UNIVERSITY OF BIRMINGHAM

INVESTIGATING THE POTENTIAL USE OF HIGHLY SENSITIVE TEAR AND SALIVA ANALYSIS TO ENABLE NON-INVASIVE DIAGNOSIS, MONITORING, AND PROGNOSTICATION IN MULTIPLE SCLEROSIS

by

CHLOE LOUISE TANNER

A thesis submitted to the University of Birmingham for the degree of

MSc BY RESEARCH IN IMMUNOLOGY & IMMUNOTHERAPY

Clinical Immunology Service

Institute of Immunology and Immunotherapy

College of Medical and Dental Sciences

April 2024

ABSTRACT

Multiple sclerosis (MS) is a progressive neurodegenerative condition characterised by chronic immune-mediated demyelination and axon loss in the central nervous system, leading to progressive decline in motor function and disability. While advancements in treatments have significantly enhanced the longevity and quality of life of individuals living with the condition, timely and accurate diagnosis remains critical. Oligoclonal banding (OCB) and elevated Kappa and Lambda free light chains (FLCs) in cerebral spinal fluid (CSF) are hallmarks of MS. However, CSF sampling via a lumbar puncture is a highly invasive procedure, requires specialist training to perform and is often an unpleasant experience for patients. This study aimed to evaluate the feasibility of non-invasive tear and saliva analysis as alternative methods for the detection of OCB and FLCs.

A cohort of 40 healthy donors (HDs), 20 MS patients and 60 non-MS neurological condition controls (NCCs) undergoing lumbar puncture investigations were recruited to the study. Blood, saliva, tear fluid, and CSF (from lumbar puncture patients) were collected and analysed utilising highly sensitive immunoassays developed by the Clinical Immunology Service. Serum reference ranges for Kappa and Lambda FLCs were established by Optilite analysis, while saliva and tear FLCs were quantified by ELISA. IgG and total free and bound immunoglobulin OCB detection was performed on all sample types using isoelectric focussing (IEF).

Significantly reduced saliva and tear secretion was observed in both MS patients and NCCs compared with HDs. Notably, FLC quantitative parameters exhibited similar trends in MS patients and NCCs when compared to HDs. Kappa FLC secretion and Kappa: Lambda ratios were elevated in serum, decreased in saliva and unchanged in tear fluid

when compared to healthy controls. OCB was absent in HDs and NCCs, but faint bands were present in 20% MS patients tears and 25% MS patient saliva.

The tear and saliva biomarkers examined in this study did not achieve sensitivity or specificity requirements to warrant an expansive follow-up study. However, the investigation provided useful insights into the collection and analysis of the sample types. Disparities in results between MS patients may mirror the heterogeneity of MS presentation and disease course, underlining the demand for further biomarker research. The ability to accurately stratify patients based on accurate biomarker profiles could transform clinical investigations for patients and clinicians, pave the way for personalised medicine and increase our understanding of the pathophysiology of the condition.

Complementary studies could aim to explore the variability in results among MS patients, particularly regarding the presence of OCBs in tears and saliva. Also, the similarity of FLC parameters between MS patients and NCCs could be investigated through a more stringent cohort analysis of age, medication usage, sample collection times and co-morbidities.

ACKNOWLEDGEMENTS

Firstly, I cannot thank Dr Jennifer Heaney and Dr Sian Faustini enough for giving me the opportunity to pursue my dream project. Throughout the past 18 months, their continual guidance, mentorship, and reassurance has been invaluable, from helping troubleshoot my experiments to pushing me to present my work in the institute away day!

The rest of the team at the Clinical Immunology Service have been exceptionally accommodating by enabling me to integrate my studies whilst working full time and for that I am incredibly grateful. I would especially like to thank Mr Bilal Jeewa and Mrs Beena Emmanuel for their time and patience in training me on the isoelectric focussing technique, and Mr Mohammed Afzal for running the serum samples through the immunoglobulin and free light chain quantification.

Additionally, I would like to thank Professor Michael Douglas for obtaining ethical approval for the study as well as Dr Pyae Aung, Professor Saiju Jacobs, and the clinical and admin staff at UHB for co-ordinating the recruitment of all the patients. I am immensely grateful for the 120 participants who consented to taking part in this study. Their generosity highlights the indispensable role of volunteers in the advancement of medical research.

Finally, none of this would have been achievable without the support and encouragement from my parents, sisters, and friends. Thank you so much for believing I could do it!

Funding

This project was supported by funding from the UKRI Medical Research Council Confidence in Concept MC PC 19029.

