, such as posters or emails, would need to be submitted for review, or could be a new substantial amendment, if not yet available.

It was anticipated that relatives/ friends/ spouses would be approached in clinic when this was considered to be appropriate, (for example if a sensitive issue were to be apparent during a consultation, recruitment to the study would not be offered. At present, it was not felt that there was a need to advertise the study, as word of mouth and cohorts available from collaborators and biobanks would be sufficient for the controls required.

The Sub-Committee noted that the consent forms for consultee, parental and non-autoimmune participant all used the phrase that 'cells may be used to study the effects in live organisms'. It was queried why the wording 'live animal studies' had not been used, as it had in the child's consent form.

It was proposed to change this wording to read "I agree for researchers to use my blood or other samples available to study their effects on living cells or animals".

The Sub-Committee noted that the participant information sheet stated that results of studies would be available but a web address for this was not provided; the inclusion of this would be helpful.

It was explained that publications and research highlights are added periodically to the following website: https://www.ndcn.ox.ac.uk/research/autoimmune-neurology-group. This information was included in the study documentation.

Finally, the Sub-Committee requested that boxes were included on the consent form, so that participants were clear on which options they were agreeing to.

It was clarified that the study documents (participant, consultee and parental consent forms, in addition to our child assent forms), had a column alongside each option for the participants to initial. It was hoped that this would suffice, and had already been working very well to ensure that participants could agree to or decline options as they wish.

The Sub-Committee was satisfied with the response provided to queries raised.

The members of the Committee taking part in the review gave a **favourable** ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Notice of Substantial Amendment (non-CTIMP)	Substantial Amendment 2, 29/05/18	29 May 2018
Participant consent form [Parental consent form Immune Factors]	3 - clean	29 May 2018
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Research protocol or project proposal [Protocol - Immune Factors in Neurological Diseases]	4 - tracked	22 June 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

16/YH/0013:	**Please quote this number on all correspondence**

Yours sincerely
pp

Sarah Prothero

Dr Rhona Bratt
Chair

E-mail: nrescommittee.yorkandhumber-leedseast@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Dr Heather House, Oxford University Hospitals NHS Trust*

Yorkshire & The Humber - Leeds East Research Ethics Committee

Attendance at Sub-Committee of the REC meeting held by correspondence

Committee Members:

Name	*Profession*	*Present*
Dr Rhona Bratt (Chair)	Retired Multimedia Project Manager	Yes
Mrs Ann Kay	Retired Special Needs Coordinator	Yes

Also in attendance:

Name	*Position (or reason for attending)*
Miss Sarah Prothero	REC Assistant

Appendix 5.4 Health Research Authority (HRA) Approval Letter 28/06/2018

IRAS Project ID 195152. HRA Approval for the Amendment

AH AMENDMENTS, Hra (HEALTH RESEARCH AUTHORITY) <hra.amendments@nhs.r

Reply

Sarosh Irani; CTRG Sponsorship Correspondence; +2 more

Dear Dr Irani,

IRAS Project ID:	195152
Short Study Title:	Immune Factors in Neurological Diseases v1
Amendment No./Sponsor Ref:	Substantial Amendment 2, 29/05/18
Amendment Date:	29 May 2018
Amendment Type:	Substantial Non-CTIMP

I am pleased to confirm **HRA and HCRW Approval** for the above referenced amendment.

You should implement this amendment at NHS organisations in England and Wales, in line with the conditions outlined in your categorisation email.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

Please contact [hra.amendments@nhs.net]hra.amendments@nhs.net for any queries relating to the assessment of this amendment.

Kind regards

Michael

Mr Michael Higgs
Assessor
Health Research Authority
Ground Floor | Skipton House | 80 London Road | London | SE1 6LH

E.hra.amendments@nhs.net
W. www.hra.nhs.uk

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Appendix 5.5 Oxford University Hospitals NHS Trust Financial Agreement 16/10/2018

Study Title: Immune Factors In Neurological Diseases
Reference: 16/YH/0013 – PID: 11669

AMENDMENT NO 1 - AGREEMENT

THIS AGREEMENT is dated 16th October 2018 and is made **BETWEEN**

(1) OXFORD UNIVERSITY HOSPITALS NHS FOUNDATION TRUST, (formerly named Oxford University Hospitals NHS Trust), whose administrative offices are at Headley Way, Headington, Oxford OX3 9DU (the "Trust");

and

(2) THE CHANCELLOR MASTERS AND SCHOLARS OF THE UNIVERSITY OF OXFORD, whose administrative office is at University Offices, Wellington Square, Oxford, OX1 2JD (the "Sponsor")

BACKGROUND

(A) The parties entered into an agreement on 27th July 2016 to **"Immune factors in neurological diseases"** (the "Original Agreement").

(B) The parties have agreed to amend the Original Agreement as set out in this agreement.

(C) This agreement is supplemental to the Original Agreement.

IN CONSIDERATION OF the sum of £1 (one pound sterling), the parties agree as follows:

AGREED TERMS

1. Definitions and Interpretations

1.1 All terms and expressions in this agreement shall have the same meaning as that given to them in the Original Agreement unless defined below.

1.2 The rules of interpretation of the Original Agreement shall apply to this agreement as if set out in this agreement save that references in the Original Agreement to 'this Agreement' shall be construed as references to this agreement.

1.4 Unless the context otherwise requires, references in the Original Agreement to "this agreement" shall be to the Original Agreement as amended by this agreement.

2. Amendments to the Original Agreement

2.1 The Original Agreement shall be amended with effect on and from the Effective Date.

2.2 The in Schedule 3 (Financial Arrangements) shall be replaced by the following:

FINANCIAL ARRANGEMENTS

	Area of Cost	Payment (£ Sterling)
1	Sampling of lymph nodes by fine needle aspirate	£150 per sample
2	Bone Marrow Aspiration	£200 per patient
3		
4		
5		
6		
7		

3. Continuity

The provisions of the Original Agreement shall, save as amended in this agreement, continue in full force and effect, and shall be read and construed as one document with this agreement.

4. Miscellaneous

This agreement may be executed and delivered in any number of counterparts, each of which is an original and which, together, have the same effect as if each party had signed the same document.

5. Governing Law and Jurisdiction

This Agreement shall be governed by English Law and the English Courts shall have exclusive jurisdiction to deal with any dispute which may arise out of or in connection with this Agreement.

Study Title: Immune Factors In Neurological Diseases
Reference: 16/YH/0013 – PID: 11669

SIGN OFF

Signed by the duly authorised representatives of the Parties on the date stated at the beginning of this Agreement

SIGNED ON BEHALF OF THE SPONSOR

Name	Position	Signature	Date
Denis Murphy	Research Contracts Manager, Research Services, Medical Sciences, University of Oxford	Denis Murphy	15 Oct 18

SIGNED ON BEHALF OF NHS ORGANISATION

Name	Position	Signature	Date
Charles A Lescott	Head of IP and Research Contracts, Oxford University Hospitals NHS Foundation Trust		16/10/18

NUFFIELD DEPARTMENT OF CLINICAL NEUROSCIENCES

Level 6, West Wing
John Radcliffe Hospital
Oxford, OX3 9DU

CI: Dr Sarosh R Irani

Immune Factors in Neurological Diseases

PARTICIPANT INFORMATION SHEET

You are being invited to take part in a *research study. Before you decide, it is important that you understand why the research is being done and what it would involve for you. Please take time to read this information, and discuss it with others if you wish.* If there is anything that is not clear, or if you would like more information, please ask us.

What is the purpose of the study?

The study aims to understand the range of neurological conditions that are caused by immune system problems. The immune system normally controls our ability to fight infection. If the immune system goes wrong it may cause diseases called 'autoimmune' diseases. We can diagnose some of these autoimmune diseases using tests developed in Oxford.

We want to be able to find out more about of the causes of these diseases and the effects they have in order to develop better treatments. To do this, we need to gather information about disease symptoms and development as well as collect samples we can study in the lab.

Why have I been invited?

Your doctor believes that you may have a condition with an underlying autoimmune tendency. We will be asking around 1500 patients who are having diagnostic tests whether they would be willing to participate. We will also obtain samples from healthy volunteers and people with neurological diseases that do not have an autoimmune illness, in order to compare results.

Do I have to take part?

No. It is up to you to decide whether or not to take part. We will talk to you about the study before you decide. If you decide to take part, you are free to withdraw consent at any time without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I decide to take part?

If you decide to take part, we will ask you to sign a consent form.

We will ask you to give samples of blood/ spinal fluid/ saliva/ stool/ urine (delete as appropriate) for research. Wherever possible, these will be taken during a routine test or a planned treatment so that providing samples will be as convenient as possible. During your blood test, for example, we may take up to 80 mls (about 4 tablespoons) more blood than is needed for your test or treatment. During your ongoing medical care we may ask permission to take further follow up samples.

If you are unable to come to Oxford or for convenience, there is the option to have a blood sample taken at your GP practice, having previously consented to taking part in the study. We will send you an EDTA tube labelled with your participant ID, a covering letter to your GP to explain the study, and a stamped-addressed envelope in order to return the sample to the research team. You should show the phlebotomist your study consent form, and your GP practice may wish to keep a copy if that is their policy.

We may ask your hospital to provide samples left over from any routine surgery, diagnostic tests or treatments that you may have had or may need in the future. For instance, if you have plasma removal treatment, we would like to use the plasma that would otherwise be discarded for research.

