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MRes Research Project

An Investigation into the Vaccine Responsiveness of Multiple Sclerosis Patients being treated with Ocrelizumab

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Abstract

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), and the most common non-traumatic disabling disease affecting young adults. The exact etiopathogenesis remains elusive however major advances in the understanding of the disease have been made in the last few decades. The traditional view of T cells being the main players in the pathophysiology of MS has evolved and focus has shifted to the complex role of B cells and their contribution to disease progression, which is highlighted by the success of B-cell depleting therapies. Ocrelizumab is a humanised anti-CD20 monoclonal antibody approved by the FDA in 2017 for the treatment of people with Relapsing Remitting MS (RRMS) and Primary Progressive MS (PPMS). With the use of ocrelizumab, over 99% of peripheral B cells in the blood are depleted, which can last anywhere between 24 and up to 175 weeks. This study aimed to examine the impact of ocrelizumab on both the quantity and quality of antibodies produced in response to vaccinations as well as its broader influence on immunological memory. Finger prick blood samples were collected from people with MS, providing precise volume of blood from which antibodies could be measured and analysed. Short-term vaccine responses were assessed using ELISAs, measuring antibody reactions to SARS-CoV-2 proteins (Spike, RBD, Nucleocapsid). To explore longer-term immune memory, a microarray platform was developed to examine responses to various antigens. We designed a microarray antigen platform, containing viral-recall antigens and pathogen associated antigens in order to assess hundreds of MS patient IgG responses in a high-throughput manner. The study revealed that ocrelizumab treatment had a significant impact on both humoral responses to recent vaccines and longer-term recall responses. Notably, despite being vaccinated, many ocrelizumab-treated people with MS exhibited inadequate antibody responses to SARS-CoV-2 vaccinations compared to untreated people with MS. Additionally, patients overall demonstrated diminished responses to recall antigens. These findings underscore the importance of continued research into ocrelizumab and its implications for the immune system. Understanding its effects on vaccine responses and immunological memory will aid in optimizing treatment strategies for people with MS and enhance our knowledge of B-cell involvement in the pathophysiology of this complex disease.

List of Abbreviations

ADCC- Antibody-Dependent Cellular Cytotoxicity
AIC- Akaike Information Criterion
ARR- Annualised Relapse Rate
BAU- Binding Antibody Units
BBB- Blood Brain Barrier
CDC- Complement-Dependent Cytotoxicity
CDP- Confirmed Disability Progression
CIS- Clinically Isolated Syndrome
CNS- Central Nervous System
CSF- Cerebrospinal Fluid
CV- Coefficient of Variability
DMT- Disease Modifying Therapy
GLMM- Generalised Linear Mixed-effects Model
ELISA- Enzyme Linked Immunosorbent Assay
FDA- Food and Drug Administration
IFN- Interferon
IQR- Interquartile Range
IR-Infrared
IRR- Infusion Related Reaction
MABs- Monoclonal Antibodies
MS- Multiple Sclerosis
N- Nucleocapsid
NEDA- No Evidence of Disease Activity
OCB- Oligoclonal Band
OCR- ocrelizumab
OD- Optical Density
OLE- Open Label Extension
PNS- Peripheral Nervous System
PPMS- Primary Progressive Multiple Sclerosis
RRMS- Relapsing Remitting Multiple Sclerosis
RBD- Receptor Binding Domain
S- Spike
SPMS- Secondary Progressive Multiple Sclerosis
TT- Tetanus Toxoid

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1. Introduction

1.1 Multiple Sclerosis

1.1.1 What is Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), and the most common non-traumatic disabling disease affecting young adults. Over 2.8 million people worldwide live with MS and due to the disease course and clinical manifestations being highly variable, thus far, diagnosis and advising the correct treatment course has proved a challenge for clinicians (Dobson & Giovannoni, 2019). Despite some variation in the way MS is classified, there are four widely accepted clinical courses of MS. The first manifestation of MS is known as clinically isolated syndrome (CIS), which consists of an episode of neurological symptoms typical of MS. Although a person that has presented with CIS has a high likelihood of developing MS, not all do (Miller et al., 2012). Approximately 85% of people with MS are initially diagnosed with relapsing remitting multiple sclerosis (RRMS), which is characterised by clearly defined attacks of neurological symptoms followed by periods of partial or complete remission. Gradually most of these cases evolve into a secondary progressive form of MS (SPMS), which sees the disease steadily become more progressive and independent from evident phases of relapse and recovery (Cree et al., 2021). Moreover, about 10-15% of patients are affected by a slow progression of the disease from the onset of symptoms, with no phases of recovery or remission, known as primary progressive MS (PPMS). There is a myriad of neurological symptoms caused by MS; optic neuritis (vision loss/eye pain), asymmetric limb weakness, bladder dysfunction and ataxia (poor muscle control) are among the most common (Huang et al., 2017). Initial diagnosis and prognosis of MS encompasses the presence of clinical symptoms cooperatively with abnormal magnetic resonance images (MRI) on both gadolinium-enhanced T1-weighted scans which show currently active areas of MS, and T2-weighted images showing older/ inactive lesions in the patient's brain (Brownlee et al., 2017).

1.1.2 Aetiology and Pathophysiology of MS

The exact etiopathogenesis of MS is unknown however research shows a combination of genetic predisposition and environmental factors, examples including smoking, Epstein-Barr virus (EBV) and vitamin D, contribute to the aetiology through complex gene-environment interactions (Barkhane et al., 2022). Furthermore, due to the heterogeneity of MS the exact pathophysiology of the disease is also not fully understood, however major advances in the understanding of the disease have been made in the last few decades (Huang et al., 2017). It's an indisputable fact that the immune system plays a central role in the pathology of Multiple Sclerosis. (Trapp & Nave, 2008). In broad terms, MS is an inflammatory autoimmune disease mediated by lymphocytes crossing the blood brain barrier (BBB) and into the CNS

causing multiple focal areas of demyelination, gliotic scarring and axonal loss, known as MS lesions or plaques.

1.1.3 Treatment of MS

As mentioned, pathogenesis of MS includes alterations in the peripheral immune system and intrinsic CNS cells, breakdown of the blood-brain barrier, demyelination, oligodendrocyte loss and axonal degeneration. These elements of disease pathogeny are the targets of therapeutic strategies designed to dampen the disease (Yang et al., 2022). There is currently no cure for MS but over the past 30 years there has been significant development in MS research which has resulted in better treatment options. Treatments can be broadly split into two categories; symptom management therapies which focus on relieving the effects of neurological symptoms/relapse, and disease modifying therapies (DMTs) which aim to slow disease progression and reduce relapse rates. Due to the considerable improvement in knowledge of the pathophysiology of the disease, treatment has rapidly evolved over recent years and the concept of an endpoint measure, where there is no evidence of disease activity (NEDA), is becoming increasingly apparent (Baecher-Allan et al., 2018).

Table 1. Examples of current DMTs used in the treatment of MS.

Administration	Medication	Mechanism of Action	Approved For
Injection	-Interferon Beta (1α/1β)	-Reduce inflammation in the central nervous system	-RRMS, SPMS
	-Glatiramer Acetate	-Modifies the immune systems response to the CNS	-RRMS, SPMS
Oral Medications	-Fingolimod	-Reduces the number of white blood cells in the blood stream	-RRMS, SPMS, paediatric MS
	-Teriflunomide	-Reduces proliferation of activated T and B lymphocytes	-RRMS, SPMS
	-Fumarates	-Decrease inflammation and prevent nerve damage	-RRMS, SPMS
	-Cladribine	-Selectively depletes monocytes and lymphocytes	-RRMS, SPMS

Infusion	-Alemtuzumab	-Monoclonal antibody directed against CD52 receptors on lymphocytes	-RRMS, SPMS
	-Ocrelizumab	-Monoclonal antibody that depletes CD20+ B cells	-RRMS, SPMS, PPMS
	-Natalizumab	-Monoclonal antibody that blocks the movement of immune cells into the CNS	-RRMS, SPMS

Table 1 provides a comprehensive overview of several treatment options for individuals with MS. Despite the therapeutic advances in MS treatment over the last three decades, there remains a lack of effective treatment for PPMS, with ocrelizumab being the first and currently the only approved DMT by the food and drug administration (FDA) for patients with primary progressive disease(Yang et al., 2022). It's important to acknowledge that the landscape of disease-modifying therapies (DMTs) is constantly evolving. New therapies are emerging, and existing ones are undergoing refinement or being phased out. Therefore, the table above does not encompass the full spectrum of DMTs available or in development.

1.1.4 The role of B cells in MS

A new paradigm of the involvement of B cells in MS has been highlighted by the success of B cell-depleting therapies in reducing relapse rates and disease progression in people with MS (Comi et al., 2021). The traditional view of T cells being the main players in the pathophysiology of MS has evolved and focus has shifted to the complex role of B cells and their contribution to disease progression. It is now understood B cells also play a dominant role in inflammation and neurodegeneration in both an antibody-dependent and antibody-independent manner (Li et al., 2018). There is still much to be understood about which B-cell subsets and what effector functions are most important for driving the disease. There is evidence that B cells contribute by presenting antigens to T helper cells via MHC class II and costimulatory molecules, producing autoantibodies, and expressing both pro and anti- inflammatory cytokines which all contribute to CNS inflammation (Figure 1) (Kanatas et al., 2023).

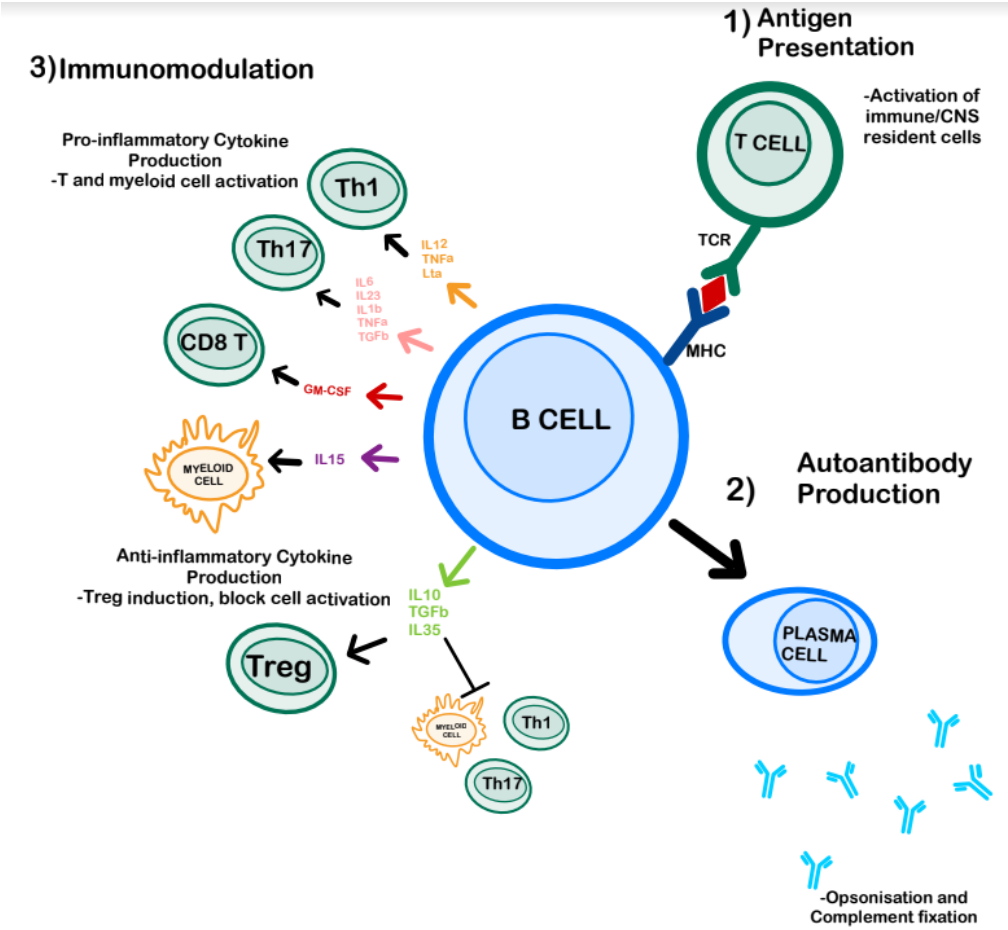


Figure 1- The role of B cells in Multiple Sclerosis. B cells act as 1) antigen-presenting cells (APCs) for the activation of T cells, 2) precursors of autoantibody secreting plasma cells and 3) producers of pro-inflammatory and anti-inflammatory cytokines.

Bidirectional interactions of B cell subsets, T cells and myeloid cells both in the periphery and CNS, and the consequences of these interactions underlie the pathology of MS (Li et al., 2018). Furthermore, the presence of IgG oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF), found in over 90% of people with MS is another demonstration of how B cells are involved in disease pathogenesis(Gharibi et al., 2020). OCBs are intrathecally produced by clonally expanded B cells, both OCBs and clonally expanded B cells are found in elevated numbers in the CSF of people with MS and are considered a hallmark of MS. Notably they correlate with conversion from CIS to RRMS and increased levels of disease activity and disability(Graner et al., 2020).

1.2 Ocrelizumab

1.2.1 Ocrelizumab Success in MS Treatment

Monoclonal antibodies (mAbs) are used for targeted drug therapy and are a key part of the treatment regimen in many diseases. Focusing on MS, mAbs have become one of the preferred treatments due to their high efficacy(Voge & Alvarez, 2019). In 2001 Rituximab, a chimeric anti-CD20 mAb designed for use in Cancer therapy showed efficacy in MS clinical trials. Based on the success of the off-label use of rituximab in RRMS, a fully humanised anti-CD20 mAb, ocrelizumab, was developed (Wesley & Hafler, 2020). OPERA I and OPERA II were two 96-week phase III, randomized, double-blind, double-dummy, parallel-group studies that evaluated the efficacy and safety of ocrelizumab compared to Interferon (IFN) β-1a in RRpeople with MS. The primary endpoint of decreased annualised relapse rate (ARR) showed a greater reduction in patients being treated with ocrelizumab ($p<0.0001$ in both). Furthermore, ocrelizumab treated patients showed fewer numbers of gadolinium-enhanced T1/ T2 hyperintense lesions on MRI scans and an increased number of patients with NEDA compared to patients being treated with IFN β-1a. Following the success of these trials ocrelizumab was approved by the FDA in 2017. Notably, a third trial, ORATORIO, resulted in ocrelizumab also becoming the first FDA-approved therapy for the treatment of patients with PPMS due to it meeting its primary endpoint: the percentage of patients with 12-week confirmed disability reduction (CDP) in ocrelizumab treated patients versus placebo was significantly lower (32.9% versus 39.3% respectively). More clinical and radiological efficacy of the trial was demonstrated by the 24 -week CDP also being significantly reduced in ocrelizumab treated patients, and the total volume of T2 weighted lesions reduced in patients treated with ocrelizumab and increased in the Placebo group (Mulero et al., 2018).

A common practice following completion of the trials investigating new DMTs is giving patients the option to enter an open-label extension (OLE) studies, to provide more long-term information about the DMT. Results from the OLEs demonstrate that years after FDA approval, ocrelizumab still continues to show a favourable benefit-risk profile. Evidence that earlier and continuous treatment with ocrelizumab had clinical and radiological benefits. This was demonstrated by ocrelizumab treated patients maintaining a significantly lower ARR than IFN β-1a treated patients. Patients that continued with ocrelizumab maintained suppression of new MRI lesions and those switching from IFN β-1a attained it. (S. L. Hauser et al., 2020)

1.2.2 Anti-CD20 Therapy/ Mechanism of ocrelizumab

CD20 is expressed by the majority of B cells starting at the pre-B cell stage of development and is only lost when B cells terminally differentiate into plasma cells (Figure 2). CD20 is a transmembrane protein thought to exist predominantly as a tetramer on the cell surface of B cells.