TABLE OF CONTENTS

ABBREVIATIONS

Abbreviation	Meaning
AHSCT	Autologous haematopoietic stem cell transplantation
BBB	Blood-brain barrier
BSA	Bovine serum albumin
BUCIS antibodies	Birmingham University Clinical Immunology Service antibodies
CBT	Cognitive behavioural therapy
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
CZE	Capillary zone electrophoresis
DMT	Disease modifying therapies
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FLC	Free light chain
GBS	Guillain-Barre syndrome
HCRW	Health Care Research Wales
HLA	Human leukocyte antigens
HRA	Health Research Authority
HRP	Horseradish peroxidase
IEF	Isoelectric focussing
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRAS	Integrated Research Application System
MGUS	Monoclonal gammopathy of unknown significance
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NCCs	Neurological condition controls
NCM	Nitrocellulose membrane
OCB	Oligoclonal bands
PBS	Phosphate-buffered saline
PNS	Peripheral nervous system
PPMS	Primary-progressive multiple sclerosis
RRMS	Relapsing remitting multiple sclerosis
SPMS	Secondary-progressive multiple sclerosis
SSPE	Subacute sclerosing panencephalitis
TMB	Tetramethylbenzidine
UHB	University Hospitals Birmingham
Kappa-FLC	Kappa free light chain
Lambda-FLC	Lambda free light chain

1. INTRODUCTION

1.1 Introduction

Multiple sclerosis (MS) is a degenerative neurological condition whereby chronic, immune-mediated demyelination and axon loss in the central nervous system leads to progressive decline of motor function and disability over time (1). Worldwide, an estimated 2.8 million people are living with MS (2). However, compared to global averages, the UK has one of the highest prevalence and incidence of MS with 130,000 currently living with the condition (3), and it is the most common, non-traumatic cause of disability amongst young adults (4). At present, there is no cure for MS, though advancements in recognition, diagnosis and treatment have made significant improvements to the longevity and quality of life for people living with the condition. Key to this, is timely and accurate diagnosis and categorisation of the disease (5). However, the invasive and specialised nature of clinical investigations act a barrier to this and have scope to improve (6).

1.2 Epidemiology

Approximately 1 in 3000 people are living with MS, however the distribution of cases varies considerably worldwide (2). MS disproportionally affects women who comprise around 70% of all cases in the UK, a proportion which fluctuates between 66% and 78% worldwide, depending on the region (2).

There is a clear geographical bias in MS cases. MS occurs mostly in the Caucasian population of Nordic heritage from higher income countries. Characteristics of which are most common in temperate Europe, North America, Australia, and New Zealand, where prevalence is as high as 1 in 300 (2). In converse, prevalence in non-white people in less

economically developed regions, such as sub-Saharan Africa, South America, and South-East Asia is as low as 1 in 30,000. This has led to a latitudinal gradient of MS occurrence (2) (see Figure 1.1).