We would ask for permission to collect and study your health records as relevant for this research alongside and for the duration of your routine clinical follow-up. This information will enable us to get a clearer picture of your symptoms over time, and how your condition responds to treatments. We may also ask for you to complete a few questionnaires to assess your health in various domains such as quality of life, and your specific symptoms. We anticipate these will not take more than ninety minutes, and will be integrated into a clinic visit wherever possible. As such, we aim to obtain this information when you attend hospital for your routine appointments, typically 3 monthly. If you are not attending clinic, for whatever reason, we will ask if we can visit you at home to assess you, if you prefer.

Are there any possible disadvantages or risks from taking part?

The disadvantages of **blood taking** are the possibility of local discomfort and bruising.

Lumbar puncture (if relevant to you). Cerebrospinal fluid (CSF) sampling is done by lumbar puncture (LP) which is generally a safe and trouble-free procedure used to remove the fluid surrounding the spine and brain. This will only be carried out by a doctor with experience and training to do so. An LP requires the injection of a small amount of local anaesthetic at the base of the back, which is frequently painful for several seconds before the anaesthetic begins to work. It is not always possible to achieve full numbing, and occasionally people feel some pain. Removal of CSF can result in a headache in about 10% of patients. This headache usually improves with bed rest over a few hours to days. Where possible, we use atraumatic needles to minimise this risk. Extremely rarely the headache can be severe enough to require hospital admission and a further invasive procedure in the form of a ‘blood patch’ to minimise CSF leakage. In exceptionally rare circumstances, an LP can result in the potentially life−threatening conditions of subdural haematoma (requiring craniotomy to relieve intracranial pressure), or meningitis (requiring intravenous antibiotics). Such severe adverse events are

very rare. All necessary expertise is available on site to deal promptly with such outcomes, if they are required.

Ultrasound-guided Fine Needle Aspiration (FNA) (if relevant to you). FNA allows us to sample immune cells in lymph nodes in the neck, under your chin or around your jaw (cervical lymph nodes). This is a site outside of the brain where immune cells learn to make antibodies that target brain molecules, contributing to autoimmune brain diseases. FNA is a well-established, quick and safe method to obtain this sample. It is used routinely to assess other diseases of the head and neck, and has a very low complication rate, equivalent to blood taking. An experienced radiologist uses an ultrasound probe to locate the node and then passes a fine needle to draw up the cells.

The main risks are local discomfort, infection, bleeding/bruising and damage to local structures. These are rare, and the radiologist minimises them by aseptic technique and precise ultrasound-guidance. The cervical nodes are not near major blood vessels or nerves which further reduces the risk of unintended damage. The technique does not damage the node and will not affect your immune system.

Bone Marrow Aspiration (if relevant to you). This procedure takes a small sample of the spongy tissue called marrow contained within your bone. Blood cells are made in the bone marrow, including white blood cells, which are the immune cells that can produce antibodies. The white blood cells can be examined from a sample of marrow, and provide useful information about the cause and potential treatments for your condition. The sample is usually taken from the ridge at the back of your hipbone. An experienced Haematologist will clean the skin with an antiseptic solution, and inject a small amount of local anaesthetic into the area around the bone. The doctor then inserts a hollow needle through the skin, and into the bone. The liquid sample of bone marrow is then drawn up into a syringe. This may cause you to feel a brief sharp pain or stinging for a few seconds. After the test, a dressing will be applied. Following the procedure, you should avoid any strenuous activity, including heavy lifting, and you can take painkillers as required. An aftercare leaflet will be provided.

The procedure may cause local discomfort, which is minimised as far as possible by the local anaesthetic. As the local anaesthetic wears off after an hour or so, some patients may feel an aching sensation over the site of the test, which can usually be relieved by taking paracetamol. Infection is a rare complication, which is minimised by use of antiseptic and other steps taken to ensure that the procedure is kept sterile. Risk of major complications due to bleeding are rare, occurring at a rate of 1 in 1000, but can be potentially life-threatening.

If you agree to participate for a bone marrow aspiration, you will be given further, detailed information sheets/ receive further counselling for this procedure.

What are the possible benefits of taking part?

You will be having tests to try to diagnose your condition, whether or not you take part in this research. Further tests we do as part of research may lead to new and better diagnoses. We anticipate the major benefit will be for other patients who develop related conditions in the next few years.

What if relevant new information becomes available?

If new information that is relevant for your health care becomes available during the course of the study, we will inform the doctor caring for you when you were referred to the study. We

will not feed back genetic information as these autoimmune conditions are not observed to run in families.

Will my taking part in the study be kept confidential?

We will follow ethical and legal best practice and all information about you will be handled in confidence.
This includes using encryption, password protection, and keeping signed documents in locked cabinets.
We will keep test results and relevant data from your medical notes in a secure research database, identified by a study code. This means that personal information will be held separately from your samples and clinical information. Personal information will only be used to contact you and your clinician, and to retrieve and match up health information in the future.
If a video has been obtained for clinical or research purposes to document aspects of your illness, you will be identifiable. We may seek your consent for the video to be used in scientific publications or presentations.

If this work is published (in scientific journals or other communications), your identity will not be disclosed other than in videos, where relevant and if explicitly agreed.

Responsible members of the University of Oxford [and the relevant NHS Trust(s)] may be given access to data for monitoring and/or audit of the study to ensure that the research is complying with applicable regulations. Only authorised members of the research team would have access to all the research details, and only very few of those would have a link to your name.

What about expenses?

If participation in the study requires extra hospital visits, we would be happy to refund your travel costs.

What will happen to the samples I give?

Laboratory tests will be performed to understand aspects of your body's immune system that may contribute to your illness. Samples used for genetic research aim to study genes that predispose you to autoimmunity. This will not have implications for you or your family as these diseases are not observed to be inherited. You may choose to make your blood or other samples available for researchers to study their effects on living cells or animals.

If you agree, the samples will be looked after by the researchers for future ethically approved research studies. Your anonymised samples would be used mainly by local researchers but ethically approved research projects may take place in hospitals, universities, non-profit institutions or commercial laboratories worldwide.

What will happen to my data?

All anonymised research data may be shared with other researchers. We will ensure that if your samples and information are used for future research it is entirely anonymously. Only researchers in the study can look at identifying details.

What if I don't want to carry on with the study?

You are free to withdraw from the study at any time. If you wish, we will destroy samples and information we hold about you and remove them from any further use.

What will happen to the results of this study?

The results of the study will be published in scientific journals and discussed at scientific meetings addressing both researchers and other patients with similar conditions. You will

have full access to this, if you wish. It will not be possible to identify you from any reports or publications. Some of the research undertaken may also contribute to fulfilment of an education requirement (e.g. a doctoral thesis). Links to our scientific publications can be find via our group's online website: https://www.ndcn.ox.ac.uk/research/autoimmune-neurology-group

What if there is a problem?

The University of Oxford, as Sponsor, has appropriate insurance in place in the unlikely event that you suffer any harm as a direct consequence of your participation in this study.

NHS indemnity operates in respect of the clinical treatment that is provided.

If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should contact Dr Irani; 01865234531; sarosh.irani@ndcn.ox.ac.uk, or Dr Sen 01865231891; arjune.sen@ndcn.ox.ac.uk or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 616480, or the head of CTRG, email ctrg@admin.ox.ac.uk.

The Patient Advisory Liaison Service (PALS) is a confidential NHS service that can provide you with support for any complaints or queries you may have regarding the care you receive as an NHS patient. PALS is unable to provide information about this research study.
If you wish to contact the PALS team please email PALSJR@ouh.nhs.uk or telephone 01865 221473.

Who is organising and funding the study? This study is being organised and led by Dr Sarosh R Irani. It is supported by funding from the Wellcome Trust, the National Institute for Health Research –Oxford Biomedical Research Centre, The Association of British Neurologists, the British Medical Association Research Grants, the UCB-Oxford Alliance, ONO pharmaceuticals, EuroImmun AG and Epilepsy Research UK.

Who has reviewed the study? This study has been reviewed and given favourable opinion by the Yorkshire & The Humber - Leeds East Research Ethics Committee.

Participation in future research We will ask if we can contact you about future studies that either we or other researchers are doing. This is additional: you can take part in the current study and decline to be contacted again. Agreeing to be contacted does not oblige you to take part in any future research. You can also withdraw your consent to future contact.

Further information and contact details:

Please contact Dr Sarosh R Irani or Dr Arjune Sen

By telephone 01865 234531 / 01865231891

By email sarosh.irani@ndcn.ox.ac.uk / arjune.sen@ndcn.ox.ac.uk

Or by post at

Nuffield Department of Clinical Neurosciences,

Level 6, West Wing, University of Oxford,

John Radcliffe Hospital,

Headington, Oxford OX3 9DS

Thank you for reading this information

Appendix 5.7 Participant Consent Form V4 22/06/2018







NUFFIELD DEPARTMENT OF CLINICAL NEUROSCIENCES
Level 6, West Wing
John Radcliffe Hospital, Oxford, OX3 9DU

Patient identification number:

PATIENT CONSENT FORM: Immune Factors in Neurological Diseases

Please Initial

I confirm that I have read the information sheet dated (.....) version (....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	
I understand that the relevant sections of my medical notes and data collected during the study may be looked at by individuals from the University of Oxford, from regulatory authorities or from the relevant NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
I agree to give samples for research and/or allow samples already collected as part of my medical care to be used for this research.	
I consider these samples a gift to the University and I understand that I will not gain any direct personal benefit from them.	
I understand genetic information will not be fed back to me as the conditions are not known to be inherited.	
I give permission for study researchers to access my health care records in order to collect and store information necessary for this research.	
I give permission for results of any research tests that are relevant to my care to be shared with my clinical team.	
I agree to take part in this study.	