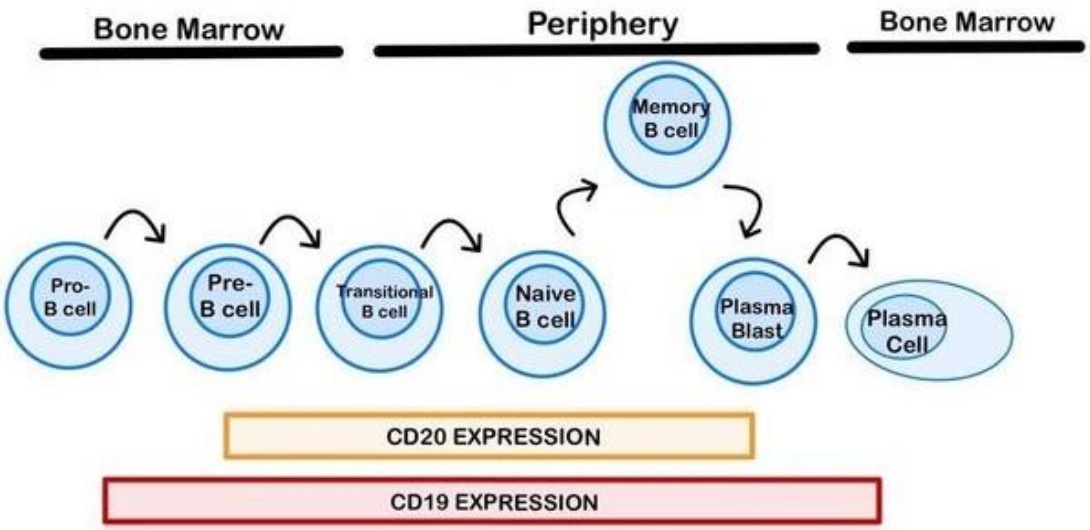


Figure 2- CD20 and CD19 expression during B cell development. During B cell maturation CD20 is expressed in pre-B cells and mature B cells which include transitional, naïve and memory B cells. CD20 expression is downregulated on Plasma Blasts and completely lost on terminally differentiated Plasma cells. Both early and late maturation stages are not depleted as they do not express CD20. CD19 expression is observed earlier in B cell maturation and is found to persist on plasma blasts and some plasma cells.

Ocrelizumab is a Type I anti-CD20 monoclonal antibody used in the treatment of RRMS and PPMS. The exact mechanism of the drug is not fully understood however ocrelizumab has complementarity determining regions (CDRs) which form a paratope (the antigen-binding site) which recognises and binds to a specific epitope on the large extracellular loop of CD20. Following binding of CD20, antibodies induce a translocation into 'lipid-rafts' and trigger at least three different effector functions: antibody-dependent cellular cytotoxicity (ADCC); complement dependent cytotoxicity (CDC); and induction of cell apoptosis (Margoni et al., 2022). The localisation of CD20 into lipid rafts is a characteristic of Type I anti-CD20 antibodies due to inter-tetramer binding in which it is hypothesised that these mAbs can bind to several molecules of CD20. This enhances the recruitment and activation of complement, resulting in ocrelizumab exerting potent CDC (Cotchett et al., 2021). Monoclonal antibodies are broadly categorised into type I and type II. Type II antibodies also bind CD20 but cause no redistribution and as such there are key differences in type I/II antibody characteristics and effector functions (Casan et al., 2018). Within the type I category, there can be variations in their binding affinity, specificity, and downstream effects. Different monoclonal antibodies target different proteins or epitopes on cells, leading to diverse effects. Additionally, factors such as the antibody's structure, pharmacokinetics, and interactions with the immune system can influence their overall behaviour and therapeutic efficacy (Marshall et al., 2017), so while type I mAbs share a common mechanism of action, they can vary significantly in their specificities

and therapeutic outcomes and therefore our results would not apply to different type I anti-CD20 mAbs.

1.2.3 B cell Depletion

Ocrelizumab, sold under the brand name Ocrevus, induces a significant depletion of several B-cell subsets. DMTs such as ocrelizumab are immunosuppressive and can impact the immune response resulting in an increased probability of infections (Winkelmann et al., 2016). Ocrevus is administered as an intravenous infusion firstly as two 300mg doses two weeks apart, followed by a 600mg dose every six months. As described, due to the mechanism of ocrelizumab, CD20+ B-cells are rapidly depleted at the time of infusion and can remain undetectable for a prolonged and variable period of time ranging from 6 months to more than 12 months. Data from the OPERA I/II trials shows the median time for B cells to return to baseline was 72 weeks after the final Ocrevus infusion with a range of 27-175 weeks (Genentech, 2017). Furthermore, Hauser and colleagues provided evidence that repeated dosing, and greater exposure with the drug is associated with greater reduction of B-cell subsets. In a study examining the relationship between ocrelizumab exposure and B-cell counts in patients, an intriguing pattern emerged. Patients with lower B-cell counts tended to exhibit lower levels of ocrelizumab exposure. Additionally, repeated dosing of ocrelizumab led to a continued decline in B-cell counts across all patient groups. To investigate this further, researchers divided the patients into four exposure quartiles (Q1 to Q4) based on the mean ocrelizumab exposure over the 96-week study period, with Q1 representing the lowest exposure to the drug. As anticipated, patients in the Q1 quartile, with the least exposure to ocrelizumab, exhibited a significantly higher median B-cell count throughout the study duration. Moreover, B-cell counts were measured every 24 weeks, just before each subsequent ocrelizumab infusion. Interestingly, regardless of the exposure quartile, all patient groups demonstrated a consistent trend: repeated dosing of ocrelizumab led to a sustained decline in B-cell counts over time. The observed decline in B-cell counts with each successive dosing highlights the ongoing impact of ocrelizumab therapy on the immune system over the course of treatment. (Hauser et al., 2021).

1.2.4 Long term use of ocrelizumab

With the use of immunocompromising drugs such as ocrelizumab there are several implications that need to be considered, especially in the context of long-term use of the drug. As discussed, treatment with ocrelizumab induces a significant depletion of all B cell subsets, leaving patients with an impaired humoral immune response. Whilst six-monthly dosing with ocrelizumab controls MS relapses, B cell depletion is maintained which can result in the inhibition of seroconversion in response to viral antigens and vaccines. Data

has shown that after 3-4 cycles of ocrelizumab only 3-5% of patients exhibit 1% B cells at 6 months following their last infusion, compared to 85-90% of patients exhibiting 1% B-cells at 12 months (Baker et al., 2022a). Studies have also suggested that despite not expressing CD20, the plasma cell population may be at risk of being reduced over time due to depletion of its progenitor cells, meaning repeated cycles of ocrelizumab may also lead to reduced plasma cell production and this could result in hypogammaglobulinemia (Laurent et al., 2023a). A 7-year follow up study of the patients in the OPERA and ORATIO trials revealed a proportion of people being treated experienced decreased immunoglobulin levels, with IgM levels dropping steadily over time and IgG levels dropping significantly after the fifth cycle of ocrelizumab (Habek et al., 2022). It's important to note that the half-life of antibody secreting plasma cells varies greatly, and they can be generally divided into two distinct categories; short lived (with a lifespan of 3-5 days) and long lived (with a lifespan varying from several months to a lifetime) (Khodadadi et al., 2019). Studies in mice and animal models have demonstrated that long-lived plasma cells can survive in the absence of memory B cells, however after memory b-cell depletion, antigen specific serum-antibody declined compared to untreated controls (Hammarlund et al., 2017). The mechanisms by which some plasma cells maintain long-term antibody production are not fully understood and the variation between some antigen-specific responses fading over time and some being essentially maintained for life can have a significant impact on the data generated when investigating the vaccine responsiveness to different antigens in ocrelizumab-treated individuals with MS.

1.3 Risks and side effects associated with B cell depleting Therapies.

1.3.1 B cells and Antibody Responses

The safety of anti-CD20 therapies have raised concerns in clinical trials and observational studies due to the consequences of immunosuppression. Anti-CD20 mAbs are prescribed as maintenance treatments, and due to the constant depletion of peripheral B cell subsets, including naïve, memory, transitional and regulatory-B cells in treated patients, raises questions regarding the long-term safety of the drug. After 6 months, the standard dose interval of ocrelizumab, Baker and colleagues showed that only 3-5% of people had repopulated to 1-3% B cell count (Baker et al., 2022b). Adaptive immunity acts through humoral and cell mediated immune responses, by recognising specific proteins which induces tools for targeting these specific pathogens. Humoral immunity is a function of B cells, which produce antigen-specific molecules known as antibodies. Lymphocytes known as naïve B cells circulate throughout the blood in the lymphatic system and differentiate into memory and effector B cells when they come into contact with a foreign antigen such as an infectious pathogen (Witman Tsur et al., 2021). With a

reduced immune response, the safety of the long-term use of ocrelizumab is a concern due to the constant increased risk of invading pathogens causing viruses and infections, and patients being unable to form an adequate antibody response (Lycke & Svenningsson, 2022).

1.3.2 COVID 19

In light of the recent COVID-19 pandemic, there were many concerns regarding the safety of using immunocompromising drugs. Examples including the potential infection risks and whether patients would be able to elicit an effective vaccine response, as well as some evidence of correlation between B cell depleting therapy and a more severe disease course (Zabalza et al., 2021) were challenges clinicians had to take into consideration. Notably, ocrelizumab was found to be associated with higher risks of hospitalisation and intensive care unit admission (Simpson-Yap et al., 2021). As such, ocrelizumab infusions were delayed by 1-3 months during the pandemic. Studies showed that extended interval dosing (EID) of ocrelizumab infusions didn't impact the effectiveness of the therapy (Baker et al., 2022d; Rolfes et al., 2021). Unsurprisingly, patients on ocrelizumab have been shown exhibit poor seroconversion following SARS-CoV-2 natural infection and vaccination (Sormani et al., 2021). Both lower ocrelizumab concentration, and higher B cell count have been associated with higher SARS-CoV-2 antibody titres and a better ability to develop a humoral response to the virus (Kempen et al., 2023). Unsurprisingly as the time goes by after an ocrelizumab infusion, the concentration of ocrelizumab in the blood decreases and B cell count increases, which in turn can ameliorate the effects of the DMT on the immune system. Clinicians have raised the question of whether EID may be beneficial to patients as it would allow them to replenish their B cells and optimise seroconversion in order to form adequate antibody responses to the SARS-CoV-2 virus (Baker et al., 2022d).

In addition to their role as mediators of the antibody response, B cells also function as antigen-presenting cells (APCs), presenting antigens to T cells to initiate cell-mediated immunity. Ocrelizumab likely induces an anti-inflammatory effect primarily through abrogating B cell functions such as antigen presentation (Holloman et al., 2021). However, Studies have shown persistence of a highly diverse T-cell repertoire suggests that aspects of adaptive immunity remain intact despite extended anti-CD20 therapy (Laurent et al., 2023b). A small study has shown that the SARS-CoV-2 virus can be eliminated by the innate and T-cell response before an efficacious IgG response has been formed and recovery from the virus can still occur in the absence of B cell activation (Cords et.al, 2022). However, while viral-clearance has been shown in B-cell depleted patients, ideally patients should be able to elicit a strong humoral response to COVID-19. There are concerns such as serial mutations developing in immunosuppressed individuals as without an antibody response, they may harbour a longer SARS-CoV-2

infection (Gibson et al., 2021), thus it would be beneficial to the have the full spectrum of immunity with innate, T, and B cell responses.

1.3.3 Antigen Specific Serology and responses to Pathogen Associated Antigens

Vaccination against infectious diseases is an important aspect of MS disease management because infections to certain diseases can exacerbate MS symptoms and cause flare-ups. As mentioned, a normal B cell response may not be crucial to patients on ocrelizumab as they will still have protection in the form of their innate and cell-mediated responses. However, there is limited data available on the responses of anti-CD20 immunocompromised patients and other virus' and moreover their ability to seroconvert and mount an immune response to several pathogens and virus' that are commonly vaccinated against at childhood and throughout life, examples being Influenza A and Tetanus Toxoid (TT). The VELOCE study was a phase IIIb study that evaluated the effects of ocrelizumab on immune response to selected vaccines in participants with RRMS. The study showed that peripherally B-cell depleted people with MS on ocrelizumab could mount humoral responses to vaccines (TT and influenza being amongst several they studied), albeit their responses were attenuated. (Bar-Or et al., 2020a). Even within the relatively short timeframe of 24 weeks, it became apparent that patient responses to vaccination were attenuated.

The role of B cells is to produce antibodies to destroy antigens and form antigen specific memory in order for the immune system to launch an effective defence for protection against future infection with the same antigen (White, 2021). In the context of our study on the effects of ocrelizumab on the immune system, we aimed to investigate how this medication may impact individuals' responses to commonly encountered antigens and pathogens, gained from both natural infection and vaccination. To assess this, we specifically examined the immune response to several well-known pathogens, including seasonal coronaviruses, herpesvirus, influenza, mumps, rubella, rubeola, and varicella-zoster virus (VZV). These pathogens were selected due to their prevalence in the population and the importance of understanding how ocrelizumab treatment may influence immune responses against them. In addition to these commonly encountered antigens, we also included some less frequently encountered antigens, such as Hepatitis E virus (HEV), Hepatitis D virus (HDV), Helicobacter pylori (H. pylori), Adenovirus, Parvovirus, and Clostridium difficile toxin A (TcdA). By including these less commonly encountered antigens, we aimed to comprehensively evaluate the effects of ocrelizumab on the immune system's ability to respond to a diverse range of pathogens. In addition to assessing responses to infectious

pathogens, our study also included the evaluation of autoantibody responses to investigate potential effects of ocrelizumab on autoimmune processes. By examining changes in autoantibody levels, we aimed to provide a comprehensive understanding of how ocrelizumab influences both immune responses to pathogens and autoimmune reactivity.

1.3.4 Methodologies for studying the effect of ocrelizumab on MS patient antibody responses

To measure secreted antibodies in blood samples, specific and sensitive Enzyme-Linked Immunosorbent Assays (ELISAs) are very well suited. While ELISAs are a powerful method for detecting and quantifying specific antibodies in a patient sample, they only perform a single parameter analysis tand therefore require more time, patient sample and can be more expensive for multi-parameter analyses. Multiplex immunoassays confer several advantages as the amalgamation of target analytes into a multiplex immunoassay panel allows for the simultaneous measurement of multiple analytes in a single patient sample, thereby reducing the cost and time of multi-parameter immunoassays (Tighe et al., 2015). Protein microarrays allow for comparative profiling as many proteins are immobilised onto a surface, exposed to treatment with a sample, and probed with detection antibodies labelled with a reporter dye which enables analysis of the fluorescent signals given for each protein. A combination of both ELISAs and protein microarrays (Figure 3) will allow for the qualitive and quantitative detection of antibodies within patient samples in order to study the effects of ocrelizumab on patient antibody responses over time. ELISAs will be used for studying patient immune responses to SARS-CoV-2 proteins and quantifying the levels of specific antibodies in patient samples for an in-depth analysis on these proteins (Figure 3A). On the other hand, microarrays will be used to study a wide range of viral-recall antigens, pathogen associated antigens and autoantibodies simultaneously (Figure 3B) to provide a broad view of antibody responses across this range of antigens. This high-throughput screening will allow us to discover whether ocrelizumab has an impact on a broad spectrum of antibody responses.

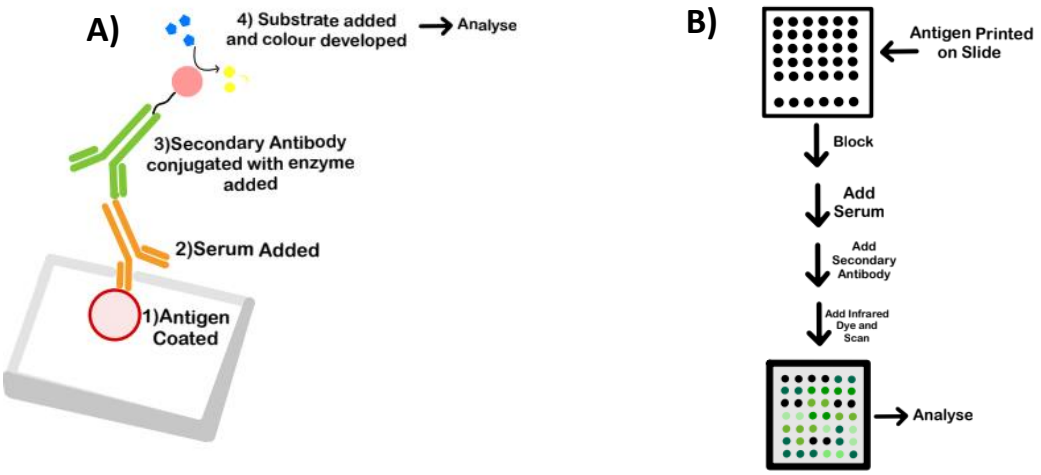


Figure 3- Methods for Antibody Detection and Analysis. A) The indirect ELISA method is one technique for antibody detection. In this schematic the steps of this ELISA are depicted: (1) Coating the plate with target antigens; (2) Serum sample incubation; (3) Secondary Antibody conjugated to an enzyme is added and binds to the primary antibodies in the sample; (4) Signal development with a substrate and (5) Analysis. B) The Microarray method is another approach for antibody profiling. The schematic shows the key steps of the method: (1) Antigens are printed onto a slide; (2) The slide is blocked; (3) The sample is added and binds to the arrayed antigens; (4) Secondary Antibodies are added; (5) Infrared (IR) dye is added, and a signal develops; (6) Analysis.