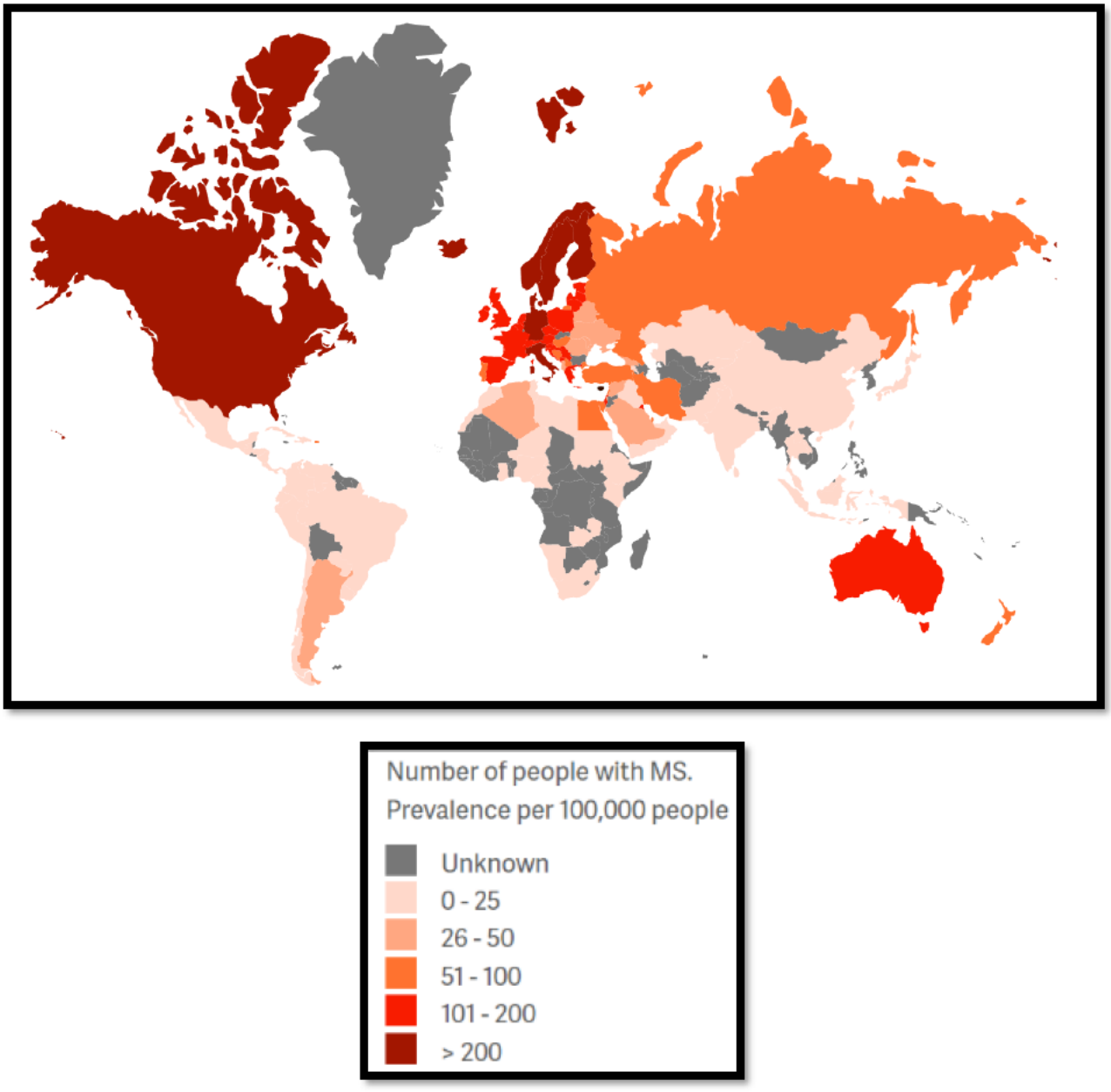


Figure 1.1 Global latitudinal gradient of MS prevalence.
This Figure was sourced from The Multiple Sclerosis International Federation Atlas of MS (2). A visual demonstration of the relationship between MS prevalence and latitudinal location. The highest global prevalence of MS is found in the northern hemisphere.

Be that as it may, there are substantial gaps in epidemiological data. “The Atlas of MS” (2), published by The Multiple Sclerosis International Federation and The World Health Organisation in 2020, is the most extensive open access data set on global MS epidemiology. The report described difficulties in obtaining comprehensive and peer-reviewed data from lower income countries. Some countries took no part in the project, leaving many regions unrepresented in updated figures. Additionally, it acknowledged that demographic characteristics such as life expectancy, ethnic diversity and access to healthcare make it difficult to make fair comparisons between countries. Furthermore, overall cases appear to be on the increase, with an average of two new cases per 100,000 annually, however it is not clear whether this correlates with increased risk and susceptibility, or factors such as the ageing population and improved recognition of the disease (7).

Although epidemiological trends in MS are mostly well documented, it is apparent that health inequalities act as barriers in the recognition and diagnosis of MS, thus impact our global understanding of MS epidemiology. The UK has a prevalence of 196 cases per 100,000 (or 1 in 500) and an incidence rate of ten new cases per 100,000 annually, ranking it relatively very high compared with other countries (2,3). This justifies considerable clinical interest into investigating the factors causing this geographical bias and whether preventative measures can be taken to control both prevalence and incidence.

1.3 Aetiology

It is thought that the uneven distribution of MS is caused by a complex amalgamation of genetic predispositions, geographically linked risk factors and socially determined lifestyle factors linked with increased susceptibility (8). However, a distinctive cause has not yet been ascertained.

The geographical bias (2), increased occurrence of MS in women (9), and inheritance patterns in some families points towards a genetic proneness of MS. Immediate family members of those with MS are 2-5% more likely to develop MS than the UK general population (10). Concordance between identical twins is around 25% (10), disproving mendelian inheritance and supporting a more complex interaction between genetic and non-genetic components.

Hundreds of genetic variations have been highlighted as candidates for increased MS susceptibility (11). Many of which code for the expression and regulation of the human leukocyte antigen (HLA) genes. HLA genes are highly polymorphic and code for cell surface proteins that aid the distinction between self and non-self-antigens during healthy immune surveillance (12). Variations in these genes are commonly implicated in other autoimmune disorders such as diabetes mellitus, rheumatoid arthritis, and coeliac disease (13). HLA-DR2, HLA-DR15 and HLA-DR16 serotype groups have been linked to increased MS susceptibility by many studies (14). Additionally, variations in non-HLA associated genes such IL2RA and IL7RA have been linked with MS (15). Both are interleukin receptor components which function to regulate T-cell regulation in immune homeostasis (16).