Turn over

Optional:

I agree to Fine Needle Aspiration (FNA) of cells in cervical lymph nodes being performed for research purposes	
I agree to bone marrow aspiration for research purposes	
I agree to stool being obtained for research purposes	
I agree to cerebrospinal fluid (CSF) being obtained for research purposes	
I agree to be videotaped and understand that I may be identifiable in videos documenting my illness.	
I agree to my videos being used in scientific publications or presentations.	
I agree for my anonymised samples to be used in future academic or commercial research, here or abroad, which has ethics approval.	
I agree to be contacted about related ethically approved research studies for which I may be suitable. I understand that agreeing to be contacted does not oblige me to participate in any further studies.	
I agree for researchers to use my blood or other samples available to study their effects on living cells or animals.	

Patient's Signature……..Date……..

Researcher's Signature……..Date……..

1 copy for patient, 1 copy for researcher, 1 for Oxford researcher, 1 copy to be kept in medical notes

Appendix 5.8 Study Protocol: Immune Factors in Neurological Diseases V4 22/06/2018

Study Title: Immune Factors in Neurological Diseases
IRAS Project ID: 195152

Ethics Ref: 16/YH/0013
Date and Version No: 22/06/2018 v4

Chief Investigator: Associate Professor Sarosh R Irani, Senior Clinical Fellow and Honorary Consultant Neurologist, Nuffield Department of Clinical Neurosciences, University of Oxford

Investigators: Dr Patrick Waters, Senior Scientist, Nuffield Department of Clinical Neurosciences, University of Oxford

Associate Professor Arjune Sen, Consultant Neurologist, Nuffield Department of Clinical Neurosciences, University of Oxford

Dr Maria Isabel Leite, Senior Clinical Research Fellow and Honorary Consultant Neurologist, Nuffield Department of Clinical Neurosciences, University of Oxford

Dr James Varley, Clinical Research Fellow, Nuffield Department of Clinical Neurosciences, University of Oxford

Dr Adam Al-Diwani, Clinical Research Fellow, Department of Psychiatry, University of Oxford

Dr Sophie Binks, Academic Clinical Fellow, Nuffield Department of Clinical Neurosciences, University of Oxford

Dr Ronan McGinty, Clinical Research Fellow, Nuffield Department of Clinical Neurosciences, University of Oxford

Dr Bettina Balint, Clinical Research Fellow, Nuffield Department of Clinical Neurosciences, University of Oxford

Dr Sophia Michael, Clinical Research Fellow, Nuffield Department of Clinical Neurosciences, University of Oxford

Dr Valentina Damato, Clinical Research Fellow, Nuffield Department of Clinical Neurosciences, University of Oxford

Dr Adam Handel. Clinical Research Fellow, Nuffield Department of Clinical Neurosciences, University of Oxford

Miss Archana Ramesh, Clinical Care Coordinator, Nuffield Department of Clinical Neurosciences, University of Oxford

Ms Rhea Zambellas, Project Manager, Nuffield Department of Clinical Neurosciences, University of Oxford

Sponsor: University of Oxford
Funders: Wellcome Trust; British Medical Association (Vera Down Grant); UCB-Oxford Alliance; Association of British Neurologists; Epilepsy Research UK; UCB-Oxford Alliance funding to Dr Irani and Dr Sen, EuroImmun funding to Dr Waters; R&D funds to Dr Irani and Dr Waters

Chief Investigator Signature:



Potential conflicts of interest
The Department of Clinical Neurology in Oxford receive royalties and payments for antibody assays: SRI and, BL and PW are coapplicants and receive royalties from patent application WO/2010/046716 entitled 'Neurological Autoimmune Disorders'. The patent has been licensed to Euroimmun AG for the development of assays for LGI1 and other VGKC-complex antibodies.

Confidentiality Statement
This document contains confidential information that must not be disclosed to anyone other than the Sponsor, the Investigator Team, host organisation, and members of the Research Ethics Committee, unless authorised to do so.

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1. SYNOPSIS

Study Title	Immune Factors in Neurological Diseases	
Internal ref. no. / short title	Immune Factors in Neurological Diseases	
Study Design	Longitudinal observation and laboratory studies	
Study Participants	Patients with neurological disease where autoimmunity is likely or being investigated as a component, and healthy and disease controls.	
Planned Sample Size	5000: 1500 participants with suspected or confirmed autoimmune component to their neurological disease. 500 anonymised samples from healthy volunteers, including environmental controls. 3000 anonymised samples and characterising data from research participants (who consented to use of their samples in further research).	
Planned Study Period	15/12/2015-15/12/2025	
	Objectives	**Outcome Measures**
Primary	Clinical and immunological characterisation of antibody positive patients with neurological conditions that may have an autoimmune component.	Clinical data from medical notes, including measures of neurological function, cognition and mood. Clinical data may be supplemented with standard questionnaires where this is necessary to achieve a standard minimal dataset. More detailed patient interviews in selected patients. Immunological findings (through routine diagnostics and research assays) from blood, CSF, lymph node aspirate, bone marrow aspirate, stool, urine and saliva. Correlations between clinical and immunological findings to determine whether there are clinical or immunological features which predict a good response to treatments [1-3]. Comparisons with healthy and disease control samples will determine cut-offs and syndrome specificity of immunological findings.
Secondary	Novel antibody discovery in patients with similar phenotypes.	Correlations between clinical and immunological findings, as performed in previous studies [1, 4], to describe phenotypes associated with novel

		antibodies, and their response to treatments. Comparisons with disease and healthy control samples will determine antibody specificity.
	Identify other immunological characteristics and markers in patients with neurological conditions.	Immunological findings, including lymphocyte types, immune-system related genes, proteins, microbes and cytokine levels, in bodily fluids, including bone marrow aspirate, and stool. The presence of serum antibodies in urine and saliva. Comparisons with disease and healthy control samples will determine assay specificity and cut-offs to allow understanding of whether these markers segregate with autoimmune neurological diseases.
	Characterise the antibodies and the cells that produce antibodies in order to understand the biology of neurological conditions and identify possible drug targets.	Cell phenotypes (protein and transcripts), responses of cells to factors in culture. In vitro and in vivo models to study the functional effects of identified patient immune factors. Comparisons with disease and healthy control samples will determine assay cut-offs to allow understanding of whether these markers segregate with autoimmune neurological diseases.
	Non-heritable genetic factors which predispose to the condition.	HLA-associations with clinical phenotype – especially in patients with LGI1-antibodies.

2. ABBREVIATIONS

ABN	Association of British Neurologists
BNSU	British Neurological Surveillance Unit
CI	Chief Investigator
CRF	Case Report Form
CSF	Cerebrospinal Fluid
CTRG	Clinical Trials & Research Governance, University of Oxford
ERUK	Epilepsy Research UK
FNA	Fine Needle Aspiration
GCP	Good Clinical Practice
GP	General Practitioner

ICF	Informed Consent Form
NHS	National Health Service
NIHR	National Institute for Health Research
NRES	National Research Ethics Service
OUH	Oxford University Hospitals NHS Trust
PI	Principal Investigator
PIL	Participant/ Patient Information Leaflet
PLEX	Plasma Exchange
R&D	NHS Trust R&D Department
REC	Research Ethics Committee
SOP	Standard Operating Procedure

3. BACKGROUND AND RATIONALE

Neurological disorders are often difficult to treat. We, and other clinical researchers, have shown an autoimmune, usually antibody mediated, cause for some such conditions in several studies.[5-10] Affected patients are of both sexes, all ages and all races. Examples include, myasthenia gravis and, most recently, novel forms of encephalitis and epilepsy. The diagnosis is usually confirmed with (serological) blood and cerebrospinal fluid (CSF) tests. Until recently CSF was not required for diagnosis, but recent publications suggest that it should be done in parallel with serum since both can be informative.[11] After the diagnosis is confirmed, these conditions are often effectively treated with immunomodulatory drugs.[2, 12, 13] There is an appreciated need to determine more fully the number of conditions that respond to immunomodulatory therapies. One way to achieve this aim is to detect more patients with autoantibodies and related abnormal immunological parameters, which will help define conditions with an immunological component, and that are often immunotherapy-responsive. Such conditions now include a variety of degenerative and other neurological conditions, in which it is beginning to be appreciated that the immune system may play a role in determining patient progression or response to treatments[14-17]. In summary, immunological parameters determined within this study can be useful in defining an immune component to these diseases, and may also suggest a role for immunotherapies in these patients.