1.4 Aims

The aim of this study was to examine the effects on both the quality and quantity of antibodies produced as a result of vaccination attempts when combined with the periodic treatment of ocrelizumab for patients with MS. Finger prick blood samples were obtained and examined from patients undergoing ocrelizumab treatment at different time points during ocrelizumab treatment and having been vaccinated against SARS-CoV-2. Assessment of recall responses to common pathogens and well-known vaccines, which a long-lasting will also be examined to observe if there is any substantial difference in these responses over time with ocrelizumab treatment. This study sought to provide insight into the dynamics of vaccine responsiveness in relation to ocrelizumab treatment and aimed optimization of delay after treatment before an effective vaccination response could be achieved. Observation of past vaccine responses provided insight into the overall effect of vaccination on long-term antibody responsiveness to pathogens and childhood vaccines in people with MS.

We hypothesized that ocrelizumab significantly modulated antibody responses in people with MS and that extended use of ocrelizumab would lead to changes in memory recall responses. A comprehensive study into these effects improved our understanding of the immunological consequences of the therapy and its implications in patient care.

2.Materials and Methods

2.1 Antigens used in ELISAs and Microarray's

Antigens used in conventional ELISAs and to produce the microarray panel are listed in Table 2 and Table 3 respectively.

Table 2. SARS-CoV-2 and Seasonal Coronavirus Antigens used in ELISA's.

Antigen	Company	Company code
Sars-CoV-2 Spike S1 (Wuhan Strain)	Sino Biological Inc.	40951-V08H
Sars-CoV-2 Nucleocapsid (Wuhan Strain)	Sino Biological Inc.	40588-V08B
Sars-CoV-2 Receptor Binding Domain (Wuhan Strain)	Sino Biological Inc.	V0592-V08H82
HCoV-OC43	Sino Biological Inc.	40607-V08H1

Table 3. Antigens used on Microarray Panel.

Antigen	Company	Code
Candida Albicans	NIBSC	77/600
Carbamylated BSA	Made in Lab	N/a
CD20	Sinobiological	11007-H07H2
CMV	Native Antigen Company	CMV-HP-100
Collagen Human Lung	Sigma	C5983
DsDNA	Diarect	12301
EBV-VCA	Meridan Life Science Inc.	8202
H.Pylori CagA	Prospec	Pro-2494-a
HCoV-229E	Sino Biological Inc.	V0601-V08H
HCoV-HKU1	Sino Biological Inc.	40021-V08H
HCoV-OC43	Sino Biological Inc.	40607-V08H1
HDV	Prospec	hdv-234-a
HEV	Prospec	hev-235-a
HSV1	Meridan Life Science Inc.	7305
HSV2	Meridan Life Science Inc.	7705
Influenza A H1N1	Fitzgerald	30R-AI038
Mumps	Meridan Life Science Inc.	8099
Native Adenovirus	BIO-RAD	0152-0507
Parovirus	NativeAntigen	PV-VP2-100
Rubella	Meridan Life Science Inc.	6075
Rubeola	Meridan Life Science Inc.	7604
TcdA	Native Antigen	CDA-TNL-50
Tetanus Toxoid	NIBSC	02/232
Thyroglobulin Human Thyroid	Sigma Aldrich	609312
VZV	Meridan Life Science Inc.	7740

2.2. Patient Recruitment and Data Collection

Participants for this study were recruited from the patient pool at the Queens Medical Centre in Nottingham, United Kingdom. Eligible participants were individuals diagnosed with multiple sclerosis (MS) who were undergoing treatment with ocrelizumab at the centre. Upon obtaining informed consent, participants were scheduled for sample collection. Samples were collected at random times during their ocrelizumab treatment and at random times relative to COVID-19 vaccine administration. Patients' medical records were reviewed to gather information on their COVID-19 history, vaccination status, and infusion schedules of ocrelizumab, with specific dates recorded for each event. Additionally, for repeated samples, they were still taken at random times relative to their ocrelizumab treatment and COVID-19 vaccine administration. Repeat samples were only taken if an individual's circumstances had changed, such as receiving another COVID vaccine or another infusion of ocrelizumab to capture any potential effects of these changes on the variables under study.

2.3. Sample Collection

Dried blood samples were collected individually by patients using a safety lancet and 10 ml Mitra-stick sampling device. Samples were stored on Mitrastix for up to 1 month. Using deep 96-well plates (neoteryx) antibodies were extracted from dried samples on the Mitra-sticks by diluting to 1:15 in 150 µL phosphate buffered saline (PBS) containing 0.1% Triton-x100 and 0.05% Sodium Azide and shaking for 2 hours. Extracted samples were stored at -20°C from 0-9 months.

2.4. Coronavirus ELISAs

2.4.1 ELISA Protocol

All steps were performed on Opentrons OT-2 Robots (Opentrons Labworks,USA). Extracted samples were diluted to 1:120 with 3% whey powder in PBS containing 0.25% tween 20, 0.05% NaN3 and 0.1% EDTA in a 96 well PCR holder (ABgene). Enzyme linked immunosorbent assays (ELISA) were performed by coating 384 well NUNC maxisorp plates (Thermofisher) with 1µg/ml of SARS-CoV-2 (Wuhan) antigens or Seasonal Coronavirus antigens (Table 2) in Carbonate Bicarbonate buffer.

Plates were sealed with foiled film and incubated overnight at 4°C. Wells were then washed with 100 µL PBS with 0.05% Tween 20 (PBST) three times using a Thermofisher Wellwash Versa plate washer. Wells were immediately blocked with 120µL of 3% whey powder in PBS containing 0.25% tween 20, 0.05% NaN3 and 0.1% EDTA and stored overnight at 4°C.

Wells were washed again 3 times as above. 20 µL of pre-diluted serum sample was added into wells with duplicate wells per sample and incubated for 15 minutes at 21°C. Wells were washed again 3 times as above followed by the addition of 20 µL of gamma chain-specific anti-human IgG-HRP conjugate (Sigma A0170–1ML) at 1:30,000 dilution in PBS-Tween and incubated for 15 minutes at 21°C. Following a final three washes, 40µL of One-Step ultra-TMB solution (Thermofisher) was added to each well and incubated for 20 minutes at 21°C. Finally, 40µL of 2N H_2SO_4 was added to each well. Absorbance was measured at 450nm using a BioTek Microplate reader (Agilent).

2.4.2 Formation of standard curve

SARS-CoV-2 antibody positive and antibody negative serum controls were obtained from the National Institute of Biological Standards and Controls (NIBSC, UK). Each assay contained a 12-point standard dilution of NIBSC 20/162 positive controls diluted two-fold from 1:120 to provide a standard curve to show very strong, moderate, and weak positive signals. A negative control from the assay verification panel and the QC standard(20/B764) were also diluted to 1:120 and added to the assay in separate wells in duplicate to create a negative cut-off and to ensure the accuracy and reliability of the assay, and two wells were used as non-serum blanks.

2.5. Microarray Protocol

2.5.1. Formation of the Microarray Antigen Panel

Each antigen (Table 3) was diluted to 100 µg/mL (4M Betaine, 50% Butanediol) and loaded onto a 384 well plate (Corning). These were printed onto nitrocellulose coated glass slides(handmade) as two sets of duplicate spots in a 13 x 13 array format using a Biorobotics Microgrid II arrayer and a contact pin (Solid Steel). Each slide was printed with 16 separate arrays, allowing the processing of 16 samples on the same slide simultaneously. The humidity of the array chamber was set to 60%, slides were left overnight on the arrayer and processed the following day.

2.5.2. Processing patient samples

Slides were loaded into 16-well slide holders, wells were washed four times with PBST and blocked for one hour with I-block (Tropix). Patient sera were diluted to 1:200 in I-block, 150 µL of diluted sera was added to each well for one hour. Following another wash, 150 µL of biotinylated IgG (Vector Laboratories inc.) at 1:5000 in I-block with 3% goat serum (Sigma, G6767) was added to each well for one hour. Wells were washed again and 150 µL of infrared dye (IR680-Streptavidin, Li-Cor) at 1:5000 in PBST was added for a final incubation (covered in foil) for 20 minutes. After a final wash, slides were rinsed in ultrapure water and dried for five minutes by centrifugation in a microarray slide spinner (Labnet). All washes and incubations were conducted at room temperature with gentle agitation on a microplate shaker. Slides were

scanned on a Licor Odyssey (M) at 700nm. Images obtained were converted from 32-bit to 16-bit TIFF images using fiji (Schindelin et al., 2012). The chemiluminescent signal was quantified using the GenePix Pro Software (Axon GenePix).

2.6. Optimisation and Validation of Antibody Microarray Platform

All optimisation and Validation experiments were carried out before final protocol described in section 2.5.

2.6.1. Antibody and Dye dilutions

We used printed nitrocellulose slides to test different concentrations of antibody and dye dilutions (see 2.4.1 for method). We used a control serum to process the slides. We tested different dilutions of antibody (company): 1:1000; 1:5000; 1:10000 with different dilutions of Streptavidin IR Dye (IR680-Streptavidin, Li-Cor): 1:1000; 1:5000; 1:10000. All steps carried out were the same as in section 2.5.2. The intensity levels of the chemiluminescent signals were compared against one another to distinguish which combination of antibody and IR dye dilution would give the best results.

2.6.2. Blocking Buffers

We printed slides with control antigens Tetanus, Candida and CMV (see 2.4.1 for method). We processed the slides with control sera. We tested several blocking buffers including I-block (Tropix), Smart block (Candor Bioscience), 3%BSA (Sigma), Low Cross Buffer (Candor Bioscience) and The Blocking Solution (Candor Bioscience). All steps carried out were the same as in section 2.5.2. The intensity levels of the chemiluminescent signals were compared against one another to distinguish which blocking buffer would give the best results.

2.6.3 Intra-assay and Inter-assay Validation

All antigens were printed as mentioned in section 2.5.1. Eight patient samples were chosen at random. All samples were processed as in section 2.5.2. To determine the precision of the Microarray platform, intra-assays were performed by processing the eight samples in duplicate on the same slide on the same day. Inter-assays were performed by processing the eight samples on two different slides three days apart. Precision was measured by calculating the Coefficient of Variability (CV). %CV was measured for each antigen by finding the standard deviation (SD) of duplicate results, diving by the duplicate mean and multiplying by 100. A CV of less than 20% was accepted.

2.7 Statistical Analysis

2.7.1. ELISA data

All ELISA data was exported into GraphPad Prism and R studio for analysis and visualisation. Antibody responses were compared between people with MS not receiving OCR treatment and patients receiving OCR treatment and were tested for significance using the Mann Whitney U test. Differences in antibody responses compared to the number of OCR infusions were tested for significance using the Kruskal-Wallis test. A Generalised Linear Mixed-Effects Model (GLMM) with normal distribution was fitted to the data using R Studio. A model was constructed with fixed effects (the covariates of interest) and a random effect of the patient sample. Stepwise deletion of the fixed effects was used to find the best model, which was determined by the model with an Akaike Information Criterion (AIC) value closest to zero. ANOVA type II tests were run on the optimised GLMM models to analyse the significance of the data and subsequent pairwise comparisons allowed us to analyse any significance in more detail. R package 'ggplot2' was used to visualise the data. It should be noted that some of these samples were repeated samples; however, for certain analyses, they were treated as independent observations. Additionally, these repeated samples were incorporated as random effects in generalized linear mixed models (GLMMs) to account for any potential biases.

2.7.2. Microarray Data

All Microarray data analysis and visualisation was performed in R Studio. GLMM models with normal distribution were fitted to the data using the same methods as described in section 2.7.1. R Package 'ggplot2' was used to visualise the data.

3. Results

3.1 Patient Demographics

This study utilised samples from 102 people with MS, of which 89 were treated with ocrelizumab (OCR) and 13 were not treated with OCR. The demographics of the patients from which the samples were taken from are shown in Table 4. The median age of the OCR treated cohort was 45(IQR=36-50), and of the control group the median age was 49 (IQR=43.5-63). Both groups were predominantly female, with 54% in the group not receiving OCR treatment and 66% in the group receiving OCR treatment. In both the OCR treated and untreated groups the majority of patients had RRMS, with 96% and 84% respectively. The other 4% of the OCR treated group presented with PPMS whereas there were no patients in the OCR untreated cohort with this type of MS and the rest of the patients had CIS or SPMS of which both were 7.5%. The control group of patients not receiving OCR treatment were not receiving any B cell-depleting therapy nor had they received any B-cell depleting therapies in the past. All patients in both the groups had at least one, and up to five SARS-CoV-2 vaccinations. Many of the people with MS in both groups, 48% and 54% had had a positive COVID-19 test in the past however for 52% and 46% we had no data on their past COVID-19 infection histories.

Table 4. Demographic characteristics of people with MS receiving ocrelizumab treatment and a control group of people with MS not receiving ocrelizumab treatment. CIS= Clinically isolated syndrome; SPMS=Secondary progressive multiple sclerosis; RRMS=Relapsing remitting multiple sclerosis; PPMS=Primary progressive multiple sclerosis.

	Samples from MS Patients Receiving OCR Treatment (n=89)	**Samples from Patients Not Receiving OCR Treatment (n=13)**
Age, median (range)	45 (24-67)	49 (39-75)
Male, n (%)	30 (34)	6 (46)
Female, n (%)	59 (66)	7 (54)
Type of MS, (%)		
CIS	0	1 (7.5)
SPMS	0	1 (7.5)

RRMS **PPMS**	86 (96) 3 (4)	11 (84) 0
Number of ocrelizumab Infusions at time of sampling, median(range)	6 (2-8)	N/A
Number of SARS-CoV-2 Vaccines at time of sampling, (%)		
1	0	0
2	4	0
3	26	46
4	43	46
5	17	78
No Data	10	0
Positive COVID-19 test in the past, (%)		
Yes	48	54
No	0	0
No Data	52	46

3.2 MS patient humoral responses to Coronavirus Proteins.

3.2.1 Antibody Responses to SARS-CoV-2 Proteins in ocrelizumab Treated people with MS.

IgG antibody responses of ocrelizumab treated people with MS were consistently low across all the SARS-CoV-2 proteins (Figure 5). The median anti-spike IgG response was lower than the negative cut-off for the assay [median 4.8 (IQR 2-89)] (Figure 5B) and similarly for the anti-RBD IgG responses [median 31.9 (IQR 20.4-102)] (Figure 5A). Remarkably only 38 (44%) samples had a positive antibody response to the spike protein and 37 (43%) had a positive RBD antibody response, surpassing the cutoff for the assay. While the range of antibody responses to the Nucleocapsid protein was narrower than that observed for Spike and RBD, with the highest value

reaching 402 binding antibody units (compared to 1000 and 1271 for RBD and Spike, respectively), there was an intriguing contrast in the case of the anti-nucleocapsid IgG responses (Figure 5C). Despite the differences in magnitude, the median anti-nucleocapsid IgG responses within the cohort [median 119.42 (IQR 82-180)] generally exceeded those seen for anti-spike and anti-RBD antibodies. The majority of the samples exhibited a positive antibody response above the assay's designated cut-off for Nucleocapsid, which came from the NIBSC.

COVID-19 vaccines are designed to specifically target the spike protein, and as RBD is a critical component of the spike protein, you would expect vaccinated individuals to have robust antibody responses to these proteins. However, despite being vaccinated the majority of people treated with ocrelizumab had insufficient antibody responses (below the assays cut off given by the NIBSC) to SARS-CoV-2 Spike and RBD. This observation is almost certainly due to the immunosuppressive effect of ocrelizumab which is compromising the MS patient’s immune system's ability to mount a robust response to the vaccine. The divergence we observed in the Nucleocapsid response patterns could be attributed to the Nucleocapsid protein's higher conservation across various coronaviruses, including SARS-CoV-2 and other seasonal coronaviruses. Consequently, the immune system may have been exposed to similar proteins from previous seasonal coronavirus infections, possibly resulting in a more robust and cross-reactive antibody response against the nucleocapsid protein in this cohort of people with MS.