As previously stated, MS occurrence is at least twice as likely in women than men. A number of studies have suggested a link to hormone levels (17). Sex hormones such as

oestrogen, progesterone and androgens have receptors on immune cells, which can have immunomodulatory effects (17). This is supported by a reported 70% decrease in relapses in pregnant women in their third trimester compared with pre-pregnancy, and up to a three-fold increase in relapses post-partum (9). However, the underlining molecular basis for these trends, and complex immune and genetic interactions are yet to be untangled.

Environmental risk factors to MS such as vitamin-D deficiency and exposure to Epstein-Barr virus (EBV) have been well-documented (8). It is suggested that they can trigger disease manifestation in the genetically predisposed. EBV is a herpes virus which is estimated to reside within 90% of the population, and 100% of MS patients. Those with EBV are 32 times more likely to develop MS in their lifetime (18). One proposed mechanism behind this is molecular mimicry between EBV and self-antigens (19). Vitamin-D deficiency, caused by lack of exposure to sunlight and natural differences in production between ethnicities, is also linked with increased risk of developing MS (20). Vitamin D is an immunomodulator (21), which may account for the concentrated occurrence of MS in the latitudinal extremes which are exposed to the least amount of sunlight.

Lifestyle choices are key social determinants of health. Unhealthy lifestyle choices are associated with more severe disease presentation, less successful responses to treatment (22), and increased risk to co-morbidities and chronic conditions such as cancers, heart disease and other immune conditions. Smoking (23) and childhood obesity (24) are main lifestyle factors linked with increasing susceptibility and exacerbating many immune diseases including MS.

Susceptibility to MS is multi-factorial and currently unpreventable. More research into the relationship between genetic, environmental and lifestyle factors is required and could lead

to implementing preventable measures. For example, the efficacy of administering vitamin-D supplementation has been explored in a number of trials (25), yet does not appear to alter the disease course (26). EBV vaccination is being investigated as a preventative measure to MS, however no EBV vaccine is currently licensed for use in the UK. Economic factors, vaccine efficacy and a 95% EBV infection rate in adults present significant barriers in clinical trials (27). In the UK as of 2021 (28), 14.4% reception school children were obese or severely obese, jumping to 25.5% in the following year. This was around 4.5% higher than pre-pandemic figures (29). Although smoking numbers are steadily decreasing in the UK, vaping is on the increase (30). It is suggested that vaping is no less damaging than smoking, yet more research is needed on the correlation between long-term vaping and MS risk. Increasing public knowledge of the significance of their lifestyle choices remains critical to public health.

1.4 Immunopathology

The nervous system can be broadly divided into the central nervous system (CNS), consisting of the brain and spinal cord, and peripheral nervous system (PNS) which innervates the rest of the body. The CNS, as well as the eyes, developing foetus and testes are commonly described as immune privileged sites (31). Immune privilege is an adaptation, evolved to tightly regulate immune activity in life-preserving and producing organs. Thus, protecting these organs from pathological damage caused by the immune system (31). This is particularly important for the CNS, given its minimal capacity to regenerate from damage. Immune privilege in the CNS was originally described as complete immune isolation, attributed to the blood brain barrier (BBB) and separation from the peripheral lymphatic system (31). More recent studies have increased our

understanding of this phenomenon, showing the CNS both interacts with peripheral immune system and has intrinsic immune surveillance (32).

In the healthy nervous system, the myelin sheath is a lipid and protein-based layer which surrounds the axon of a nerve cell, similar to the insulation around an electric cable. The myelin is synthesised by glial cells and oligodendrocytes in the CNS and serves to protect the neurones and facilitate efficient neurotransmission (33) (see Figure 1.2).