This research builds on a previous project, *Immune and genetic factors in neurological disorders* (REC Ref: 07/Q1604/28) where consent was obtained to use clinical details and research blood and cerebrospinal fluid (CSF), sent for routine clinical purposes to the University of Oxford Department of Clinical Neurosciences Neuroimmunology Laboratory (often through the Oxford University Hospitals NHS Trust Clinical Neuroimmunology Service), for novel and established immunological markers. Also, additional blood, CSF, urine and saliva were obtained in order to enable us to study immunological markers in more detail. In addition, patients were able to consent to having these bodily fluids collected directly from clinical encounters (typically outpatient appointments). The previous project has been successful in defining new antibody targets, generating associated patents, translating rapidly to clinical practice and offering patients worldwide appropriate treatments with good outcomes: many of these outputs are described in the reference list. [2, 6, 8, 18-20] This current study aims to examine clinical and immunological laboratory features in patients for whom autoimmunity is a part of their neurological disease. In particular, we are interested in symptoms, signs, investigation results, autoantibodies, cytokine and lymphocyte profiles of

these patients. The presence of an autoantibody often means that the patient receives a diagnosis of an autoimmune neurological condition and that the patient may respond to immunologically-directed therapies ('immunotherapies'). We intend to continue to characterise the detailed clinical profile of patients with these disorders in order to allow identification of immunotherapy-responsive diseases. This approach has been successful in us identifying novel clinical phenotypes which respond to immunotherapies. Identification is markedly enhanced with videos of patients and accounts of disease from their spouses, relatives, or friends. [1, 18, 19, 21]

Since some patients will not have the defined autoantibodies, systematic clinical characterisation may help define a syndrome that may respond to known therapies. These clinical-laboratory correlations are a major focus of our research and we, and others within the field, have already helped define new phenotypes and offered many patients new, immunotherapy-responsive diagnoses using these approaches, as reviewed in our published work. [8, 9, 20] Our research aims to discover immune markers, including immune-cell and genetic signatures, which define immunotherapy-responsive diseases, predict responses to treatment, inform causation, and help characterise the clinical features of the affected patients. Our laboratory investigations of patient samples – with and without antibodies – will help us understand the biology of these neurological conditions with an autoimmune component by identifying the mechanisms which operate *in vitro* and *in vivo* [22]and the cells which produce the antibodies,[23] and which may prove to be viable targets for future drug development.

Indeed, our recent published studies have improved our understanding of cells in circulation which make the autoantibodies. Therefore as a natural extension of this work, we aim to:

1. Characterize if these cells are present in the bone marrow, a potential niche for cells which make antibodies, [24] which will have implications on identifying the cell types and their naïve precursors which produce antibodies, and future targeted therapies.
2. Further study causality, which was identified as the major unmet research need by our patients and relatives from our recent PPI day. We propose investigating environmental factors, including gut microbes (the microbiome), which is known to influence many major diseases [37].]We have also very recently ascertained HLA genetic factors which will have implications for disease causation, as originally proposed (Binks, Varley…and Irani, in press), and we would like to extend these studies to study further patients.

4. OBJECTIVES AND OUTCOME MEASURES

Objectives	Outcome Measures
Primary Objective Clinical and immunological characterisation of patients with neurological conditions that may have an autoimmune component.	Clinical data from medical notes, including measures of neurological function, functional capacity, quality of life, cognition, mood, and environmental exposures. Clinical data may be supplemented with standard questionnaires where this is necessary to achieve a standard minimal dataset. More detailed patient interviews may be performed in selected consenting patients. Immunological findings (through routine diagnostics and research assays, including in vitro and in vivo models) from blood, lymph node aspirate, bone marrow aspirate, stool, urine, saliva and CSF. Correlations between clinical and immunological findings in particular autoantibody detection to determine whether there are

	clinical or immunological features which predict a good response to treatments. Comparisons with healthy and disease control samples will determine cut-offs and syndrome specificity of immunological findings.
Secondary Objectives Novel antibody discovery in patients with similar phenotypes.	Correlations between clinical and immunological findings, to describe phenotypes associated with novel antibodies and their response to treatments. Comparisons with disease and healthy control samples will determine antibody specificity.
Identify other immunological characteristics and markers in patients with neurological conditions.	Immunological findings, including lymphocyte types, immune-related proteins, genes, microbes and cytokine levels, in bodily fluids, including bone marrow aspirate, and stool. The presence of serum antibodies in urine and saliva. Comparisons with disease and healthy control samples will determine assay specificity and cut-offs to allow understanding of whether these markers segregate with autoimmune neurological diseases.
Characterise the antibodies and the cells that produce antibodies in order to understand the biology of neurological conditions and identify possible drug targets.	Cell phenotypes (protein and transcripts), responses of cells to factors in culture. Examination of the bone marrow compartment may further inform on the cells which produce the antibodies, and further define pathogenic mechanisms. Use of *in vitro* and *in vivo* models to understand effects of immune factors, especially antibodies. Comparisons with disease and healthy control samples will determine assay cut-offs to allow understanding of whether these markers segregate with autoimmune neurological diseases.
Genetic studies to understand non-heritable genes which may predispose to the neurological condition.	Presence of genetic factors including polymorphisms or mutations which are found in patients more frequently than controls (data from publically available databases). Our recent finding of strong HLA associations in patients with LGI1/ CASPR2 autoantibodies demonstrates a need for this line of investigation (Binks, Varley….Irani, in press).

5. STUDY DESIGN

This is an exploratory study correlating longitudinal observational clinical data from patients with a possible autoimmune component to their neurological condition, with sequential immunological findings from standard and novel assays. It is designed to minimise burden on participants by drawing primarily on information from medical notes and either diagnostic samples or samples taken at the same time as those obtained for clinical purposes. This clinical and immunological characterisation will allow description of associated phenotypes, biomarkers and responses to treatments. This will be true for both well-established and novel antibodies or immunological parameters. Further cellular and other immunogenetic

laboratory work will seek to understand the biology of neurological conditions, predisposing factors and to identify possible targets for future drug development.

6. PARTICIPANT IDENTIFICATION

6.1 Study Participants

1. Patients in whom their clinician has established or suspects an autoimmune component to their neurological disease: this is anticipated to be 1500 participants over 10 years. Healthy controls: 500 samples from healthy volunteers will come from clinic, word-of-mouth, e-mail, and cohorts available from collaborators and biobanks (e.g. www.oxfordbiobank.org.uk) and be consented within our study.
2. 3000 anonymised samples and characterising data from research participants (who consented to use of their samples in further research).

6.2 Inclusion Criteria

Patients:

- There is or may be an autoimmune component to their neurological disease.
- Individual and/or parent/guardian willing to give informed consent for participation in the study
  OR:
- where an individual is deemed to lack capacity to consent, a declaration from a designated consultee.

Controls (from biobanks and within this study):

- Disease controls with a range of neurological conditions that share some overlapping clinical features to the patients with autoimmune neurological diseases. These will act as stringent controls.
- Healthy volunteers - age-matched as closely as possible to adult patients who are recruited to studies and those who have lived in close contact with/ a similar environment to a patient.

6.3 Exclusion Criteria

An individual may not enter the study if ANY of the following apply:

- For competent adults: unwilling to provide informed consent
- For children or those lacking capacity: expressing dissent
- For designated consultees: unwilling to provide a declaration in support of participation.
- For controls: consent not present for further research use of samples.

7. STUDY PROCEDURES

7.1 Recruitment

Patients:

Recruitment of patients with established or suspected autoimmune component to their neurological disease is summarised in Appendix A Flowchart. As many of these diseases are rare (estimated to be around 100 patients per year in the UK), identification and recruitment will incorporate three methods:

1. **Clinician Identification.** Patients with autoimmune neurological conditions may be identified by their UK clinician. Where appropriate arrangements are in place (collaboration, delegation, Trust approval) clinicians may obtain consent from their patient for participation in the research. Alternatively, they can obtain verbal consent for their patient's details to be passed on to Oxford researchers who will contact the patient to provide the PIS, discuss the study in greater detail, and obtain consent if the patient wishes to participate.

2. **British Neurological Surveillance Unit (BNSU).** British Neurological Surveillance Unit (BNSU). The BNSU (http://www.theabn.org/what-we-do/bnsu.html) currently provides a portal by which UK neurologists can declare having seen a patient with a specified condition. This is especially useful in rare diseases. Via the BNSU, we have permission to survey UK neurologists with patients that have NMDAR or VGKC-complex antibodies (Appendix B). Clinicians can provide the PIS and/or obtain permission to pass on patient details to Oxford researchers to discuss potential research involvement and consent the interested patients. Telephone numbers and patient details will be conveyed using a secure email address and patients will be coded as rapidly as possible to maximize confidentiality.

3. **Diagnostic Referral.** Patients with suspected autoimmune neurological conditions may be identified after their blood/CSF test positive after NHS-based testing for autoimmune neurological conditions, frequently performed in our Oxford NHS or University laboratories. We would contact the clinician referring the sample, as above.

Disease/ Healthy Controls

- Disease controls will be approached via Neurology clinics and ward inpatients within Oxford University Hospital.
- Healthy controls may be approached though word-of-mouth, or inter-departmental advertising through, for instance, e-mail. We anticipate that a large number of healthy controls will be recruited alongside patients in clinic (eg. friends or relatives), and in the case of partners/ spouse/ relatives who have lived with the patient for a substantial period of time, recruitment of such individuals may serve to act as important environmental controls.

In addition, we aim to study:
3000 anonymised samples and characterising data from research participants (who consented to use of their samples in further research). These patients will have a range of neurological disorders, with clinical or immunological features which overlap with the patients.
Healthy control anonymised samples recruited from cohorts available from collaborators and biobanks (n=500).

7.2 Screening and Eligibility

Patients are eligible if their clinician has established or suspects an autoimmune component to their neurological disease. Results will be compared to age and sex-matched adult healthy controls and disease controls throughout, to generate reference values. Disease controls will be those with a low likelihood of having an autoimmune component to their neurological disease but often have a few clinical features that are comparable to the patients. Control samples will be obtained from cohorts consented elsewhere, such as biobanks (e.g. http://www.oxfordbiobank.org.uk) or via collaborators who hold research samples for which there is consent for their further research use.