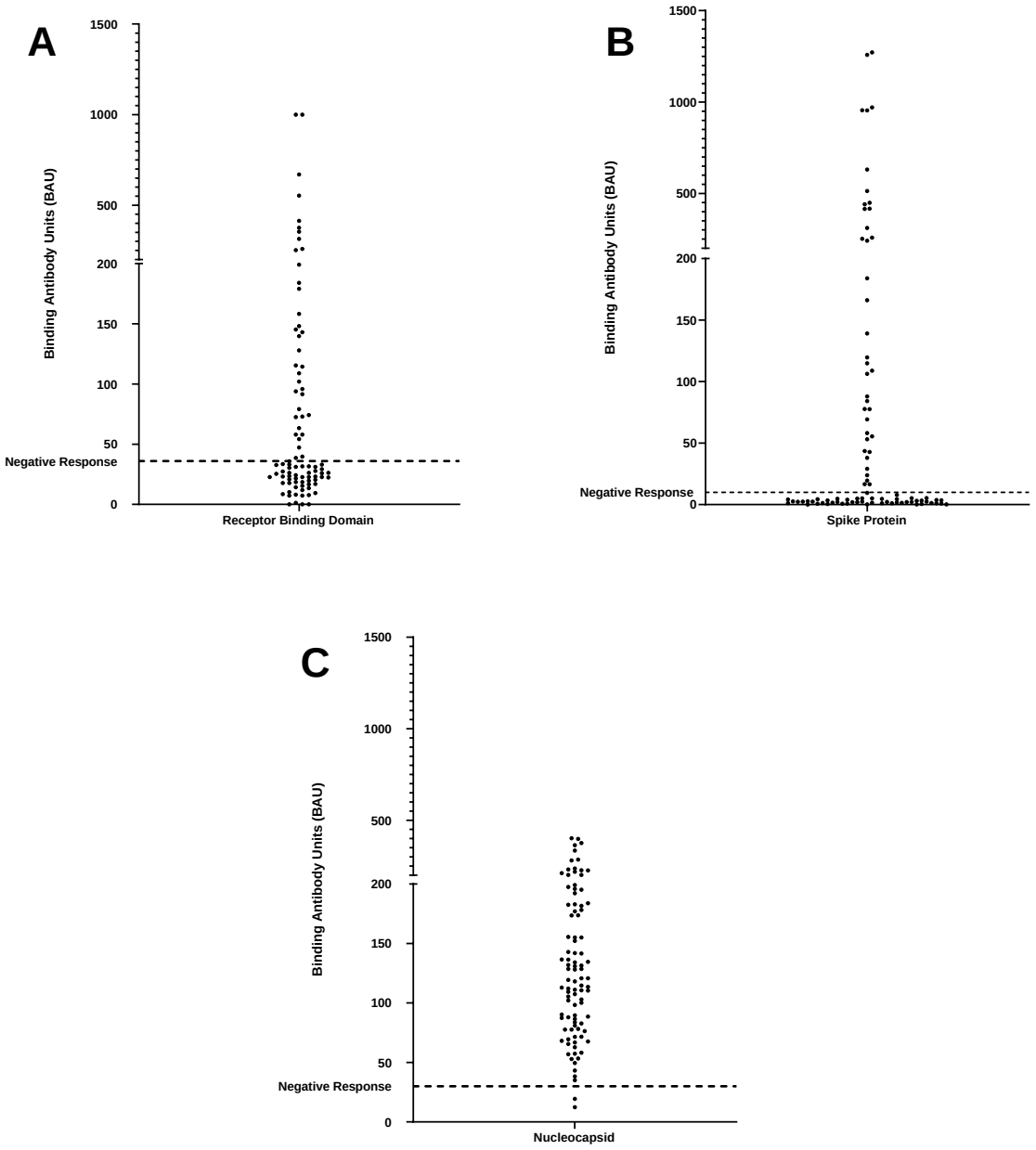


Figure 5- people with MS receiving ocrelizumab treatment IgG antibody responses to SARS-CoV-2 Proteins. Indirect ELISAS were used to quantify the binding antibody units (BAU) of A) Receptor Binding Domain (RBD), B) Spike (S) and C) Nucleocapsid (N) IgG in the blood of people with MS receiving ocrelizumab treatment. The dashed line labelled “negative response” represents the cutoff for the assay, which was taken from the NIBSC for the S and N data. For the RBD there was no data available from the NIBSC so the “negative response line” is taken from the average of sera samples from the NIBSC which are negative for SARS-CoV-2.

3.2.2 Antibody responses to Coronavirus Proteins in ocrelizumab treated people with MS and people with MS not receiving ocrelizumab.

To investigate whether ocrelizumab was affecting the vaccine response in our MS patient cohort, we conducted a comparative analysis of SARS-CoV-2 spike responses between OCR patients and a control group of people with MS not receiving the treatment. The comparison between the SARS-CoV-2 anti-spike IgG responses of the ocrelizumab treated people with MS and control group are visualised in Figure 6. 13 (100%) of the samples from people with MS not receiving ocrelizumab treatment had a positive antibody response above the negative cut-off for the assay to the SARS-CoV-2 spike protein compared to 38 (44%) in the ocrelizumab treated group. The median of the untreated group was significantly greater than that of the ocrelizumab treated group [median 1018 (IQR 720-1335)] and the large statistical difference between the groups ($p<0.0001$), indicates that ocrelizumab is clearly having a negative effect on the vaccine responses of the patients.

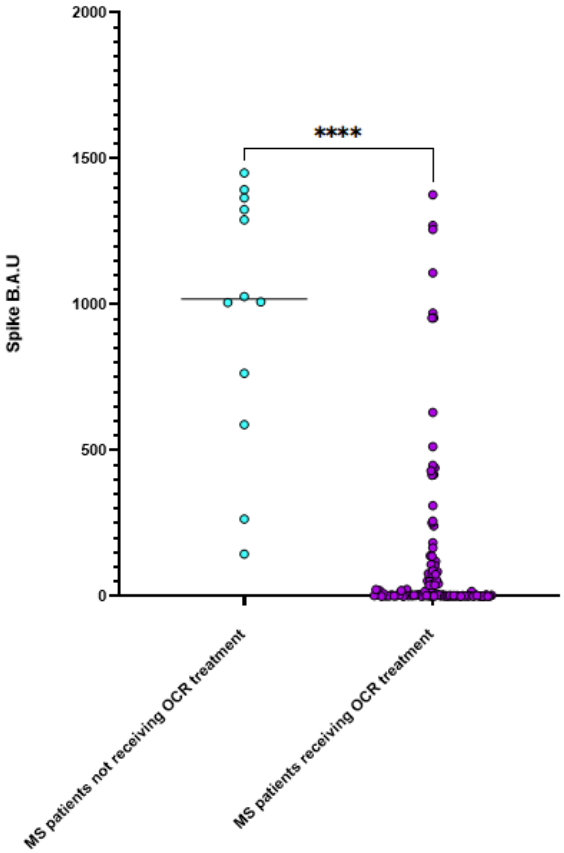


Figure 6- SARS-CoV-2 Spike IgG Responses of people with MS. Comparison of antibody responses to SARS-CoV-2 spike protein in people with MS not receiving OCR treatment (blue) and people with MS who are receiving OCR treatment (purple). An Indirect ELISA was used to investigate MS patient antibody responses to the SARS-CoV-2 Spike Protein. Statistical significance between groups is shown with **** symbolizing $p < 0.0001$ (Mann-Whitney U Test) (OCR=ocrelizumab, BAU= Binding Antibody Units).

After observing the weak responses to SARS-CoV-2 RBD and Spike proteins, and the distinctive responses to the Spike protein between OCR treated and untreated patients, we wanted to investigate whether the observed immune suppression is specific to SARS-CoV-2 or whether it extended to other coronaviruses. Contrastingly, when investigating MS patient responses to a Seasonal Coronavirus, namely HCoV-OC43, there seems to be little disparity between the patients receiving ocrelizumab treatment and the patients not receiving ocrelizumab treatment (Figure 7).

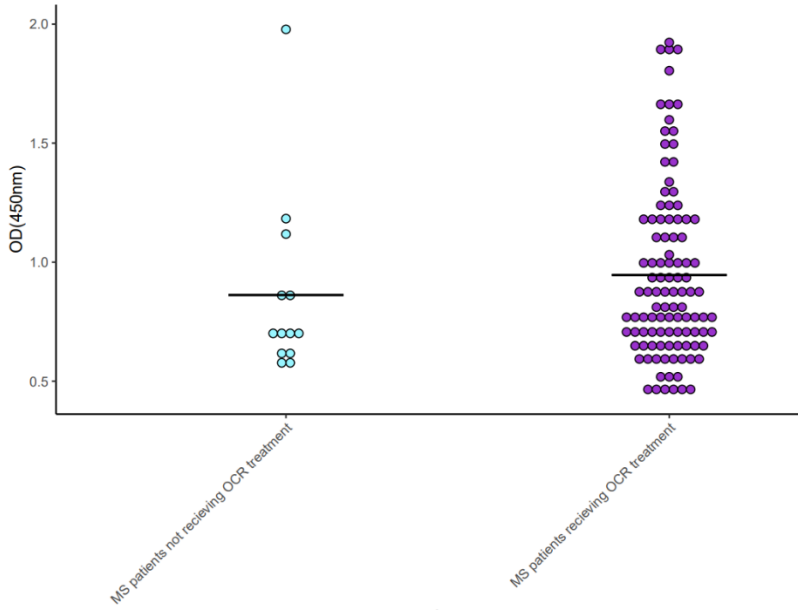


Figure 7- HCoV-OC43 IgG Responses of MS Patients. Comparison of antibody responses to Seasonal Coronavirus OC43 in people with MS not receiving OCR treatment (blue) and people with MS who are receiving OCR treatment (purple). An Indirect ELISA was used to investigate MS patient antibody responses to the HCoV-OC43 protein. The black lines represent the medians of the groups. A Mann Whitney U test revealed there was no statistical difference between both groups. (OD=Optical Density; OCR=ocrelizumab)

There are no widely established standards for the seasonal coronaviruses, therefore we were unable to convert our results into binding antibody units or establish a definitive assay cutoff. Nevertheless, upon examining the antibody responses of the two groups, it is clear to see that responses are fairly similar across the both the ocrelizumab treated and untreated cohorts. The median OD of the ocrelizumab treated group (0.705) showed no statistical difference to the median of the untreated group (0.851). Interestingly, it's worth mentioning that the median OD of the ocrelizumab treated group slightly exceeded that of the group not receiving ocrelizumab, which contrasts greatly from the SARS-CoV-2 Spike responses seen in Figure 6, and what we may expect, however as this did not reach statistical significance this observation may be attributed by the relatively small size of the untreated group.

3.2.3 Does ocrelizumab have a more substantial effect on antibody responses over time?

We wanted to investigate whether the effects of ocrelizumab were more detrimental as time increased and the number of infusions they had also increased. As patients receive an infusion approximately every six months, to see if time had an effect on antibody responses of ocrelizumab patients, the MS patient samples were split into groups corresponding to the number of OCR infusions they had at the time their blood was sampled (Figure 8). People with two infusions had been receiving OCR treatment for a year compared to people with eight who have been receiving treatment for approximately four years.

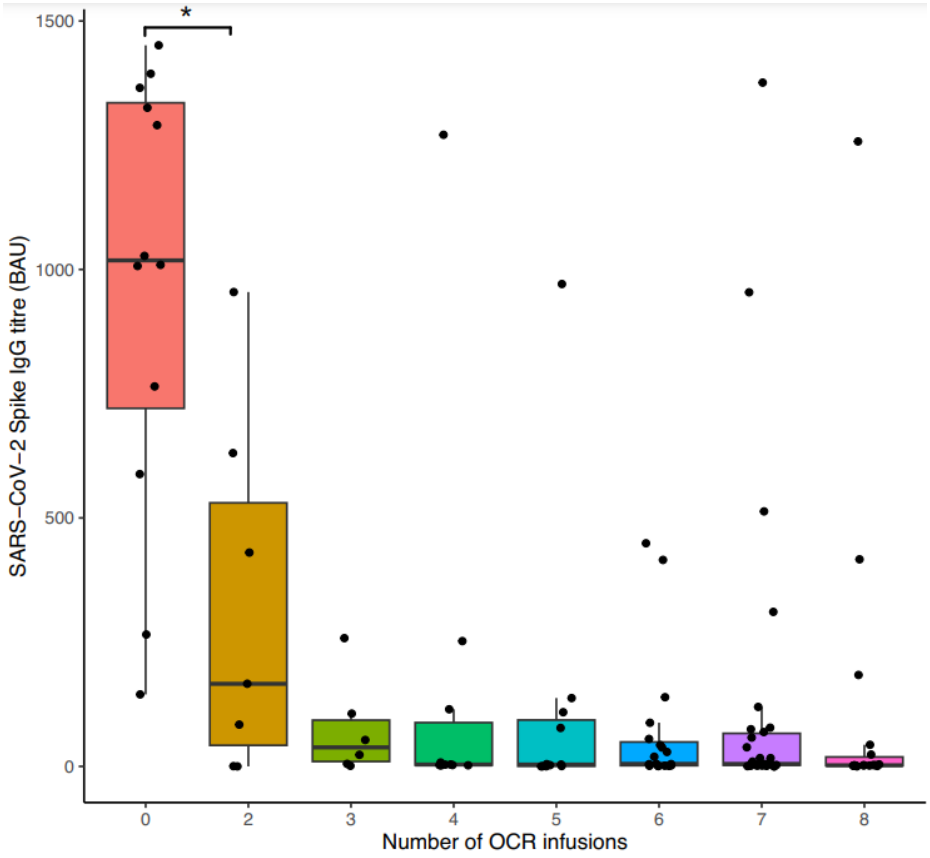


Figure 8- SARS-CoV-2 Spike IgG responses of OCR treated people with MS. An Indirect ELISA was used to investigate MS patient antibody responses to SARS-CoV-2 Spike protein. The box plot shows MS patient spike antibody responses corresponding to the number of ocrelizumab infusions they had at the time their blood was sampled. A Kruskal-Wallis followed by Dunn's multiple comparison test revealed that the difference between zero and two OCR infusions is statistically significant (p=0.0197). Statistical significance between groups is shown with * symbolising p<0.05. (OCR= ocrelizumab; BAU= Binding Antibody Units)

From the figure above it looks the number of OCR infusions does have an effect on the spike antibody responses of the people with MS. A Kruskal-Wallis test revealed that there was significance in the data (p=0.000075),

however Dunn's multiple comparison test showed there was only a significant difference (p=0.0197) from zero OCR infusions to two OCR infusions. After two infusions there is no further significant change by adding any further infusions. Notably, there were no patients with only one ocrelizumab infusion in our sample cohort, preventing us from assessing whether there is a difference from zero to one or one to two ocrelizumab infusions. While it may appear from the Figure that a substantial difference occurs between two and three infusions, the limited sample size of the cohort (comprising of only seven patients with two infusions), it's difficult to suggest whether the drop seen from two to three ocrelizumab infusions could be statistically meaningful or if it's there just due to the small sample size. A larger sample size would allow us to know if the change we observe in Figure 8 is truly there. Furthermore an assay with more resolution would allow us to detect whether there were reductions at very low levels.

3.2.4 Factors that may influence the SARS-CoV-2 Vaccine Response in ocrelizumab treated patients.

We examined the effects of the different factors that may have a detrimental effect on the vaccine response of ocrelizumab patients. Upon examination of Figure 9, a more clear pattern emerges when focusing on people with MS who are receiving ocrelizumab treatment and removing patients who have not received ocrelizumab treatment. As we added a factor investigating the OCR infusion relative to when patients had their Covid vaccine, we had to remove some patients from this analysis as we did not have this information. It becomes evident that the number of infusions does not significantly impact the antibody responses in vaccinated patients undergoing ocrelizumab treatment. Our analysis also explored additional factors potentially influencing the antibody responses among ocrelizumab-treated patients. Notably, as seen in Figure 9, the timing of SARS-CoV-2 vaccinations, and whether they were administered before or after the last ocrelizumab infusion at the time their blood was sampled, appears to exert a substantial influence on the Spike antibody responses of these patients. As you can see in the figure, the majority of patients who had not had an ocrelizumab infusion after their Covid vaccine formed a much stronger antibody response, compared to those who did receive an ocrelizumab infusion after their vaccine. Out of 69 patients, 29 had an infusion after their last SARS-CoV-2 vaccine and only 4 (13.7%) had an antibody response above the cut off for the assay. Contrastingly, whilst many of the patients who didn't have an infusion after their vaccine were unable to mount a sufficient antibody response to the SARS-CoV-2 spike protein, 22(55%) of the patients who didn't have an ocrelizumab infusion after their vaccine formed a sufficient response above the cut off for the assay. It's clear that the timing of ocrelizumab infusion in relation to vaccination affects the antibody response of patients. This observation aligns with what we would expect as being an immunosuppressive medication, ocrelizumab is known to

affect the immune system's ability to mount a robust antibody response to the vaccine.