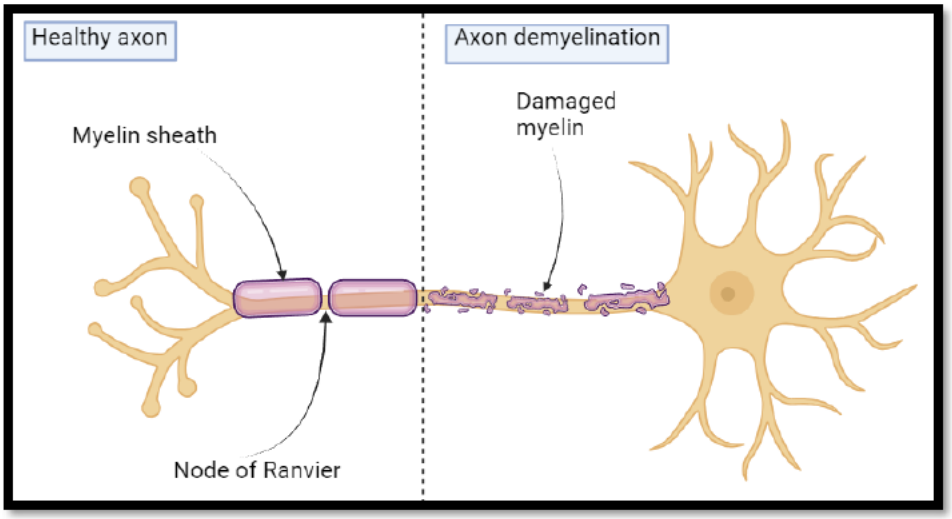


Figure 1.2 Axon demyelination in MS.
Left: In healthy people, the myelin sheath is an insulating layer which protects the axon and offers insulation. This allows for efficient nerve transmission. Right: in MS patients, the immune system attacks and destroys the myelin in the CNS, resulting in symptoms consistent with neurological decline. This figure was created with Biorender.com.

In MS, the myelin sheath surrounding neurones in the CNS is attacked by the immune system. This damages the ability of the neurones to transmit signals efficiently (34) (see Figure 1.2). The exact immunopathology of MS development is yet to be deciphered (35), however there are two generally accepted models which require further investigation. The "outside-in" model of disease development considers MS to start in the periphery, the "inside-out" model suggests the autoimmune reaction begins in the CNS (36). In either

case, this auto-immune reaction is a failure of the central tolerance mechanisms designed to negatively select autoreactive T-cells and B-cells. Due to molecular mimicry, the immune system recognises the myelin as a foreign antigen and an autoimmune reaction is triggered which infiltrates the CNS (37).

The CNS has processes of remyelination to repair the damage made by MS (38). However, over time, chronic demyelination results in permanent damage to the CNS over time, leading to physical, cognitive, and psychological symptoms.

1.5 Symptoms

The symptoms of MS manifest as evidence of CNS damage and typically appear between the ages 20-40. The range, severity and pattern of symptoms differ between individuals depending on their disease classification (39).

Patients are categorised into one of three main types of MS: relapsing remitting (RRMS), secondary progressive (SPMS) or primary progressive (PPMS) (39) (see Figure 1.3). Relapsing remitting MS describes the disease course for 80% of MS patients and is characterised by cycles of remission and relapse episodes which worsen over time and often develop into secondary progressive MS (39). Secondary progressive MS is characterised by gradual progression of symptoms without obvious relapses (40). 65% of RRMS patients will progress to this classification. Primary progressive MS is gradual progression of symptoms with no remissions at all (41). This occurs in 10% MS cases.

During an episode of remission, symptoms improve, and the patient stabilises. During relapse, common symptoms include, but are not limited to the following: Loss of vision, muscle atrophy, numbness and tingling, spasms, stiffness, weakness, fatigue, depression

and anxiety, bladder and bowel problems, sexual dysfunction, mobility difficulties, speech and swallowing difficulties, pain, and cognitive difficulties (see Figure 1.4) (42). There is a very high variation between patients of relapse patterns and severity (43).

Some lifestyle risk factors are associated with more frequent and severe relapses. These include smoking, vitamin D deficiency and obesity (8). Furthermore, studies investigating MS in ethnic minority groups found that although the occurrence of MS is lower in these groups, disease progression is significantly worse (44). This highlights the impact of health inequalities on MS disease severity (45).

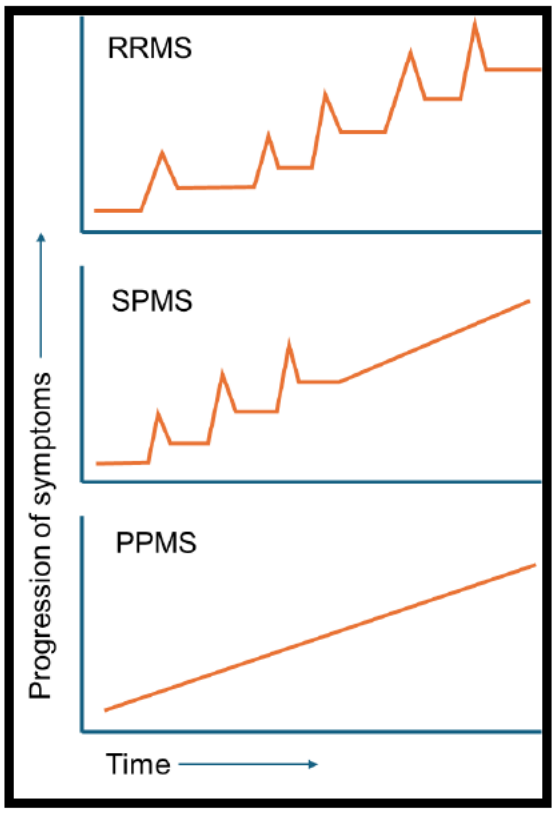


Figure 1.3 Disease progression for different categories of MS
Schematic of disease course for different categories of MS. Top: Relapsing remitting MS is characterised by cycles of remission and relapse episodes which worsen over time and often develop into secondary progressive MS. Middle: Secondary progressive MS is characterised by gradual progression of symptoms without obvious relapses. 65% of RRMS patients will progress to this classification. Bottom: Primary progressive MS is gradual progression of symptoms with no remissions at all.