Informed Consent

The participant and individual obtaining consent will personally sign and date the latest approved version of the Informed Consent form before any study-specific procedures are performed.
The participant will be allowed as much time as wished to consider the information, and the opportunity to question the investigator or other independent parties to decide whether they will participate in the study. This may occur in person or over the telephone. Subsequently,

written and verbal versions of the Participant Information Sheet and Consent Form will be presented to the participants. These will detail the exact nature of the study; what it will involve for the participant; the implications and constraints of the protocol; any risks involved in taking part. Informed written consent to study participation will be obtained by a member of the participant's clinical team who is suitably qualified and experienced, and has been authorised to do so by the Chief Investigator.
Individuals obtaining consent will ensure that the study is explained in a manner and at a level appropriate for the potential participant. Where participants are minors, informed consent will be sought from the responsible parent or guardian. A separate information sheet will be provided for children aged 10 upwards. Children under age 16 will be asked for assent. For adults with cognitive impairments so severe as to render them incapable of consenting for themselves, advice will be sought from a designated consultee, as per the provisions of the Mental Capacity Act 2005.
The consent form clearly states that the participant is free to withdraw from the study at any time for any reason without prejudice to current or future care, and with no obligation to give the reason for withdrawal.
A copy of the signed Informed Consent will be given to the participant. The original signed form will be retained at the study site and a copy sent by secure email to AProf Irani to retain in Oxford.

7.3 Study Assessment

The clinical details and immunological findings from blood, CSF, lymph node aspirate, bone marrow aspirate, urine, saliva and stool are the parameters to be measured. These will be compared to healthy and disease control samples, although CSF and bone marrow will not be requested from healthy and disease controls.

Clinical / in vivo data

Clinical data obtained as part of clinical care will be collected from the patient medical record. These will include the patient's demographics, presenting symptoms and signs, subsequent clinical features, test results, treatment and responses to treatment. If clinical features are assessed using established tools and questionnaires (such as the modified Rankin Scale (mRS), Addenbrooke's cognitive examination revised (ACER)[25, 26], Hospital anxiety and depression scale (HADS)[27], formal neuropsychology, and Quality of life scores (e.g. Quality of life in epilepsy 31 (QOLIE-31))[28], this will also be recorded. Where possible, in order to obtain this minimal dataset, consenting participants will be asked complete assessments which had not been completed as part of routine practice.

In addition to routine practice, patients with unusual problems will be consented to longer interviews to understand certain complaints, environmental exposures or qualitative symptoms in greater depth. These are anticipated to last 30-90 minutes. Occasionally, if it is inconvenient for the patient to visit researchers, the researcher will ask to visit them with their permission.
Video recordings for research purposes will be used to document the precise clinical phenotypes of selected patients and controls, as has been essential in previous studies.[21]

Samples for Laboratory/experimental studies

Blood is routinely obtained for clinical purposes. We aim to obtain additional volumes of blood at the time of clinical blood sampling during a single venepuncture. We anticipate blood (up to 80mls), will be sampled at all baseline visits. Baseline visits will also involve CSF for clinical purposes in around 20% of patients, and for all consenting patients additional CSF will be

obtained during the same procedure (up to 30mls). In addition, CSF will be sampled in about 10% of adult patients with capacity to consent for themselves, for research purposes.
For participants unable to come to Oxford or for convenience there is the option to have a blood sample taken at their GP practice. Consent would have been obtained prior to this. The study team will send the participant an EDTA tube labelled with their participant ID, a covering letter for their GP to explain the study, and a stamped-addressed envelope for its return to the research team. We will advise participants to show the phlebotomist their study consent form, and the GP practice may wish to keep a copy if that is their policy.
Self-collection of faecal samples will follow IHMS standard procedures (SOP 05 V1), and online YouTube videos are available to assist participants (see: www.goo.gl/iq9Nm4). For convenience, participants will have the option of collecting their sample from home and posting their sample to us. Participants will be sent a stool collection pack via post in advance, including a stool container labelled with their study ID, and a stamped addressed envelope for return of their sample.

Additionally, for research purposes in adult patients with capacity to consent, we will request a donation of cells from cervical lymph nodes using a technique called ultrasound-guided Fine Needle Aspiration (FNA). A radiologist experienced in head and neck ultrasound-guided FNA will obtain cells from cervical lymph nodes which can then be characterised by immunophenotyping, genetic sequencing and cell culture. FNA is used routinely in the assessment of diseases of the head and neck, and has a very low complication rate, equivalent to routine peripheral venous blood sampling and is lower than lumbar puncture. It has been used successfully to investigate the role of cervical lymph node cells in Multiple Sclerosis for example [36].
The main risks are local discomfort, infection, bleeding/bruising and damage to local structures. These are offset by aseptic technique, targeting nodes far removed from major blood vessels or nerves, and precise ultrasound-guidance.
Similarly, for research purposes around 40 adult patients with capacity to consent, bone marrow aspiration will be possible. A trained Haematologist will obtain the bone marrow aspirate, which will be used to study the immune cells which are known to make antibodies. The procedure is used routinely in Haematology practice, and has few major risks.

Urine (up to 50mls), saliva (up to 5mls), and stool are non-invasive collection procedures and may be requested from patients with 20 minutes extra to spare (anticipated 30%). We aim to understand whether 20 patients with high and low levels of serum antibodies have detectable antibodies in non-invasive bodily fluids (urine and saliva). If successful, we may extend this to other patients, especially children in whom routine invasive procedures can be difficult. Stool samples will be used to explore the gut microbiome.

Samples from disease and healthy controls (prospectively recruited to our study and from biobanks or collaborators with permissions to share samples) will be used to determine cut-offs for the assays, typically at a stringent level of the mean plus three standard deviations. The samples requested will reflect the phenotypes of the participants found to have immune markers. For example, if a patient with a new antibody had an Alzheimer's like presentation, we may ask to test cohorts with Alzheimer's disease.

Clinical-laboratory correlations and impact on stated objectives
Correlations between clinical and laboratory observations are ongoing projects with constant evaluations which guide our subsequent thinking, and future directions. [8, 29, 30] Typically,

after 10 patients with similar clinical or immunological findings, we will begin to analyse the data in batches. The recruitment for a particular analysis is estimated to take 1-2 years.
We will use the clinical assessments to evaluate whether there has been a response to treatment over time and observe whether the presence of antibodies or other immune factors are enriched within the responders (primary objective). In patients with suspected autoimmune neurological diseases and novel antibodies or other immune factors including cytokines and immune cells, we will observe whether clinical assessments improve with immune-system directed therapies (second objective, first three points). This will help us understand whether the immune factors we detect segregate closely with clinical phenotypes. We will further explore HLA and other genetic associations in patients and by comparison to controls.

7.4 Subsequent Visits

Patient participants will typically have clinical appointments at 3-monthly intervals with a major aim being to understand whether there has been a clear response to treatments. Further clinical details (including neurological follow-up, the tools and questionnaires mentioned above, and videos) would be obtained at these times to assess clinical progress and allow us to determine the effect of treatment in patients with autoimmune neurological diseases. At these follow-up appointments, we will also obtain repeat biological samples to assess the alterations in measured immunological parameters in parallel with the change in the clinical features. The aim is to longitudinally assess the correlation of immune factors and clinical features, especially in patients administered immune-system directed therapies as part of their clinical care (primary objective). We intend to follow them for the duration of their routine clinical follow-up, typically between 2 and 5 years. Occasionally, when inconvenient for the patient to visit Oxford, we will ask to visit the patient at home, or conduct follow-up via the telephone.

7.5 Sample Handling

We anticipate samples will be analysed using a variety of immunological and cellular/molecular techniques including immunochemistry (including cell-based assays), western blotting, flow-cytometry, ELISAs, cell culture, sequencing and polymerase chain reaction. See table 1, below.
The samples, which will be stored in the John Radcliffe Hospital, Nuffield Department of Clinical Neurosciences. Biological samples will be frozen at -20˚C, -80˚C or on liquid nitrogen for use in current and future studies. Disease control and healthy control samples will be stored under the same conditions. A selection of the samples will be sent to companies who can perform the above assays. A selection of samples including sera, plasma, CSF, urine and lymph node/bone marrow aspirates may be used to study their effects in in vivo and in vitro systems comprising cellular and live animal models.

Table 1. Samples

Sample	Purpose	Volume and potential burden
Blood	For serum, plasma, cytokines and immune-cell studies. A single sample may be taken for genetic studies.	Up to 80mls. Obtaining this volume in addition to any clinical sampling causes no adverse effects and is usually possible from a single needle. We may require large volumes (e.g. 80mls) in order to study rare antibody-secreting cell populations in selected patients.

		Previous research practice has established that this amount can be obtained without additional adverse affect to the participant.
CSF	To examine antibodies, cytokines and cells found within the central nervous system, not just in the periphery (blood)	Up to 30 mls. Obtaining this additional volume during procedure causes no adverse effects. Previous research practice has established that this amount can be obtained without additional adverse affect to the participant.
Lymph node aspirate	To examine extra-CNS development of CNS Antigen-specific lymphocytes.	Ultrasound-guided Fine Needle Aspiration of cervical lymph nodes. Volume is negligible, cells fill tip of 23 gauge needle during aspiration. Procedure is routinely performed by radiology colleagues to assess head and neck disease. Local complication rate, largely localised bruising <5% is equivalent to phlebotomy.
Bone Marrow Aspirate	To study the bone marrow compartment to answer questions generated by our recent studies about whether the earliest and latest B-lineage cells (both found in bone marrow) have capacity to produce autoantibodies in patients.	Up to 20mls Procedure performed as a routine Haematological investigation for assessment of many Haematological conditions. Complications include localised pain, and infrequently, infection, and major bleeding (1 in 1000). See 'Appendix D'.
Urine	To study the presence of immunological markers, especially antibodies, in non-invasively obtained fluids	Up to 50mls
Saliva		Up to 5mls
Stool	To explore the effect of the gut microbiome on antibody production, cytokines, and T cell interactions, and longitudinal alterations in individuals.	One unit, requested on up to 3 occasions per consenting patients

Additional residual samples

Patients with immune related neurological disorders often have plasma exchange (PLEX) as part of their treatment. This involves removal of plasma, and this is a useful resource for large quantities of antibodies which could be used in laboratory studies. The plasma filtrate would otherwise be discarded.