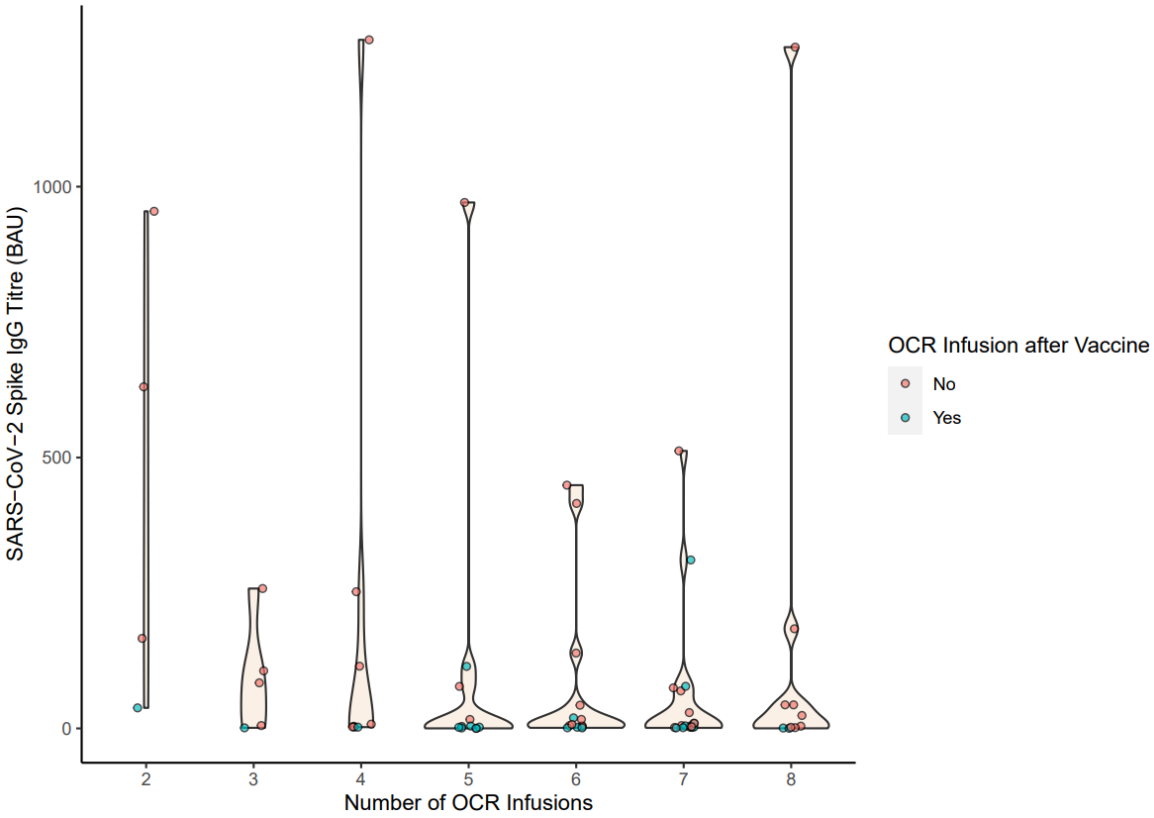


Figure 9- SARS-CoV-2 Spike IgG antibody responses of OCR treated patients, with a dot plot showing whether patients had an OCR infusion after a SARS-CoV-2 vaccine at the time of blood sampling. An Indirect ELISA was used to investigate MS patient antibody responses to SARS-CoV-2 antibody responses. The violin plot shows MS patient spike antibody responses corresponding to the number of ocrelizumab infusions they had at the time their blood was sampled. The dots represent individual MS patient samples and are coloured corresponding to whether they had an OCR infusion after their most recent SARS-CoV-2 vaccine (pink=no, blue=yes). (OCR=ocrelizumab; BAU=Binding Antibody Units).

Taking into consideration the fact that there are many different factors that could affect SARS-CoV-2 antibody responses, we used a generalised linear mixed model (GLMM) to explore these factors that may have a significant effect on patient antibody responses, and the results are summarised in Table 5. Mixed effects models combine both fixed and random effects to analyse data. We chose this statistical modelling approach due to the fact that some of the samples in our cohort were repeated samples from the same patient at different times. GLMMs are particularly useful for handling data with repeated measurements by incorporating them as random effects and properly accounting for these samples by not treating them as independent

observations, which helps mitigate the pseudo-replication we may get by using a traditional linear model.

After observing that there was an initial effect of ocrelizumab treatment on SARS-CoV-2 antibody responses in people with MS, highlighted by the significance seen between zero and two OCR infusions in Figure 8, we wanted to use this model to investigate any other factors that may affect vaccine responses in OCR treated patients whilst undergoing treatment. On account of this, we removed the patients not being treated with ocrelizumab from our model. In addition, as we were investigating the relationship between timing of vaccination and OCR infusions on Spike responses, several patients for which we did not have this information for were also removed from the analysis. As there are many ways we could have fit this model, when creating the GLMM we chose the model with the lowest Akaike Information Criterion (AIC) of the models being compared, due to this providing us with a model best fit to the data whilst using the fewest parameters.

Table 5- Summary of mixed effects model results for Anti-spike IgG antibody responses of people with MS being treated with ocrelizumab. The table provides a summary of the results obtained from a mixed effects model analysis assessing the relationship between the fixed effects (shown in the table) and the Spike BAU of people with MS, whilst incorporating random effects (the patient sample) to properly account for the correlated data. Statistical significance is shown with ** symbolising $p<0.01$.

Fixed Effects	Chisq	Df	P-Value	Significance
Number of ocrelizumab Infusions	8.2624	6	0.219501	
Days between blood test and last OCR infusion	2.6166	1	0.105753	
Infusion after SARS-CoV-2 vaccine at the time of blood sampling	6.6811	1	0.009744	**
Time interval between SARS-CoV-2 vaccine and OCR infusion	0.0343	1	0.853129	

We investigated several fixed effects such as the number of ocrelizumab infusions at the time of blood sampling; the duration between patients' blood sampling and their last OCR infusion; whether they had an infusion after their most recent SARS-CoV-2 vaccine at the time of blood sampling; and the time interval between their last SARS-CoV-2 vaccine and ocrelizumab infusion, with all the information gathered from the patients' medical records as described in Section 2.2. This analysis aimed to determine whether any of these factors had an effect on the Spike binding antibody units of the people with MS, to conclude whether they had an effect on the vaccine responsiveness of the OCR treated patients. The only factor that reached

statistical significance($p<0.01$) was whether patients had an infusion after their SARS-CoV-2 vaccine at the time their blood sample was taken.

3.3 Development and Validation of Microarray Protocol

3.3.1 Optimisation of optimal Blocking Buffer

We used three control antigens to test five blocking buffers in order to ensure we chose the blocking buffer with minimal non-specific binding (background) and optimal signal intensity (Figure 10).

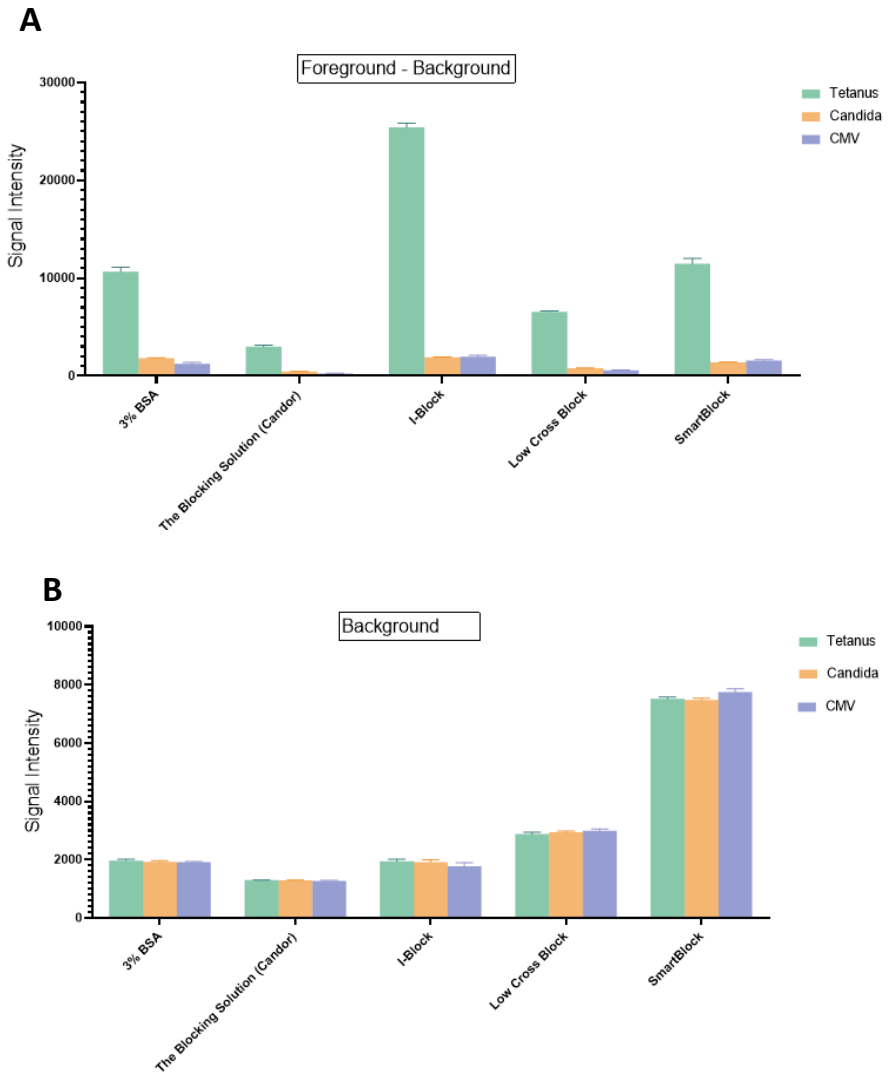


Figure 10- Microarray blocking buffer validation. Five blocking buffers: 3%BSA; The Blocking Solution; I-Block; Low Cross Block; Smart Block were tested on three different control antigens (Tetanus, Candida and CMV) printed onto a nitrocellulose

slide. Control plasma was used to process the slide. The signal intensities of A) Foreground minus Background and B) Background are shown in the Figure.

We compared several popular blocking buffers to determine the optimal choice to use in our microarray assay. We wanted to minimise background interference and low background signals were produced by 3% BSA, The Blocking Solution and I-Block as demonstrated in Figure 10. In addition, I-block had the highest signal intensities across the tested antigens as illustrated in Figure A, followed by 3% BSA. Although Smart Block also displayed high signal intensities it also produced significantly higher background signals than any of the other blocking solutions. This background noise would result in a low signal to noise ratio which is suboptimal for our assay. We chose I-block due to the combination of high signal intensities and low background interference which will result in the largest signal to noise ratio and ensure optimal performance of our antibody microarray assay.

3.3.2 Intra-assay and Inter-assay microarray validation

To determine the precision of the microarray platform, we performed intra-assay and inter-assay validation experiments. Both assays were performed on the same slide with the same eight patient samples, intra-assays were performed repeatedly over the same day and inter-assays were performed over a period of three days.

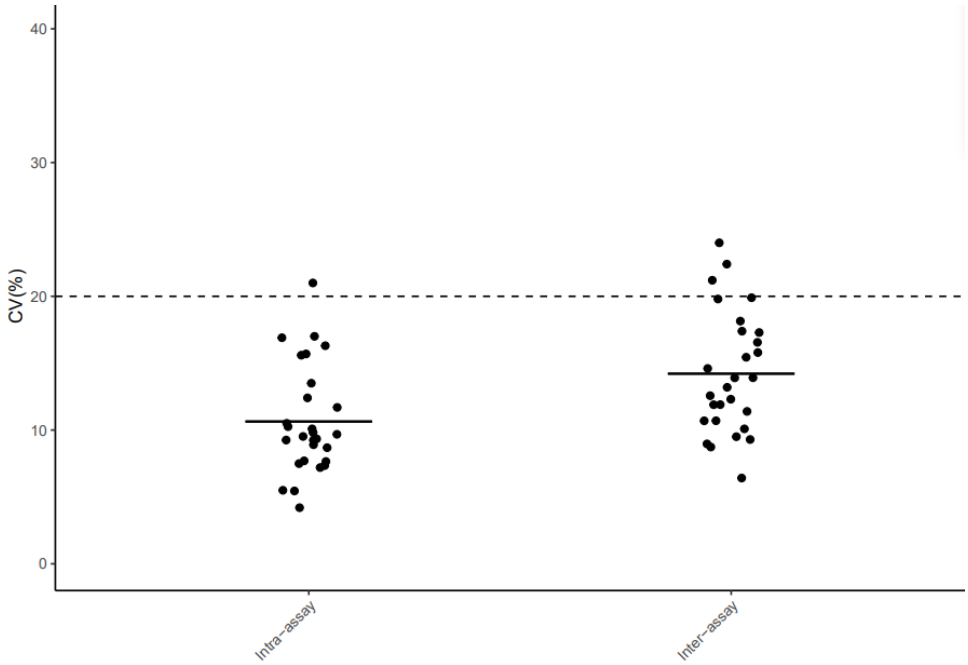


Figure 11- Validation of Microarray Antigens. The CV (%) was calculated to assess intra-assay and inter-assay precision of 28 proteins. The dashed line

represents the acceptable limit of precision for the microarray (20%). The solid lines represent the median for each group. CV= Coefficient of Variation.

28 Antigens were tested for precision through both intra-assay and inter-assay experiments. The median of the intra-assay experiment was found to be lower than that of the inter-assay experiment, which was expected, however both medians fell within the acceptable limit of precision. As displayed in Figure 11, in the intra-assay experiment, one out of the twenty-eight proteins exceeded the acceptable limit of precision, and for the inter-assay this number rose to three. Its's important to note that the protein that exceeded the limit in the intra-assay test was the same protein as one of the three proteins that exceeded the limit in the inter-assay test, meaning a total of only three of the proteins we tested were outside the acceptable limit of precision. Consequently, these proteins were removed from the microarray to exclude them from any further analysis and our experiment only used antigens that met our criteria for precision.

3.4 The effect of ocrelizumab on MS patient antibody responses

3.4.1 Positive Controls

We used Tetanus and Candida as positive controls for the microarray assay due to their high immunogenicity. Researchers have typically used Tetanus and Candida as positive controls to validate microarray assay performances as they induce potent immune responses, and their immunogenicity is well studied and understood. It's important to note that the fluorescence we observed for these proteins was remarkably higher than those produced by the other proteins (Figure 12). We used these signals to serve as a reference point for interpreting and validating the results of our protein microarray and the antibody responses we observed to other antigens on the array.

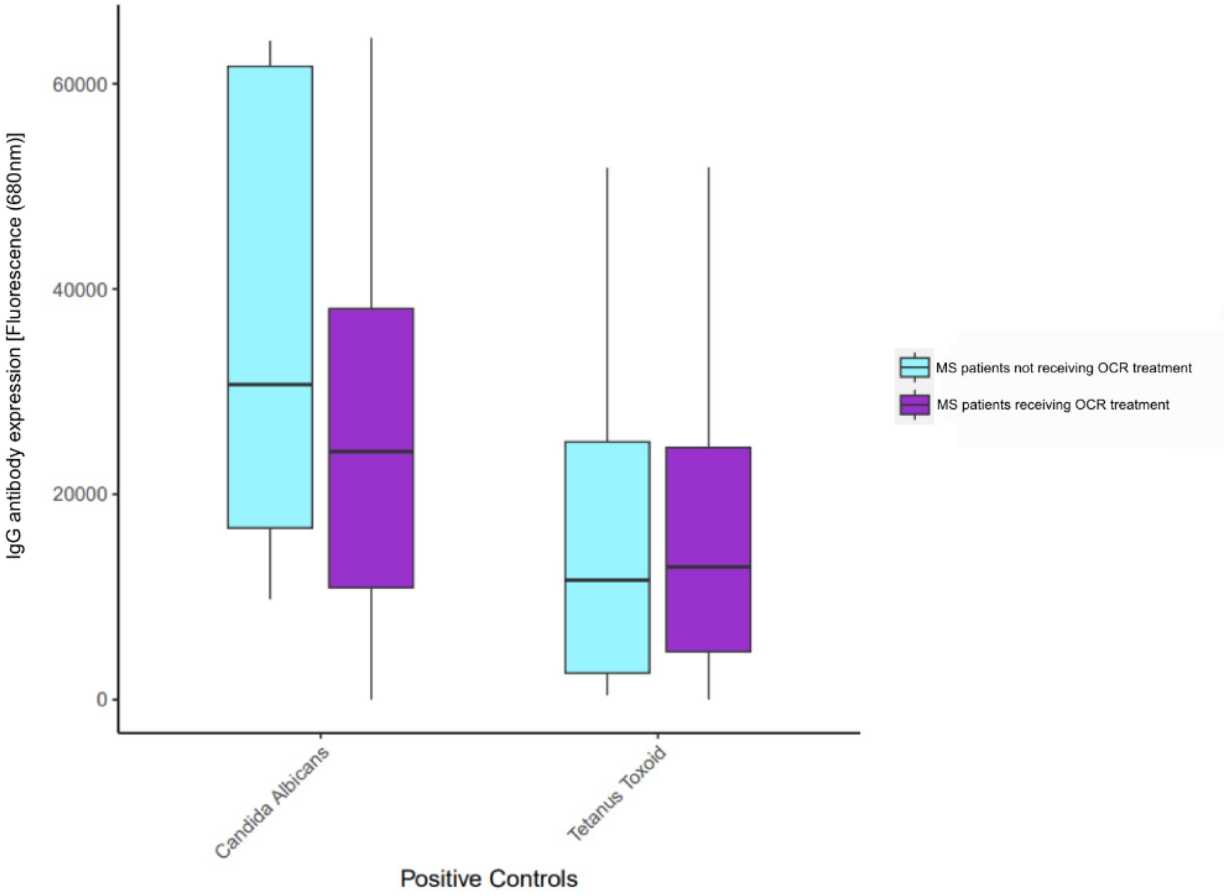


Figure 12- Antibody responses of people with MS to Positive Controls. Comparison of antibody responses to Positive controls on a Microarray Assay Panel (Tetanus Toxoid and Candida Albicans) in people with MS not receiving OCR treatment (blue) and people with MS who are receiving OCR treatment (purple). Pairwise comparisons revealed there was no statistical differences between groups.