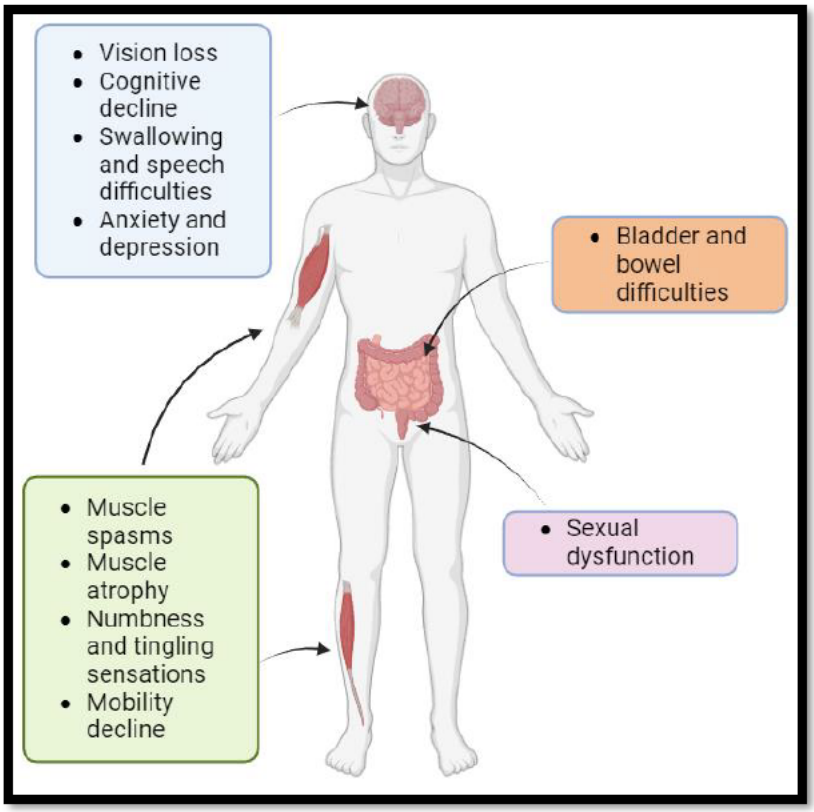


Figure 1.4 MS symptoms.
Symptoms of MS can affect the whole body, from physical, cognitive, and emotional health. The presentation of MS can be vastly different between patients but may include the following and more: Loss of vision, muscle atrophy, numbness and tingling, spasms, stiffness, weakness, fatigue, depression and anxiety, bladder and bowel problems, sexual dysfunction, mobility difficulties, speech and swallowing difficulties, pain, cognitive difficulties. This figure was created with Biorender.com.

1.6 Diagnosis

There is no singular definitive test for MS. Instead, diagnosis is based on a combination of evidence accumulated from clinical, molecular, and imaging data. At present, the McDonald criteria, first published in 2001 (46), revised in 2005 (47), 2010 (48) and 2017 (49), is the gold standard diagnostic criteria and its usage has been adopted by 87% countries worldwide (2). The newest revision in 2017 (49) bases diagnosis on evidence that CNS damage has disseminated in space and time. In other words, the development of

CNS damage in new anatomical locations which spread and increase over time. A typical clinical investigation is as follows.

The diagnostic process tends to begin at primary care whereby individuals present with new neurological symptoms such as atypical sensations, vision decline, changes in bladder and bowel function (42). Following referral to secondary care, a specialist neurologist will confirm whether clinical symptoms are consistent with CNS decline (42). This includes a physical examination to evaluate any deteriorations of eye function, movement in arms and legs, speech, and coordination.

A magnetic resonance imaging scan (MRI) is performed to detect any lesions in the white matter of the brain and spinal cord which are indicative of MS-related CNS damage (50). It was this hallmark symptom of MS which coined its name early in the characterisation of the condition (51) which was derived from the Latin for multiple scarring. When MS is suspected, two different forms of MRI scan are used: T2 and T1. A T2 MRI scan, which is more routinely used, is used to visualise lesions on the brain and spinal cord which appear as bright white marks. A T1 MRI scan uses a contrast dye called gadolinium. Dead or damaged nervous tissue appears as black (See Figure 1.5) (50). The presence of lesions in the brain or spinal cord are considered to be high prognostic factors of MS development (52).