Similarly, consent will be obtained for any samples or tissue surplus to diagnostic need resulting from other clinical treatments or investigations.

7.6 Discontinuation/Withdrawal of Participants from Study

Each participant has the right to withdraw from the study at any time. Their data and samples may be withdrawn from the study and destroyed at their request. In addition, the Investigator may discontinue a participant from the study at any time if the Investigator considers it necessary for any reason including:

- Ineligibility (either arising during the study or retrospectively having been overlooked at screening)
- Withdrawal of Consent
- Loss to follow up

The reason for withdrawal will be recorded in the CRF.

7.7 Definition of End of Study

The end of study is the date of the last visit of the last participant.

8. INTERVENTIONS

Lumbar puncture. This will be taken once or twice in most cases for clinical purposes and, in about 10% of consenting adult patients, specifically for research.

9. SAFETY REPORTING

9.1 Definition of Serious Adverse Events

A lumbar puncture can result in the potentially life−threatening conditions of subdural haematoma (requiring craniotomy to relieve intracranial pressure), or meningitis (requiring intravenous antibiotics). Such severe adverse events are very rare (based on the practice from an experienced neurological ward where such procedures are carried out on a daily basis typically without any complications). A bone marrow aspiration may result in potentially life-threatening major haemorrhage, which may require resuscitation and blood transfusion. This is extremely rare, occurring at a rate of <1 in 1000 cases. Patients will be advised in advance to be familiar with their aftercare leaflet, avoid any strenuous activity post-procedure, and in the instance of any noted bleeding through their dressing site, to apply pressure and seek medical attention immediately. All necessary expertise is available on site to deal promptly with such outcomes, if they are required.
Other ‘important medical events’ may also be considered serious if they jeopardise the participant or require an intervention to prevent one of the above consequences.

9.2 Reporting Procedures for Serious Adverse Events

A serious adverse event (SAE) occurring to a participant should be reported to the REC that gave a favourable opinion of the study where in the opinion of the Chief Investigator the event was ‘related’ (resulted from administration of any of the research procedures) and ‘unexpected’ in relation to those procedures. Reports of related and unexpected SAEs should be submitted within 15 working days of the Chief Investigator becoming aware of the event, using the HRA report of serious adverse event form (see HRA website).

10. STATISTICS AND ANALYSIS

10.1 Description of Statistical Methods

Databases will be maintained on Microsoft Excel and Access. T-tests, ANOVA, correlation coefficients and other statistical analyses, typically available using GraphPadPRISM software or SPSS will be used to compare patients with disease and healthy controls. Ongoing analyses are planned, for the observational data collected.

10.2 The Number of Participants

We plan to study:

1. 1500 patients in whom their clinician has established or suspects an autoimmune component to their neurological disease.
   Healthy controls: 500 anonymised samples from healthy volunteers will come from clinic, word-of-mouth, e-mail, and cohorts available from collaborators and biobanks (e.g. www.oxfordbiobank.org.uk).
2. 3000 anonymised samples and characterising data from research participants (who consented to use of their samples in further research).

Novel antibodies will be looked at on a case-by-case basis for around the first 10 cases, to establish clinical correlations. For questions examining established antibodies, other immunological markers, cells or genetic findings at least 10 patients are typically required to understand trends. [2, 31] An equal number of age- and sex-matched controls will be compared throughout.
For DNA assessments, we anticipate 300 patients will be required and control data derived from available public databases.

10.3 Analysis of Outcome Measures

The laboratory work will help determine the presence of an autoantibody (using brain tissue staining, cell based assays and other related techniques), the properties of the patient white blood cells from blood and lymph and bone marrow aspirates (especially using flow cytometry and cell culture techniques), the cytokines found in patients' blood and CSF, and DNA signatures found in the patient's blood. Throughout, healthy control and disease controls samples will be used to calibrate the baselines for assays, and determine rigorous cut-offs.
We hypothesize that, in addition to stool samples, saliva and urine may be non-invasive fluids from which to determine antibody levels. We aim to trial this on 20 patients with high and low levels of serum antibodies, and 20 healthy controls to compare antibody levels, and proceed to further collections if successful.
The clinical outcome measures will be analysed using the statistical methods described above, as already established in the literature[4,9,11,17]. These are often simply descriptive but often utilize statistical techniques such as Chi-Squared, correlation coefficients and t-tests available in GraphPad PRISM. Values may be compared to those available from disease and healthy controls to establish unique features of the participants.
Data from participants who have withdrawn will be excluded from analyses.

11. DATA MANAGEMENT

11.1 Access to Data

Direct access will be granted to authorised representatives from the Sponsor and host institution for monitoring and/or audit of the study to ensure compliance with regulations.

11.2 Data Recording and Record Keeping

Participant details will be held on a password-protected Microsoft Excel file within the CI's laboratory / offices on University of Oxford premises. Participants will be identified by a unique, study-specific code number. Paper records (including consent forms) will be held in locked cabinets in the CI's laboratory/offices.

Retrieval of data from medical records will generally be effected by means of NHS numbers and is subject to participant consent.

Participant identifying data will be retained for the duration of the study's ethical approval and then destroyed.

12. QUALITY ASSURANCE PROCEDURES

The study may be monitored, or audited in accordance with the current approved protocol, GCP, relevant regulations and standard operating procedures.

13. ETHICAL AND REGULATORY CONSIDERATIONS

14.

14.1 Declaration of Helsinki

The Investigator will ensure that this study is conducted in accordance with the principles of the Declaration of Helsinki.

13.2 Guidelines for Good Clinical Practice

The Investigator will ensure that this study is conducted in accordance with relevant regulations and with Good Clinical Practice. Dr Irani has completed his GCP certification.

13.3 Approvals

The protocol, informed consent form, participant information sheet and any proposed advertising material will be submitted to an appropriate Research Ethics Committee (REC), and host institution(s) for written approval.
The Investigator will submit and, where necessary, obtain approval from the above parties for all substantial amendments to the original approved documents.

13.4 Reporting

Upon request, the CI shall submit a Progress report to the REC Committee, host organisation and Sponsor. In addition, an End of Study notification and final report will be submitted to the same parties.

13.5 Participant Confidentiality

The study staff will ensure that the participants' anonymity is maintained. The participants will be identified only by a participant ID number on all study documents and any electronic database, with the exception of the CRF, where participant initials may be added. The ID number will contain the initials of hospital of recruitment (e.g. JR for John Radcliffe) followed by a randomly generated six-digit number, starting from 000000. As few researchers as possible in Dr Irani's team will have access to the name and identification numbers of the patients for initial data inputting and code generation. All documents will be stored securely and only be accessible by study staff and authorised personnel. The study will comply with the Data Protection Act, which requires data to be anonymised as soon as it is practical to do so. Identifiable videos will be kept on laboratory computers used only by the research team. Consent forms will be emailed using Dr Irani's secure nhs.net email address.

13.6 Expenses and Benefits

Reasonable travel expenses for any visits additional to normal care will be reimbursed on production of receipts, or a mileage allowance provided as appropriate.

13.7 Other Ethical Considerations

Vulnerable Participants: As many patients with autoimmune neurological diseases have cognitive impairment, their participation will be supported, where appropriate, through

involvement of a designated consultee, in keeping with the provision of the Mental Capacity Act, 2005.
Children will be included in this study as patients where there is suspicion of an autoimmune component to their neurological disease. We have developed information aimed at children aged 10 and above only. This was decided on the basis of the study's theoretical complexity balanced against the minimal anticipated intervention (at most, a blood, urine or saliva sample, if these have not already been collected in clinical care). Age 10 will be used as a guide; attempts will be made to engage all children in decisions about participation as appropriate to their understanding.

Incidental Findings: Findings considered to be of clinical significance for patients will be discussed with their referring clinician / GP and fed back to collaborators. For control samples, feedback pathways will comply with biobank and source study protocols.
Genetic findings will not be fed back to participants because the conditions under investigation are not known to be heritable.

14. FINANCE AND INSURANCE

14.1 Funding

Dr Irani has a Wellcome-Trust funded intermediate fellowship, BMA Vera Down and Margaret Temple, ONO pharmaceuticals, and UCB-Oxford University Alliance grants to study neurological diseases with an autoimmune component. Dr Irani and Dr Waters are supervising two DPhil students with ABN fellowships to study autoimmunity in neurology and have R&D funds. Dr Waters is supported by EuroImmun AG, and by the NIHR Oxford BRC. Dr Sen and Dr Irani are co-PIs on a grant from the UCB-Oxford Alliance. Dr Varley is supported by an ABN Clinical Research Training Fellowship. Dr Al-Diwani has a Wellcome Trust DPhil Training Fellowship supported by the NIHR Oxford BRC and Oxford University Medical Sciences Doctoral Training Centre. Dr Binks is supported by the Oxford University Clinical Academic Graduate School and a Wellcome / BRC DPhil. Dr McGinty and Dr Michael are supported by the UCB-Oxford University Alliance. The lab is supported by BRC Oxford.