Although we used Tetanus and Candida as positive controls in our assay, we still investigated any potential differences between the people with MS who were receiving ocrelizumab compared to those who weren't. As shown in Figure 12, both ocrelizumab treated and untreated groups had similar responses and our analysis revealed no statistically significant difference between the groups for either antigen.

3.4.2 Multivariate Analysis

Firstly, we used a generalised linear mixed model (GLMM) to explore whether ocrelizumab had an effect on MS patient antibody responses to our comprehensive microarray panel of proteins (Table 3) and the results are summarised in Table 6. We used a GLMM for the same reason as discussed in section 3.2.3, as we aimed to mitigate the influence of repeated patient samples introducing pseudo-replication in our results.

Table 6- Summary of mixed effects model results for antibody responses to microarray panel of proteins of people with MS. The table provides a summary of the results obtained from a mixed effects model analysis assessing the relationship between the fixed effects (shown in the table) and the MS patient antibody responses to the proteins on the microarray panel, whilst incorporating a random effect (the patient sample) to properly account for the correlated data. Statistical significance is shown with * symbolising $p<0.05$ and **** symbolising $p<0.0001$.

Fixed Effects	Chisq	Df	P-Value	Significance
ocrelizumab treated patient (yes/no)	3.9988	1	0.04553	*
Antigen	549.19	23	-2.2e-16	****

We used a GLMM to examine the overall effect of ocrelizumab treatment on the antibody responses of people with MS. As highlighted in the table above, whether the MS patient has been treated with ocrelizumab has a statistically significant effect($p=0.04553$) on the antibody response across the diverse range of antigens analysed on our microarray. It's important to note that the Antigen shows a very significant effect ($p<2.2e-16$) on the value in the GLMM. This is expected as all the antigens on the microarray in our study are distinct and elicit varying antibody responses, and the significance shown here is a result of the diversity in immune reactivity of the antigens we tested. From this multivariate analysis we could conduct pairwise comparisons to see if there were any statistically significant differences in the responses to antigens between ocrelizumab treated and un-treated patients.

3.4.3. Commonly Encountered Antigens

Our microarray panel of antigens included a selection of 12 common Viral antigens. We used these to explore whether ocrelizumab treatment affected MS patient antibody responses to these specific antigens and the results are depicted in Figure 13 below.

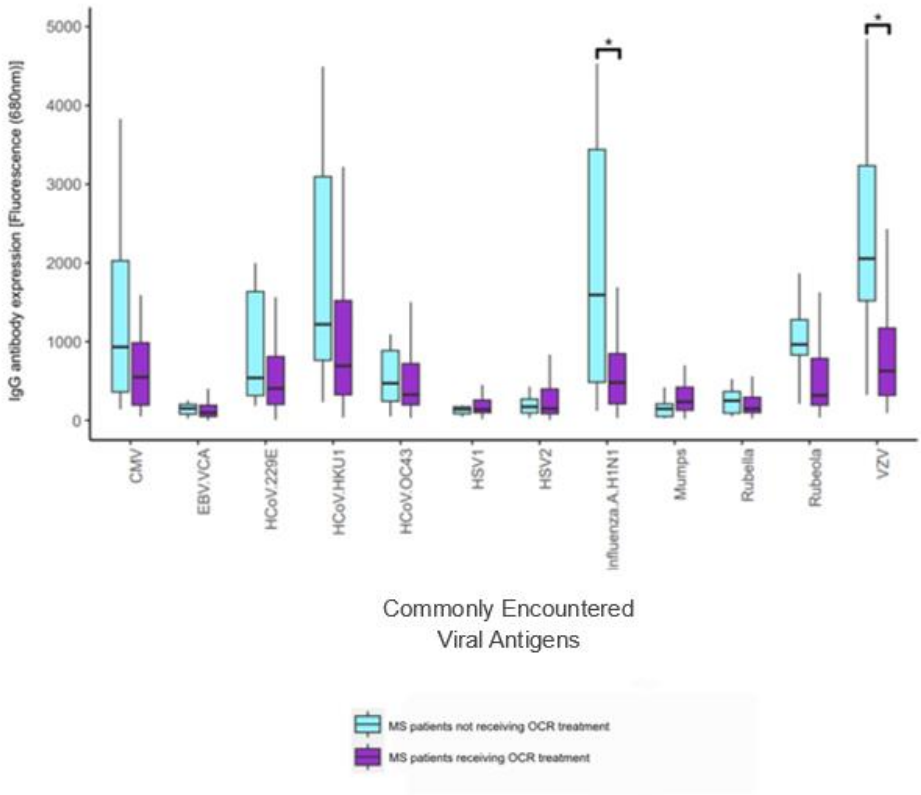


Figure 13- Antibody responses of people with MS to Commonly Encountered Viral Antigens. Comparison of antibody responses to viral recall antigens from 13 samples of people with MS not receiving OCR treatment (blue) and 89 samples from people with MS who are receiving OCR treatment (purple). A Protein microarray was used to investigate MS patient responses to several viral recall antigens. Pairwise comparisons revealed there was statistical differences between groups. Statistical significance between groups is shown with * symbolizing $p < 0.05$.

As you can see in the graph, there seems to be a trend in that people with MS who have not been treated with ocrelizumab seem to overall have higher responses to the viral recall antigens we included on our microarray. We conducted pairwise comparisons based on the results from our GLMM to explore whether there were any significant differences between the two groups and the results are summarised in Table 7.

Table 7- Pairwise Comparisons of Antibody Responses to Viral Antigens between ocrelizumab-Treated and Untreated MS Patients. The table shows the pairwise comparisons derived from the GLMM results assessing the differences in antibody responses to various viral antigens between people with MS receiving ocrelizumab treatment and people with MS who have not received ocrelizumab treatment. Statistical significance is shown with * symbolising $p<0.05$.

Antigen	Contrast	Estimate	SE	Df	P-value	Significance
CMV	OCR vs no OCR	-873.21	373.01	2472	0.975568	
EBV-VCA	OCR vs no OCR	82.23	373.01	2472	1	
HCoV-229E	OCR vs no OCR	470.44	373.01	2472	1	
HCoV-HKU1	OCR vs no OCR	541.69	373.01	2472	1	
HCoV-OC43	OCR vs no OCR	-24.82	373.01	2472	1	
HSV1	OCR vs no OCR	-58.52	373.01	2472	1	
HSV2	OCR vs no OCR	-4.38	373.01	2472	1	
Influenza A-H1N1	OCR vs no OCR	1528.76	373.01	2472	0.032348	*
Mumps	OCR vs no OCR	-92.45	373.01	2472	1	
Rubella	OCR vs no OCR	-226.80	373.01	2472	1	
Rubeola	OCR vs no OCR	579.94	373.01	2472	0.999998	
VZV	OCR vs no OCR	1588.18	373.01	2472	0.017491	*

Our pairwise comparisons revealed that there were some significant differences in the antibody responses of ocrelizumab-treated and untreated patients for influenza ($p=0.032348$) and Varicella-Zoster-Virus (VZV) ($p=0.017491$). This is an indication that for these antigens the effect of ocrelizumab is certainly decreasing the levels of antibodies people with MS are producing and affecting their humoral responses. This observed decrease in antibody levels raises concerns regarding ocrelizumab treated patients' susceptibility to infections related to these antigens. Ocrelizumab treated patients may have a heightened risk of developing these diseases, as their antibody responses may not be reaching optimal levels, such as those of the people with MS who have not undergone ocrelizumab treatment or any other B-cell depleting therapy.

It's important to note that while statistically significant differences in antibody response were observed for Influenza and VZV, none of the other differences in the responses to other viral recall antigens reached statistical significance. However, when examining the Figure there seems to be a trend, and ocrelizumab- untreated patients seem to exhibit higher antibody responses than the ocrelizumab treated group across several of the proteins. The ones that stand out include CMV, HCoV-229E, HCoV-HKU1 and Rubeola. Notably

in 10 out of the 12 antigens, the medians of the untreated cohort are higher than that of our treated. While statistical significance was not reached in these cases, it's important to acknowledge the relatively small size of our untreated group compared to the treated group, which could influence the results. A larger control group would offer us a greater perspective on the impact of ocrelizumab/ the impact when patients have not received this treatment.

3.4.4. Pathogen Associated and Hepatitis Antigens

In addition, we included 4 pathogen associated antigens and 2 Hepatitis virus antigens on our microarray panel. We explored the differences in antibody responses to these antigens between ocrelizumab treated and untreated people with MS and the results are illustrated in Figure 14 and Figure 15.

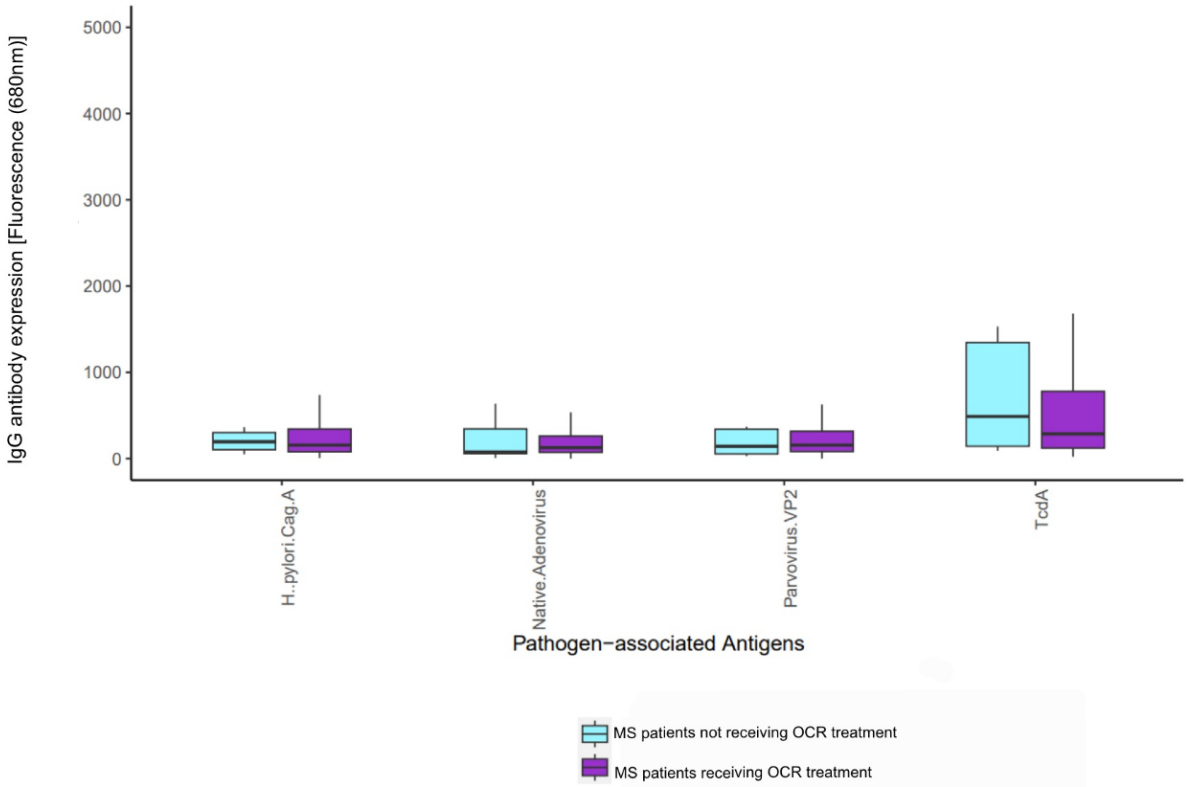


Figure 14- Antibody responses of people with MS to Pathogen Associated Antigens. Comparison of antibody responses to Pathogen Associated Antigens in 13 samples from people with MS not receiving OCR treatment (blue) and 89 samples people with MS who are receiving OCR treatment (purple). A Protein microarray was used to investigate MS patient responses to several pathogen associated antigens. Pairwise comparisons revealed there were no statistical differences between groups.

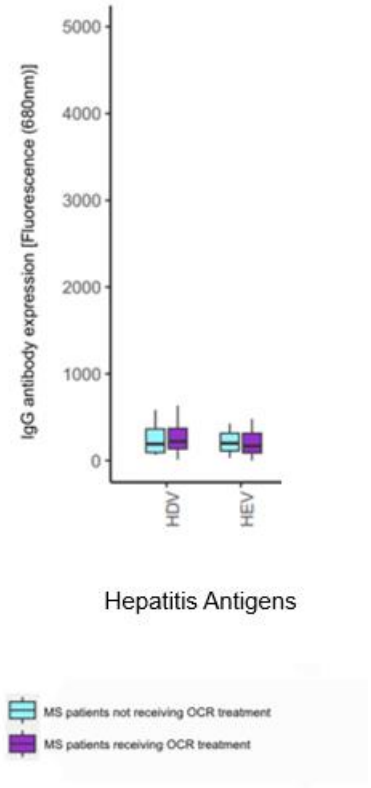


Figure 15- Antibody responses of people with MS to Hepatitis Antigens. Comparison of antibody responses to Hepatitis Antigens in 13 samples from people with MS not receiving OCR treatment (blue) and 89 samples from people with MS who are receiving OCR treatment (purple). A Protein microarray was used to investigate MS patient responses to several pathogen associated antigens. Pairwise comparisons revealed there were no statistical differences between groups.

As depicted in Figure 14 and Figure 15, the responses of both ocrelizumab-treated and the untreated cohort are low across all of the less commonly encountered pathogen-associated antigens we tested. Notably, the only antigen that induced an antibody response in both the groups was TcdA. The TcdA antibody response appears to be higher in the untreated cohort, however pairwise comparisons revealed there was no statistical significance (Table 8).

Table 8- Pairwise Comparisons of Antibody Responses to Pathogen Associated and Hepatitis Antigens between ocrelizumab-Treated and Untreated MS Patients. The table shows the pairwise comparisons derived from the GLMM results assessing the differences in antibody responses to various Pathogen Associated Antigens between people with MS receiving ocrelizumab treatment and people with MS who have not received ocrelizumab treatment. Comparisons revealed there were no statistically significant differences between the groups in any of the antigens tested.

Antigen	Contrast	Estimate	SE	Df	P-value	Significance
H. Pylori CagA	OCR vs no OCR	60.93	373.01	2472	1	
Native Adenovirus	OCR vs no OCR	-17.58	373.01	2472	1	
Parvovirus	OCR vs no OCR	21.92	373.01	2472	1	
TcdA	OCR vs no OCR	-11.5753	373.01	2472	1	
HDV	OCR vs no OCR	21.55	373.01	2472	1	
HEV	OCR vs no OCR	21.24	373.01	2472	1	

These findings suggest that among the tested pathogen-associated antigens and Hepatitis antigens on our microarray panel, there is no difference in responses between people with MS who have been treated with ocrelizumab and those who have not. This result was expected as these pathogens associated antigens aren't commonly encountered and individuals in the UK aren't vaccinated against them.

3.4.5. Autoantibodies

Finally, our microarray panel of antigens also included two autoantibodies. We analysed whether there were any differences between patient antibody responses with respect to these autoantibodies, and their results are illustrated in Figure 16.