Cerebral spinal fluid (CSF) analysis is undertaken to diagnose a number of neurological conditions. CSF is a clear and colourless liquid which surrounds the brain and spinal cord within the intrathecal space and serves many purposes in the CNS. CSF acts as a shock absorber to physical injury, a medium for substance exchange, and a structural support for the brain (53). The presence of oligoclonal banding (OCB) in CSF has been shown to be a

significant biomarker for developing MS (52,54) and indicates intrathecal IgG synthesis. Intrathecal IgG synthesis is indicative of inflammation in the CNS. Inflammation markers in the CNS are indicative of either an infection or autoimmune activity. In addition, elevated Kappa free light chains (FLC) in CSF have been observed in MS patients (55), though this is not tested as standard practice in NHS diagnostics (56).

CSF is extracted via a lumbar puncture (57). During the procedure, a clinician administers local anaesthetic and inserts a needle between the L3-L4 or L4-L5 vertebral interspace to extract the CSF (57). Isoelectric focussing (IEF) is the gold standard method used to detect IgG oligoclonal banding (OCB) patterns. CSF and serum OCB is tested in pairs to ascertain whether OCBs are being produced systemically or restricted locally to the CNS (See Figure 1.6). Kappa FLCs can be measured using the Binding Site Optilite apparatus (58).

Despite a seemingly comprehensive testing process, the combination of results does not necessarily lead to a straightforward diagnosis (6). For example, there is vast heterogeneity in the symptoms, severity, and relapsing patterns between patients. OCB presence in the CSF is not exclusive to MS. CSF OCBs appear in other systemic inflammatory diseases, CNS infections and some hereditary disorders. Subacute sclerosing panencephalitis (SSPE) Guillain Bare syndrome (GBS), CNS infections and cerebrovascular incidents can all cause the appearance of OCB and Kappa FLC (59). Therefore, the diagnosis of MS and/or ruling out differential diagnosis often takes years and can be highly stressful for patients (60).

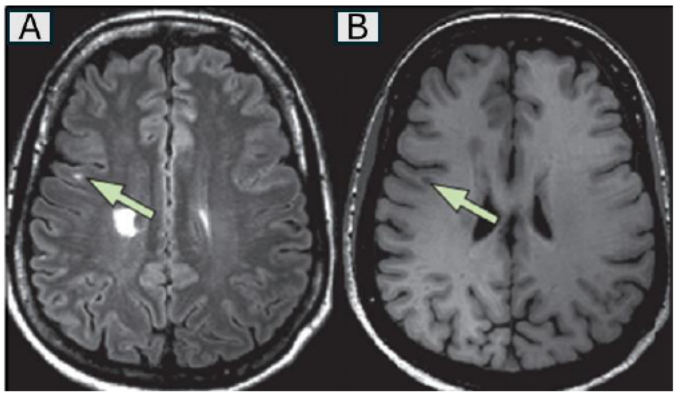


Figure 1.5 MRI as a tool for MS diagnosis.
MRI images were sourced from (50). A: A T2 MRI scan where lesions appear as bright white marks. B: A T1 MRI scan where lesions appear as dark marks.

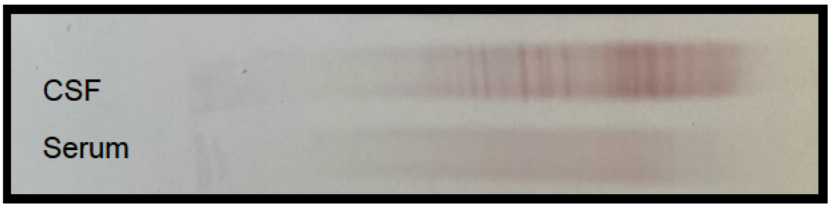


Figure 1.6 OCB in MS
Example IEF immunoblots from this study. OCBs are restricted to the CSF, indicating intrathecal synthesis of IgG and likely MS.

1.7 Treatment

There is currently no cure for MS, therefore treatment aims to decrease the immune-mediated damage to the CNS, target specific symptoms, reduce the risk of comorbidities, and improve psychological wellbeing (42). Treatment options are increasing with a particular focus on personalised medicine, tailored to the patients' disease categorisation and symptom pattern and progression. Current clinical guidance includes the following:

A. Steroids

During a relapse, the first line of treatment in the UK is usually a course of corticosteroids, in tablet form or infusion (42). Steroids are generic, non-specific inhibitors of the immune system and are effective at mitigating the severity of relapses. However, they cannot be prescribed for extended periods of time due to systemic adverse effects associated with immunosuppression and other conditions such as osteoporosis and diabetes (61).