14.2 Insurance

The University has a specialist insurance policy in place, which would operate in the event of any participant suffering harm as a result of their involvement in the research (Newline Underwriting Management Ltd, at Lloyd's of London). NHS indemnity operates in respect of the clinical treatment that is provided.

15. PUBLICATION POLICY

The Investigators will be involved in reviewing drafts of the manuscripts, abstracts, press releases and any other publications arising from the study. Authors will acknowledge that the study was funded by the Wellcome Trust. Authorship will be determined in accordance with the ICMJE guidelines and other contributors will be acknowledged.
To ensure results are available to study participants, they are often presented to patient charities/groups (e.g. myaware and encephalitis society) and published as open access journals.
Links to our scientific publications can be find via our group's online website:
https://www.ndcn.ox.ac.uk/research/autoimmune-neurology-group

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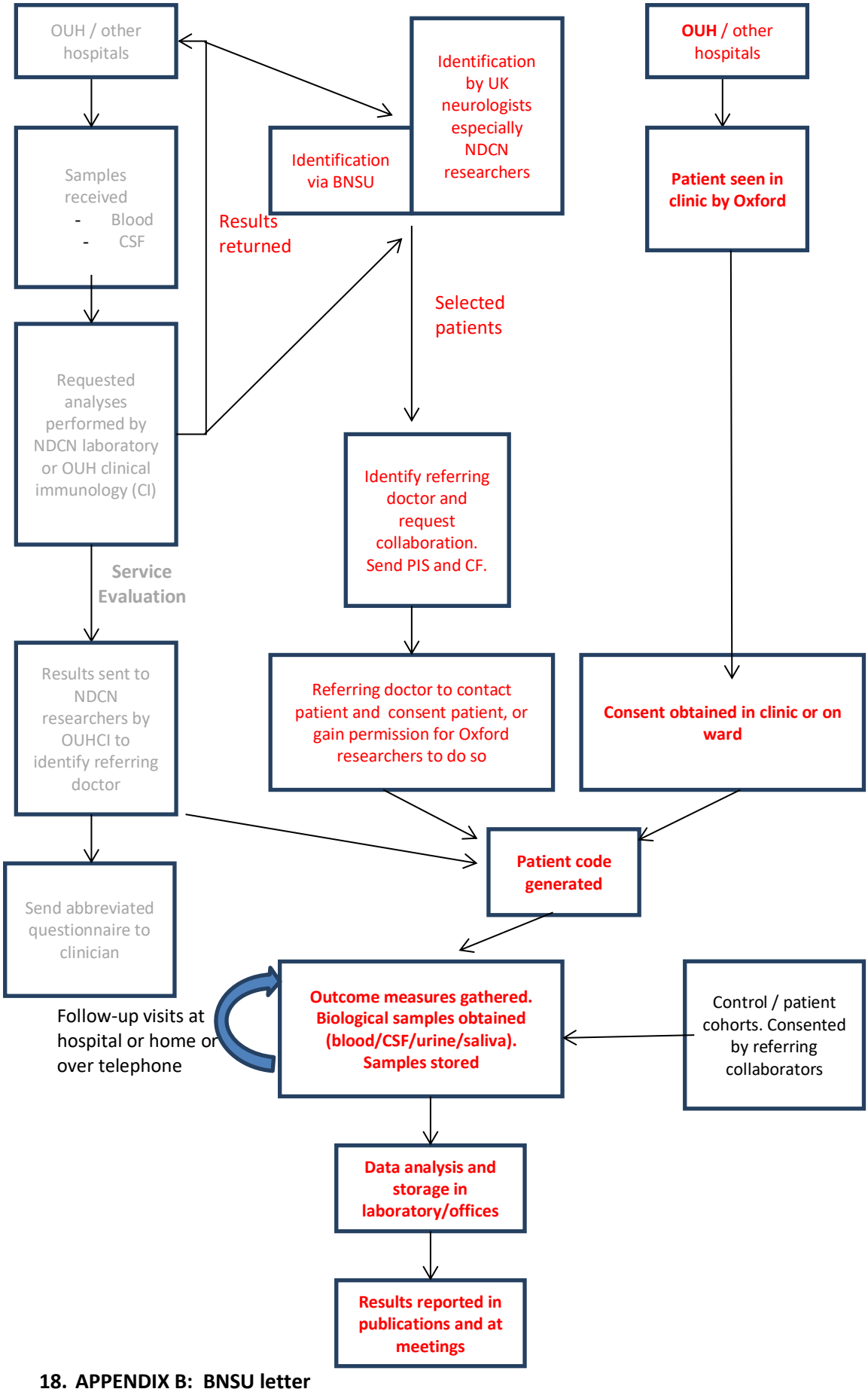
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17. APPENDIX A: STUDY FLOW CHART

*the grey text indicates the routine NHS laboratory work which will be a source of patient recruitment for NDCN researchers. It will be a route by which researchers will retrospectively and prospectively identify clinicians looking after patients who may be eligible research participants. In addition, the data generated by the NHS service provided by NDCN researchers will be regularly audited to understand service provision, outside of the auspices of this research project. OUH = Oxford University Hospitals; OUHCI = Oxford University Hospitals Clinical Immunology laboratory; NDCN = Nuffield department of Clinical Neurosciences; BNSU = British Neurological Surveillance Unit.

* the text in red indicates the direct research



18. APPENDIX B: BNSU letter

Association of British Neurologists

From the British Neurological Surveillance Unit

Dr Sarosh Irani
Nuffield Department of Clinical Neurosciences
Level 6, West Wing, John Radcliffe Hospital
Oxford. OX3 9DS

Tuesday, 25 June 2013

Dear Sarosh

British Neurological Surveillance Unit application for clinical syndromes associated with antibodies to either the VGKCc or the NMDAR

Thank you for your application, which has been reviewed by members of the ABN's CRAC. I am very pleased to let you know that CRAC would like the BNSU to support your proposed surveillance subject to the resolution of the following issues:

1. **Sample size.** We note that the REC application does not mention a target sample size, though BNSU likes to have guidance on this so that it can gauge for how long surveillance may be required. Can you estimate your target? I wanted to warn you that your estimate of 50-100 cases notified to BNSU is more than I would expect given BNSU's tendency to under-ascertainment. Because CRAC wants to support studies that have demonstrated feasibility by successfully identifying and recruiting participants using BNSU, we would only want to commit to five years of recruitment after seeing your first year report. We also wondered how many cases you think will not be ascertained by the neuroimmunology laboratory in Oxford?
2. **Portfolio adoption.** Please could you tell me whether this study has been adopted by the UKCRN, or whether it will be in future because it is funded by a partner organisation?
3. **REC approval**. At the moment the BNSU is not mentioned as a source of ascertainment in the REC application. Could you submit an amendment to the REC application mentioning BNSU?
4. **Approach for consent.** We note that in section 7 of the REC application that the research requires SSAs and that ethical approval has been given for "senior (post-membership) neurologists" and "local, referring clinicians" to obtain consent. However Part B Section 1 names only one research site. Have others been added? CRAC is concerned that these days research governance approval is currently required for every site at which participants are approached, even if only at a Participant Identification Centre (PIC) - http://www.crncc.nihr.ac.uk/Resources/NIHR%20CRN%20CC/CSP/PICs%20leaflet_DL_FINAL.pdf. We are concerned that neurologists who approach patients for you (even if they do not seek consent) would be expected – in the current regulatory environment – to have been approved by their local R&D department on an SSI form. We wonder if you can obtain approval for neurologists to offer patients information about your research for which they may be eligible, then the patients can contact you for formal eligibility assessment, provision of the information leaflet and completion of consent by post, which would avoid the need for a vast number of local PICs?

Once you have addressed these issues and I confirm approval of your application, please liaise with Josie Shew in the ABN office to create a page of information on the BNSU's section of the ABN website (http://www.abn.org.uk/StudyInformation/StudyInformation.aspx), modelled on the "white card" that you sent but also including further helpful information (you may want to take a look at some of the other studies' pages). We could begin surveillance from January 2014 as you suggest, or sooner if you wish.

Yours sincerely

Rustam Al-Shahi Salman

Prof. Rustam Al-Shahi Salman MA PhD FRCP Edin
MRC senior clinical fellow and honorary consultant neurologist

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APPENDIX C: AMENDMENT HISTORY

Amendment No.	Protocol Version No.	Date issued	Author(s) of changes	Details of Changes made
1	3	19/01/2017	Dr Adam Al-Diwani	Addition of Lymph node aspiration Addition of option of participant blood sampling at GP practices Addition of investigators
2	4	22/06/2018	Dr Sophia Michael AProf Sarosh Irani	Removal and addition of investigators, and amendment of current investigators' titles Updated CTRG telephone number – Participant and Parental Information Sheets Inclusion of use of in vitro and in vivo models to study the effects of patient immune factors. Adjustment of planned sample size to account for anticipated increased number of samples Amended specificity of lymph node sample site from 'submental' to 'cervical' Addition of bone marrow aspiration and stool collection and creation of 'Appendix D'. Expansion of control groups, with new information and consent sheets for 'Non-autoimmune participants' Study funders updated to include ONO Pharmaceuticals

Appendix D

Oxford Radcliffe Hospitals NHS
NHS Trust

Clinical Haematology Department

Having a Bone Marrow Test

Information for patients

You may find this leaflet helpful if you or someone close to you is going to have a bone marrow test.