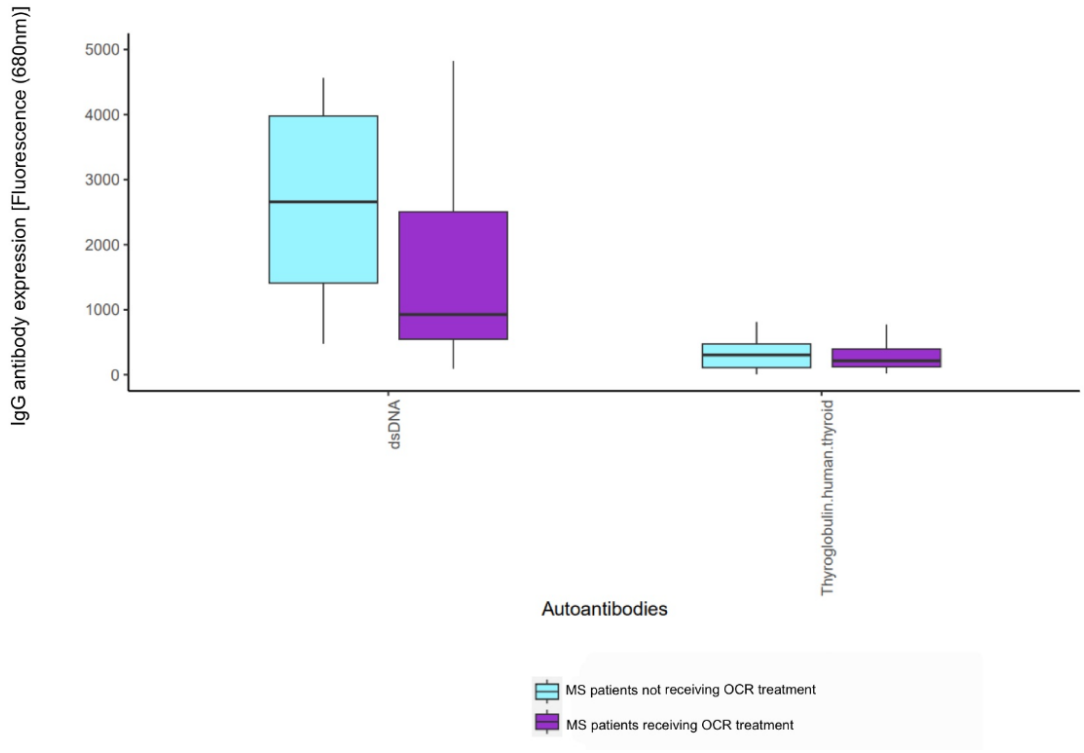


Figure 16- Antibody responses of people with MS to Autoantibodies. Comparison of antibody responses to autoantibodies in 13 samples from people with MS not receiving OCR treatment (blue) and 89 samples from people with MS who are receiving OCR treatment (purple). A Protein microarray was used to investigate MS patient responses to several autoantibodies. Pairwise comparisons revealed there we no statistical differences between groups.

As you can see in Figure 16, patient antibody responses to Thyroglobulin (human thyroid protein) were little across all patients and there is no difference between groups. This suggests that this autoantibody does not trigger any response in our cohort. On the other hand, the responses to double-stranded DNA (dsDNA) were present in most patients and there seems to be a noticeable distinction between the ocrelizumab treated and un-treated groups. Pairwise comparisons of these proteins revealed there was no significant difference between the groups (Table 9).

Table 9- Pairwise Comparisons of Antibody Responses to Autoantibodies between ocrelizumab-Treated and Untreated MS Patients. The table shows the pairwise comparisons derived from the GLMM results assessing the differences in antibody responses to various Autoantibodies between people with MS receiving ocrelizumab treatment and people with MS who have not received ocrelizumab treatment. Comparisons revealed there were no statistically significant differences between the groups in any of the antigens tested.

Antigen	Contrast	Estimate	SE	Df	P-value	Significance
dsDNA	OCR vs no OCR	1444.062	373.01	2472	0.072367	
Thyroglobulin Human Thyroid	OCR vs no OCR	801.6062	373.01	2472	1	

The observed difference in dsDNA antibody responses implies that ocrelizumab treatment may have an influence on autoantibody responses in people with MS, however it's important to acknowledge that this difference did not reach statistical significance, as shown by our analysis in Table 9. This may be because of the small size of our control group and warrants further investigations into the influence of ocrelizumab on autoantibody responses. Expanding the size of the control group may provide more robust insights into this influence.

3.4.6. Factors that may influence Antibody Responses in ocrelizumab treated patients.

We ran another GLMM focusing exclusively on the ocrelizumab treated people with MS so we could include additional information, namely the Number of OCR infusions they had and the timing of their infusion in relation to when their blood was sampled. We removed the people with MS who had not been treated with ocrelizumab from this GLMM, as this would allow for a more detailed analysis within the ocrelizumab treated group and see if any of these specific factors affect the patient responses. The results are summarised in Table 10.

Table 10- Summary of mixed effects model results for antibody responses tin ocrelizumab Treated MS Patients. The table provides a summary of the results obtained from a mixed effects model analysis assessing the relationship between the fixed effects (shown in the table) and the MS patient antibody responses to the proteins on the microarray panel, whilst incorporating a random effect (the patient sample) to properly account for the correlated data. Statistical significance is shown with **symbolising p<0.01 and **** symbolising p<0.0001.

Fixed Effects	Chisq	Df	P-Value	Significance
Days Between Blood Sampling and Infusion	7.6494	1	0.005679	**

Number of OCR Infusions	1.2798	6	0.972762	
Antigen	441.1259	23	< 2.2e-16	****

This GLMM allowed for a more detailed analysis within the group to assess whether any variables relating to ocrelizumab treatment, specifically the days between blood sampling and Infusion and number of ocrelizumab infusions they had at the time of blood sampling had an effect on the antibody response. As mentioned before, the antigen shows a very significant effect ($p<2.2e-16$) on the value in the GLMM which is expected as all the antigens on the microarray in our study are distinct and elicit varying antibody responses. Similarly, to our previous GLMM when we focused on the spike protein (shown in Table 5), the number of ocrelizumab infusions had no statistically significant effect on the antibody responses of patients. As we previously found this was the case for the spike protein antibody responses, we expected that the number of OCR infusions, which was in the range of two to eight infusions would also not have an effect on the range of antibody responses to the wider antigen panel. However, the days between when their patients had their blood sampled and their last infusions was significant($p=0.005679$). This indicates that time has an impact on the humoral immune response of ocrelizumab treated patients to a wide array of antigens and provides insights into treatments immunological effects.

All of the samples from out cohort were taken at varying times during the patients ocrelizumab treatment cycle. This posed a challenge for us in analysing the effect of time on antibody responses in ocrelizumab treated patients. As mentioned, some of our samples were repeated samples which were taken at different time points over the course of treatment. Three of the MS patient samples were taken at two different time points after their most recent ocrelizumab treatment, without any intervening ocrelizumab administrations, and these are shown graphically in Figure 17.

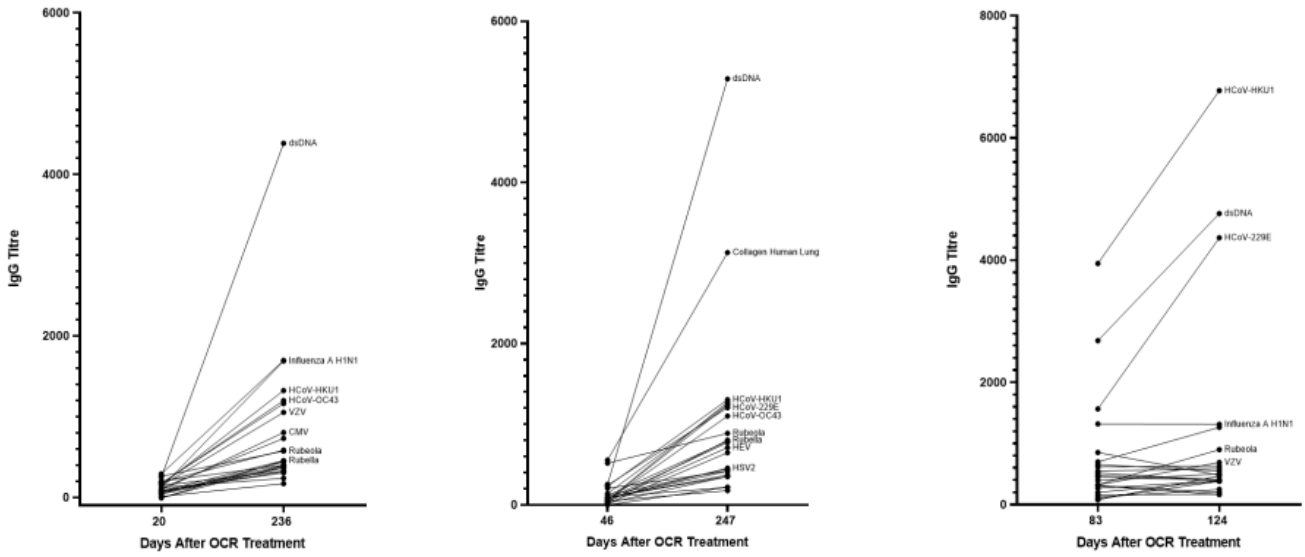


Figure 17- Antibody Responses to a Microarray panel of Antigens at different times Post-ocrelizumab Treatment. Three individual people with MS had their blood sampled at different times-points after treatment with ocrelizumab. The Figure shows the changes among individual people with MS antibody responses after ocrelizumab treatment. Each data point represents an antibody response to an antigen from the microarray panel at a specific number of days after ocrelizumab treatment.

The graphs in Figure 17 allow us to see the temporal aspects of antibody responses to the microarray panel of antigens following ocrelizumab treatment. As illustrated, in all three graphs in Figure 17, as the days after ocrelizumab treatment increases, the antibody IgG titres across the microarray panel generally increase and a trend is observed. This observation highlights the effect ocrelizumab has on the humoral response. Interestingly in graphs (1) and (2) of Figure 17, every antibody response to the proteins in our microarray panel increases. The graphs go up to 236 and 247 days respectively, which exceeds the typical number of days between ocrelizumab infusions (as it is usually administered within 6 months or around182.5 days) and within this time frame, all antibody responses increased. In graph C, the second time the blood was sampled was after 124 days, which is within the 6-month window between when ocrelizumab is usually administered. Whilst most of the antibody responses are increasing, 7 out of the 20 proteins we analysed actually exhibited decreased antibody responses within this timeframe. This is an intriguing observation that may suggest that on several of the antibody responses in this patient, the influence of ocrelizumab treatment is still affecting their humoral responses and certain antibody responses are affected differently over time.

4. Discussion

This study was split into two parts where we aimed to provide a comprehensive understanding of the effects of ocrelizumab on the humoral response of MS patient, looking at both immediate and longer-term implications. We firstly aimed to explore the immediate effects of ocrelizumab on MS patient humoral responses by looking at patient responses, particularly in the context of the SARS-CoV-2 proteins. In light of the recent COVID-19 Pandemic, the opportunity arose to explore the effects of this B-cell depleting therapy, and how it may affect immune responses following recent vaccinations. In addition, we further aimed to elucidate the effects of this immunocompromising therapy and the longer-term effects it may have on the immune system. We assessed whether ocrelizumab had an effect on past vaccinations and pathogens that we would expect people to have a strong humoral response to, and we included several other pathogens less commonly encountered to provide insights into the potential consequences of the B-cell depletion induced by ocrelizumab on people with MS' immune memory and long- term protection.

4.1 The effect of ocrelizumab on Coronavirus-Protein Antibody Responses in MS Patients.

We firstly analysed ocrelizumab treated MS patient responses to several SARS-CoV-2 proteins. The antibody responses to the Spike and RBD proteins were consistently low across the ocrelizumab treated cohort, with the majority of patients not forming a sufficient antibody response. All current vaccines that are approved and authorised for general use rely on the spike protein (Heinz & Stiasny, 2021), and as the RBD is a subunit of the Spike proteins S1 subunit, vaccinated individuals should elicit strong antibody responses to these proteins. As all of our patient samples had received prior vaccinations against SARS-CoV-2, it's clear to see that ocrelizumab has a detrimental effect on recent vaccine responses of people with MS receiving the drug. Our findings are consistent with several other studies that have found ocrelizumab treated patients to exhibit low and almost undetectable levels of Spike protein antibodies following SARS-CoV-2 vaccination. (Katz et al., 2022) (Nytrova et al., 2023). Being a component of the Spike S1 subunit, the low responses we found for RBD align with our findings and further support the notion that ocrelizumab is affecting the SARS-CoV-2 vaccine responsiveness of the patients.

Interestingly our observations of the antibody responses to the Nucleocapsid differ from those of the Spike and RBD in that most of our patient cohort form a sufficient response above the assays cut off. This could be as a result of the Nucleocapsid protein being relatively conserved among other coronaviruses, such as the seasonal coronaviruses that commonly circulate among the population. The effect of ocrelizumab on the Nucleocapsid proteins may be

less than that of the spike and RBD, due to the fact that the Nucleocapsid antibodies are produced as a result of infection and have been found to be long-lived(Swartz et al., 2023), possibly avoiding anti-CD20 depletion due to being produced by long-lived plasma-cells. Studies have suggested that not all B cells are created equally (Cho et al., 2021), while vaccination produces greater amounts of circulating antibodies, natural infection gives rise to memory B-cells that give rise to highly potent antibodies. It does raise a question regarding the effects of ocrelizumab. Specifically, whether ocrelizumab primarily affects recent vaccinations by depleting B-cells from the blood stream, meaning patients are unable to form a lasting antibody response, whilst sparing antibody responses to antigens that have been encountered in the past, that are produced by long lasting plasma cells.

We saw a more definite confirmation of the effect of ocrelizumab on SARS-CoV-2 Antibody responses when we compared Spike responses of our ocrelizumab treated group, with a control group of people with MS who were not receiving ocrelizumab treatment or any other form of B-cell depleting therapy. While our study did not include any healthy controls without MS, it is noteworthy that other studies have reported similar findings and have also demonstrated that people with MS not on ocrelizumab have similar antibody responses to Healthy control patients without the disease(Brill et al., 2021). We observed 100% of our control group formed an antibody response to the SARS-CoV-2 spike protein compared to only 44% on the ocrelizumab treated group. In addition, we found that there was a profound difference between the medians of both groups ($p<0.0001$). These results corroborate findings from other studies and contribute to the growing body of evidence of the influence of ocrelizumab on SARS-SoV-2 vaccination attempts.

From the onset of ocrelizumab treatment initiation, SARS-CoV-2 antibody responses are affected. Studies have suggested that the number of cumulative ocrelizumab infusions prior to vaccination weakly correlates with decreasing antibody titres (van Kempen et al., 2022) (Räuber et al., 2022). Whilst we did see a slight weak correlation, there was no statistically significant difference in antibody titres between two and eight infusions. This suggests that the degree of peripheral B cell reduction does not substantially worsen with each cumulative infusion. An assay with more resolution would allow us to detect the changes at lower levels.+ We observed a statistically significant difference ($p=0.04$), between the group with zero infusions, and the group with two infusions, which aligns with what we expected from our previous analysis on the difference between our OCR treated and untreated groups. One caveat of our analysis is that it did not include any patients who had only had one infusion, and a very small number of patients who had 2 infusions before their covid vaccines and blood sampling. Adding more patients to our study with one and two ocrelizumab infusions would allow for a more comprehensive analysis into the effects of whether the effect of ocrelizumab becomes more pronounced after the initial infusions. Nevertheless, our research depicts that after two infusions the effect of

ocrelizumab does not appear to worsen significantly, however it remains consistently impactful.

From our multivariate analysis, we found that when patients had their infusion in relation to when they had their vaccine at the time their blood was sampled was a significant factor ($p=0.0097$) in whether the patient could form a robust antibody response, more specifically whether the patient had an infusion after their most recent SARS-CoV-2 vaccine. Of the patients who had a vaccine after their infusion, at the time their blood was sampled, 55% were able to form an antibody response above the assays cut off, compared to only 13.7% in the group who had an infusion after their most recent vaccine at the time their blood was sampled. This finding is not unexpected as when patients have an infusion after their vaccine, it appears to deplete the B cells that are involved in the formation of new antibodies. Moreover, this finding underscores the importance of gaining a deeper understanding of how ocrelizumab affects the immune system. As infusions are administered on an ongoing basis, there will always invariably be an infusion scheduled after a vaccine, and understanding when to vaccinate patients in relation to their infusions can offer them a better chance at mounting a robust immune response and achieving antibody protection against SARS-CoV-2. In addition, in relation to their most previous vaccine. at the time of sampling, 45% of patients who had it after their most previous infusion did not mount an antibody response above the assays cut off, however as mentioned a notable 13.7% still managed to form robust antibody responses. These findings imply two key takeaways. First, having a vaccine after an OCR infusion does not ensure that a patient will form an antibody response. Secondly, they are indicative of a possible window of time for OCR treated patients to receive their COVID-19 vaccinations in order to elicit sufficient antibody responses. The optimal timing for COVID-19 vaccination in people with MS receiving ocrelizumab treatment is still heavily debated with some studies suggesting patients get vaccinated as soon as possible and 2-4 weeks prior to Ocrevus treatment has been suggested (Wolf & Alvarez, 2021) with others suggesting waiting up to nine months after an infusion, which would mean extending the recommended time between infusions (Achiron et al., 2021). Unfortunately, as our samples were not taken at set times, our research faced several challenges which hindered our ability in finding an ideal timing for vaccination for ocrelizumab patients (something we aimed for in our initial hypothesis). This posed a challenge as a lack of timed data at predetermined intervals (i.e., immediately after infusion; 3 months after infusion; 6 months after infusion) caused it to be difficult to accurately analyse the antibody response over time. Secondly, we were constrained by the relatively small cohort size, and not having the vaccination dates of all the patients in our study. In an ideal scenario, we would vaccinate ocrelizumab treated patients at different pre-determined time points to meticulously assess antibody responses in relation to the vaccine. Our findings warrant further research with larger

cohorts and more precisely times samples to provide a clearer set time for ocrelizumab-treated patients to be vaccinated.

Our research showed that the timing of ocrelizumab infusion in relation to recent vaccination (before or after), is an important factor in the outcome of the humoral response of the patient. It's important to note that while none of the other variables we tested in our multivariate analysis reached statistical significance, we didn't investigate every potential variable that could impact the humoral response in ocrelizumab patients. For example, one that is known to be of importance is the percentage of B cells at the time of Covid vaccination. Studies show the number of B cells increase in the months after infusions and are correlated with whether patients can form a humoral response to the vaccine (Rico et al., 2021). Another parameter that has been shown to predict humoral immunity is the concentration of ocrelizumab in the blood (Kempen et al., 2023). B cell count and concentration of ocrelizumab increase and decrease over time respectively, however there is inherent variability in these trends among individual patients (Baker et al., 2022c). Incorporating these to our multivariate analysis would have revealed a deeper understanding of seroconversion after vaccination during ocrelizumab treatment, and potentially inform clinical strategies such as the consideration of extended interval dosing (EID), or the development of more personalised treatment plans.

For future work, it would also be beneficial to research the immune response following different kinds of recent vaccinations and focus on vaccines other than SARS-CoV-2. As CD20 expression on T cells is low, researchers have investigated cellular immunity in people with MS receiving ocrelizumab. Some studies have suggested that even though patients have attenuated humoral responses to the disease and may not form antibodies in response to being vaccinated, they still have protection in the form of their T cell response (Madelon et al., 2021), and in the case of SARS-CoV-2, having a robust antibody response from vaccination is not as important as once thought. However, it's important to emphasize that not all vaccines produce the same cellular immune responses. Furthermore, the responses and safety of different types of vaccines in ocrelizumab treated people is still largely unknown. People with MS on ocrelizumab are still advised to not receive live attenuated vaccines (Bar-Or et al., 2020b), as they carry a risk of viral replication which could be problematic. This underscores the importance of gaining a comprehensive understanding of diverse vaccine responses that extends beyond SARS-CoV-2 vaccinations. It's important to gain a deeper understanding of more vaccine responses so clinicians can make more informed decisions regarding vaccination during ocrelizumab treatment. This shift in focus extends beyond SARS-CoV-2 vaccinations and encompasses other vaccines that patients may require such as those that are live attenuated or travel vaccines to give patients the best chance of protection.

4.2. The effect of ocrelizumab on Humoral immune responses from Antigen Microarray analysis

In hopes to provide a clearer picture on the long-term use of ocrelizumab, we wanted to analyse patient responses to a range of antigens. To gain a more holistic understanding of the treatments impact on the immune system, we wanted to assess whether ocrelizumab exerts prolonged effect on the humoral response particularly with regard to its impact on past vaccinations or pathogens, which we would anticipate people to have a sustained antibody reaction. This would not be feasible if we used conventional ELISA, so we designed a microarray panel in order to examine a wide range of antigens simultaneously, to see if we could uncover specific changes or patterns in our cohorts' immune profiles compared to the patients who weren't receiving ocrelizumab treatment. This method gave us the opportunity for a high-throughput analysis of the patients' antibody reaction to the antigens we chose. We optimised our protocol for the correct dilutions and buffers and ensured low coefficients of variation (CVs) for all antigens (<20%), to ensure the correct precision of our assay with minimal noise from other components of the plasma.

We observed a pattern between ocrelizumab treated and untreated patients when we compared their responses to viral recall antigens. In general, patients who hadn't been treated with ocrelizumab displayed stronger antibody responses to the viral recall antigens on our array. This is what we hypothesised due to the mechanism of ocrelizumab depleting B-cells which we believed could potentially leave patients with a reduced ability to form antibodies in response to common pathogens. Notably, for 12 out of the 14 viral recall antigens on our array, people with MS not receiving ocrelizumab treatment exhibited stronger antibody responses. Remarkably, in the case of two antigens, Influenza A-H1N1 and Varicella-Zoster Virus (VZV), the difference in response between the ocrelizumab-treated and un-treated cohorts reached statistical significance, demonstrating ocrelizumab can in fact have an effect on the past humoral immunity and recall responses.

Influenza A-H1N1 is a subtype of the influenza A virus that circulates constantly in human populations worldwide, and in general people have antibody responses to this subtype (and other influenza subtypes) through either natural infection, vaccination, or a combination of both(Krammer, 2019). Our results show ocrelizumab treated patients have a decreased antibody response to this pathogen ($p= 0.03$). The effect of ocrelizumab could be leaving patients more susceptible to catching influenza A. This is worrying as without optimal antibody protection, ocrelizumab patients are more at risk of serious illness due to the virus. Furthermore, prolonged viral shedding can increase the risk of transmitting the virus to others, and the virus' ability to mutate which could lead to the emergence of more virulent strains. Other studies have shown that after receiving Influenza A vaccines,

ocrelizumab treated patients can form humoral responses to the virus, however these responses are significantly attenuated (Bar-Or et al., 2020c), although patients are still encouraged to get vaccinated. This underscores the significance of determining an optimal vaccination timing to maximise humoral immunity in ocrelizumab treated patients.

MS aetiology is multifactorial and viral infections have been considered as significant environmental risk factors for a long time (Pignolo et al., 2021). Many studies have focused on VZV and its association with MS, suggesting that reactivation of the disease could act as a trigger during relapses, and may participate in the pathogenesis of MS (Sotelo et al., 2007)(Oskari Virtanen & Jacobson, 2012). VZV usually occurs at childhood and causes an infection that typically results in lifetime immunity (Bianchi et al., 2021). Our data shows a significant difference between antibodies against VZV in ocrelizumab treated and untreated patients. This observation raises concerns as ocrelizumab is possibly rendering patients to be more susceptible to VZV re-activation, which could counteract the benefits of ocrelizumab treatment and contribute to MS relapses or disease progression. This observation underscores the importance of closely monitoring the immune responses of ocrelizumab. Implementing serological testing for antibodies of ocrelizumab patients (such as the microarray analysis used in our study) could provide insights into immune profiles of all patients and identify potential vulnerabilities caused by the depletion of B-cells. Furthermore, as it is causing an effect, revaccination strategies may be an important factor to consider during ocrelizumab treatment and this reinforces previous points on finding an optimal time to vaccinate ocrelizumab patients so they can maintain robust antibody protection.

As previously mentioned, no other statistically significant differences were observed from the pairwise comparisons of the rest of the viral antigens on our array, however interestingly, there was an intriguing trend observed between ocrelizumab treated and untreated patients, with the untreated cohort consistently exhibiting higher antibody responses. Several that clearly stood out were Cytomegalovirus (CMV), HCoV-229E, HCoV-HKU1 and Rubeola. Similarly, to VZV, CMV has also been amongst viruses that have been researched as possible epigenetic factors linked to MS(Sedighi et al., 2023), and although these aren't confirmed, it's still imperative we monitor ocrelizumab viral recall responses to mitigate potential risk. Monitoring responses to these viruses is a proactive approach to better understand how ocrelizumab treatment can affect humoral immunity and minimize any unforeseen adverse events associated with the treatment. With seasonal viruses like the seasonal coronaviruses, and the previously mentioned influenza A, it's imperative that patients have a potent antibody response as without, ocrelizumab treated patients are more at risk of serious illness due to these pathogens. Furthermore, optimal antibody protection in the population is crucial for these viruses due to their high rates of mutation and antigenic drift, potentially giving rise to the emergence of more harmful

strains, posing additional health risks. It's important to note that while no other difference in antibody response on our array reached statistical significance, the size of our control group was small (n=13) compared to our ocrelizumab treated group (n=89). This discrepancy in group size may have impacted our ability to detect more differences in antibody responses, and a larger control group may have unveiled more significant insights from our data set with greater statistical power.

We also extended our microarray analysis to include less commonly encountered antigens and other pathogens to see if there were any other potential differences in people with MS' humoral responses due to the effects of ocrelizumab treatment. Regarding the pathogen associated antigens and Hepatitis antigens, we saw no statistically significant differences in the responses across all the pathogens we tested. However, it is important to note that for five out of the six pathogens we had on our array (Pylori CagA, Native Adenovirus, Parvovirus, HDV and HEV), many of the patients did not display detectable antibody responses suggesting that most of the cohort may not have encountered these pathogens before. Furthermore, Clostridium Difficile Toxin A (TcdA) was the only pathogen which induced a response, however this again this was relatively weak, and interestingly although the difference was not statistically significant, the cohort of patients not receiving ocrelizumab exhibited slightly stronger responses, which possibly suggests out of the people that had been exposed to this toxin, patients who had not been treated with ocrelizumab could mount more robust antibody responses. A limitation of this analysis is that we did not possess information regarding what pathogens patients in our cohort had been exposed to in the past (acquiring such data is quite challenging) however knowing this information would have allowed us to tailor our array to include a broader range of pathogens and explore more potential differences that may occur due to ocrelizumab.

In addition, out of the autoantibody responses we tested, we also didn't find any statistically significant results. However again, interestingly, Double Stranded DNA (dsDNA) was the only autoantigen that patients exhibited responses to, and once again patients who were not treated with ocrelizumab had higher responses. It's worth noting this observation is consistent with previous findings associated with other anti-CD20 therapies (Rituximab, Obinutuzumab), which have been shown to reduce dsDNA autoantibodies (Rovin et al., 2012) (Wang et al., 2021). Although dsDNA autoantibodies have not been found to be associated with MS pathogenesis, autoantibodies have long been thought to contribute to the complex pathogenesis of the disease (DeMarshall et al., 2017). These finding may suggest a potential positive aspect of ocrelizumab therapy in depleting B cells responsible for producing autoantibodies. Although our primary focus was not on autoantibody responses, expanding the analysis to include more autoantigens, and a larger cohort of patients, could provide a more

comprehensive understanding of how ocrelizumab influences autoantibody responses.

Our multivariate analysis showed that the days between blood sampling and infusion was a significant factor ($p=0.0057$) in the antibody responses of the people with MS receiving ocrelizumab to the antigens on our microarray. This finding aligns with our expectations, as in the time following ocrelizumab infusion, B cells would repopulate which correlates with stronger antibody responses. This shows that B cell depletion is transient and is consistent with studies on B cell replenishment after anti-CD20 therapy. B cells repopulate with new naïve cells, replenishing plasma cells that can secrete antigen specific antibodies (de Bourcy et al., 2017). Unfortunately, we didn't have many samples taken from the same patient, and out of the ones we did only three were taken without and interfering ocrelizumab infusions. Interestingly, two of these were taken at intervals exceeding the recommended treatment schedule of six months, and both exhibited a consistent pattern, every antibody response had increased over this time. Conversely, the other sample was taken in a shorter timeframe and within the six-month window and showed a mixed pattern with most responses growing stronger however some of the antibody responses remained stable or diminished slightly. Even though most of the B cell depletion due to ocrelizumab occurs soon after the treatment initiation, given the relatively long half-life of the antibody, and the fact that B cells move to areas that would be accessible for depletion, its thought that additional depletion occurs in the subsequent weeks (Bar-Or et al., 2020c). This could explain why not all antibody levels increased uniformly in the shorter time frame; however, our dataset is very limited in the number of examples, making it difficult to draw conclusions. Expanding the dataset would enable a more in-depth understanding of these dynamics, as well as explore individual variability in the responses, and how different antigens respond to treatment and repopulate differently. Our research has revealed that ocrelizumab does have a wider effect on humoral immunity, (not specific to recent vaccinations) however it seems humoral responses improve with time. In summary, our analysis underscores the crucial role in the time interval after infusion and warrants further analysis into the humoral responses of patients after ocrelizumab treatment, to shed light on the dynamics of ocrelizumab treatment and its implications for optimizing therapy for people with MS.

4.3. Limitations and Future Research

Our study had limitations, the most important one being the very limited sample size. A larger sample size, encompassing both the ocrelizumab-treated and untreated group would have allowed us to detect more subtle effects of the treatment and be more confident in our statistical reasoning. Another limitation was the lack of standardized sampling times. Collecting

samples at pre-determined intervals would have enabled more precise and informative comparisons between antibody responses, and as such, we could have incorporated B cell counts, precise timings of vaccination, and the concentration of ocrelizumab in the blood as more variables into our research. Another limitation is the lack of investigation into the time elapsed between the most recent COVID-19 vaccine administration and the date of sampling. This variable is crucial as the duration between vaccination and sampling may influence antibody responses, with longer intervals potentially leading to waning antibody levels.

Furthermore, to comprehensively understand the effects of ocrelizumab treatment on antibody responses to pathogens and vaccines, incorporating antibody binding data from pre-treatment samples for every individual in the study would be the most effective way of demonstrating any effects. A longitudinal analysis would allow us to track changes in antibody levels within individuals over time, providing a more accurate depiction of the dynamic alterations induced by ocrelizumab. By including pre-treatment samples, baseline antibody levels can be established prior to ocrelizumab administration, enabling direct comparisons with post-treatment responses. This would also offer insights into the kinetics of antibody reconstitution following B cell repopulation post-ocrelizumab treatment. Understanding the temporal patterns of antibody recovery can inform optimal timing for vaccination strategies and guide clinical decision-making regarding the management of infectious risks in individuals undergoing ocrelizumab therapy. Future studies involving pre-treatment samples would provide a more comprehensive understanding of the impact of ocrelizumab on humoral immunity.

Another limitation in our study investigating the effects of ocrelizumab was the very small number of repeat samples, and again, the lack of standardized timing in which these samples were taken. As such, we encountered a challenge due to the absence of repeat samples. In the initial phase of our study investigating the effects of ocrelizumab on COVID-19, we made the decision to treat these few samples as independent samples given the changed circumstances such as COVID-19 vaccination status and the number of ocrelizumab infusions, this led us to incorporate certain data points as independent samples, albeit against statistical conventions. Notably, in the subsequent phase of our research, we rectified this methodological limitation. In the latter part of our study, we addressed this issue by integrating these samples into Generalized Linear Mixed Models (GLMMs) as random variables. This adjustment was important in mitigating bias and ensuring the robustness of our analytical approach.

Additionally, it's important to acknowledge the limitation regarding the strain of SARS-CoV-2 utilized in our study. While we employed the Wuhan strain for our investigations, it's essential to recognize that the emergence of new variants may introduce confounding factors into our results. Variations in viral strains could potentially influence antibody responses and vaccine

efficacy, thus impacting the interpretation of our findings. Future research endeavours should consider incorporating multiple strains of SARS-CoV-2 to capture a more comprehensive understanding of ocrelizumab's effects on antibody responses across different viral variants.

4.4. Conclusions

To conclude, our research revealed the multifaceted impact of ocrelizumab on both short-term humoral immunity and vaccine responses, and longer-term responses to viral recall antigens. Our findings demonstrate that ocrelizumab significantly influences people with MS' ability to mount robust antibody responses following recent vaccinations, and this underscores the importance of finding an optimal time to vaccinate patients to maximise chances of patients eliciting robust antibody responses. Additionally, we have shown that ocrelizumab treatment can modulate patients' antibody responses to viral recall antigens and warrant further investigation to fully determine the implications of these alterations. Ocrelizumab is very successful in the treatment of MS and given that it's a relatively recent addition to the treatment options of the disease, and it's designed as a long-term therapeutic intervention which needs to be administered over an extended duration, it's important we continue researching the effects to gain a more comprehensive understanding of how to optimize treatment for the benefit of patients with Multiple Sclerosis.

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