It explains:

- what bone marrow is
- what a bone marrow test is and why you might need one
- what a bone marrow test involves.

What is bone marrow?

Bone marrow is a sponge-like material found inside certain bones of the body. Blood cells are made in the bone marrow. Blood is made up of three main types of cells: red blood cells, white blood cells and platelets. Disorders affecting any of these cell types can be assessed by examining a sample of the bone marrow.

What is a bone marrow test?

In a bone marrow test a sample of bone marrow is taken from inside the bone. This is usually taken from the back of the hip bone, as there is plentiful bone marrow here and it is usually easy to reach. The sample can then be examined under a microscope in a laboratory.

Why do I need a bone marrow test?

Some of your symptoms or abnormalities on your blood tests suggest that there might be a problem with the way your blood cells are made. A bone marrow test is the best way of investigating this to help the doctors to find a diagnosis.

How is the bone marrow test done?

The doctor or nurse will discuss the procedure with you, give you an opportunity to ask questions and ask you to sign a consent form.

You will be asked to lie on your side with your knees bent up and your clothing loosened to expose the top of your hipbone. Your skin will be cleaned with an antiseptic solution. Local anaesthetic will then be injected into the skin over the back of the hip bone to numb the area where the sample is to be taken.

Once the area is numb, a needle will be passed through the skin into the bone and a sample of liquid marrow will be drawn up into the syringe. This is called a bone marrow aspirate. Sometimes a second needle is used to take another sample of the more solid bone marrow tissue: this is called a bone marrow trephine or biopsy.

After the test the nurse will place a small dressing over the site. You may shower or bathe and remove the dressing after 24 hours.

Are there any risks?

It is exceedingly rare for a bone marrow test to be complicated. As with any procedure involving a tissue biopsy, there is a small risk of bleeding from the puncture site: if you are taking medications to thin your blood (e.g. warfarin, aspirin) it is important to discuss this with your doctor before attending for the bone marrow test.

There is also a small risk that the needle puncture site could become infected after the procedure. We take extreme care to keep the procedure sterile to minimize this risk.

Please note that there are additional risks if you choose to be sedated for the procedure. These are described in a separate information sheet and must be discussed with your doctor well in advance of the procedure.

Medication

It is important to tell the nurse or doctor about any tablets or injections you are taking, as they may have to be stopped for a short while before having the test. This is especially important for medications that thin your blood, including warfarin.

How long does it take?

It usually takes about 10-15 minutes to take the sample.

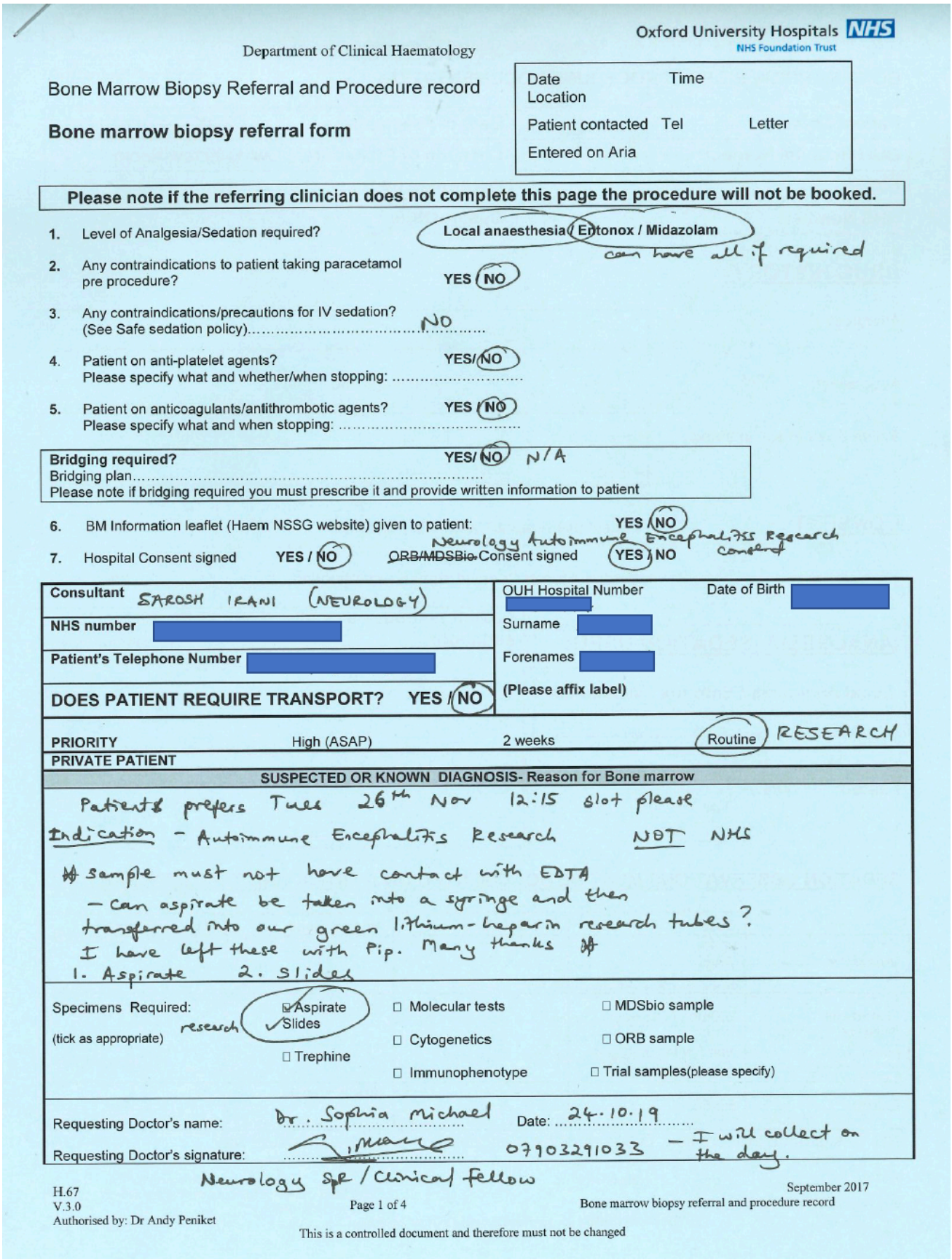
Where is it done?

The test can be done in the Day Treatment Unit.

When will I get the results?

It will take approximately two working days to get the bone marrow aspirate result. A trephine result takes considerably longer (a minimum of ten days) due to the nature of the tests which are done on this sample. We realize that it is a stressful time waiting for results, so your doctor will let you know as soon as possible.

Appendix 5.9 Bone Marrow Referral Form



Department of Clinical Haematology

Oxford University Hospitals NHS
NHS Foundation Trust

Bone Marrow Biopsy Referral and Procedure record

Bone marrow biopsy referral form

Date	Time
Location	
Patient contacted	Tel Letter
Entered on Aria	

Please note if the referring clinician does not complete this page the procedure will not be booked.

1. Level of Analgesia/Sedation required? **Local anaesthesia / Entonox / Midazolam** can have all if required
2. Any contraindications to patient taking paracetamol pre procedure? YES / **NO**
3. Any contraindications/precautions for IV sedation? (See Safe sedation policy)..... NO
4. Patient on anti-platelet agents? YES/ **NO**
   Please specify what and whether/when stopping: .....
5. Patient on anticoagulants/antithrombotic agents? YES / **NO**
   Please specify what and when stopping: .....

Bridging required? YES/ **NO** N/A
Bridging plan.....
Please note if bridging required you must prescribe it and provide written information to patient

6. BM Information leaflet (Haem NSSG website) given to patient: YES / **NO**
7. Hospital Consent signed YES / **NO** ~~ORB/MDSBio~~ Consent signed Neurology Autoimmune Encephalitis Research consent **YES** / NO

Consultant SAROSH IRANI (NEUROLOGY)	OUH Hospital Number [redacted] Date of Birth [redacted]
NHS number [redacted]	Surname [redacted]
Patient's Telephone Number [redacted]	Forenames [redacted]
DOES PATIENT REQUIRE TRANSPORT? YES / **NO**	**(Please affix label)**

PRIORITY High (ASAP) 2 weeks **Routine** RESEARCH
PRIVATE PATIENT

SUSPECTED OR KNOWN DIAGNOSIS- Reason for Bone marrow

Patient prefers Tues 26th Nov 12:15 slot please
Indication - Autoimmune Encephalitis Research NOT NHS
** sample must not have contact with EDTA
- Can aspirate be taken into a syringe and then transferred into our green lithium-heparin research tubes? I have left these with Pip. Many thanks **
1. Aspirate 2. Slides

Specimens Required: (tick as appropriate) research
- ☑ Aspirate ✓ Slides
- ☐ Trephine
- ☐ Molecular tests
- ☐ Cytogenetics
- ☐ Immunophenotype
- ☐ MDSbio sample
- ☐ ORB sample
- ☐ Trial samples(please specify)

Requesting Doctor's name: Dr. Sophia Michael Date: 24.10.19
Requesting Doctor's signature: [signature] 07903291033 – I will collect on the day.
Neurology Spr / Clinical Fellow

H.67
V.3.0
Authorised by: Dr Andy Peniket

Page 1 of 4

September 2017
Bone marrow biopsy referral and procedure record

This is a controlled document and therefore must not be changed