B. Symptom based therapy

As summarised in Figure 1.4, symptoms of MS are vast, therefore, management of MS can be multi-disciplinary (42). Specific symptoms including but not limited to vision problems, sexual dysfunction, pain, incontinence, and muscle spasms can be treated for separately according to the individual (42).

C. Disease modifying treatments (DMTs)

DMTs are specifically targeted, immunomodulatory drugs designed to decrease the frequency and severity of relapse attacks (62). Around 18 different DMTs are currently licensed for use by the NHS in the UK (63,64). They take many forms such as monoclonal antibodies, nucleotide analogues and receptor antagonists and engage in different mechanisms of action.

For example, Alemtuzumab is a monoclonal antibody which binds to CD52, flagging mature lymphocytes for cell death (65). Interferon beta-1a and interferon beta-1b are naturally occurring cytokines which dampen inflammation which can be manufactured and administered (66). Cladribine is a purine analogue which, when incorporated into B-cells

and T-cells, interferes with DNA replication, thus triggering cell death (67). Fingolimod, Siponimod and Ponesimod block the sphingosine-1-phosphate receptor which aids the translocation of leukocytes out of lymph nodes (68).

DMTs are tailored to the individual's disease category and symptom patterns, with the common aim to target the immune system precisely to reduce CNS damage whilst minimising adverse effects of more aggressive and non-specific immunotherapy (69). Choosing a DMT also must also consider factors such as pregnancy status or family plans, methods of drug delivery which a patient is comfortable with and regional use of certain medications (42,70).

D. Autologous haematopoietic stem cell transplant (AHSCT):

Patients which fulfil certain disease criteria living in certain regions may be eligible for an AHSCT which aims to "reset" the immune system (71). This involves harvesting haematopoietic stem cells from the patient's blood, administering an intensive course of chemotherapy which eradicates remaining white blood cells, and reinfusing the patient's own cells (71). Sometimes DMT medications are used in combination with an AHSCT. The aggressive nature of the procedure invokes high risk of severe and long-term side effects such as early menopause, and an increased likelihood of infections, cancers, and other auto-immune diseases (72). In the UK, 1 in 300 patients die as a result of complications associated with this treatment (72).

E. Lifestyle alterations and holistic treatment

As previously mentioned, lifestyle choices are key social determinants of health (45). Quitting smoking, eating a balanced diet and frequent exercise or physiotherapy are proven to increase receptiveness to treatment, lessen the severity of relapse and decrease the chances of developing co-morbidities (42). This will not only to improve physical health but also mental wellbeing, which can be further assisted by support groups, counselling, and cognitive behavioural therapy (CBT) (73).

The vast variation in disease course between individuals and lack of a specific therapeutic target makes MS management complicated. Treatment inevitably comes with controlling side effects and as with any immunotherapies, patients can be left vulnerable to infections. Therefore, MS patients are placed in the vaccine priority groups alongside the elderly, the young and those with other conditions which make them vulnerable to infection. Additionally, the availability and access to DMTs and AHSCT are limited to regions (74) or countries that can afford them. As there is geographical bias of MS occurrence, there is also a bias of access to MS treatment. Around 70% low-income countries have no access to DMTs (2). Global drug-affordability schemes are needed to resolve these health inequalities.

In countries where DMTs are licensed for use, the importance of early intervention for patient outcomes has been well studied (75). Additionally, accurate prescription of DMTs prevents wasted time and resources on using less-effective therapies. Choosing the most appropriate DMT for each MS patient relies on timely and accurate diagnosis and categorisation of MS. Ultimately, this leads to faster intervention thus better prognosis (76).

1.8 Investigating new biomarkers

A biomarker can be defined as a measurable indication of health. In the context of clinical investigations, biomarkers are used to predict, diagnose, prognose, monitor a condition, and predict treatment compatibility. Biomarkers typically take the form of specific genetic, molecular, histological, or physiological characteristics. A successful biomarker must be sensitive and specific and correlate with the presence, absence, or severity of disease. Sensitivity refers to the percentage of patients with the disease, and test positively for the biomarker. Specificity refers to the percentage of patients without the disease, and test negatively for the biomarker. Important to the adoption of a biomarker is method of sample collection, ease of application to clinic or community, and sample storage conditions. There is always a clinical interest in finding novel biomarkers, whether that be for financial, ethical, or clinical reasons. Regardless, the shared goal is to give the patient the best chance of personalised, predictive, and preventative medicine, in a timely and accurate manner.

1.9 Study rationale

As previously described, biomarkers for MS include clinical, imaging, and molecular data (49). OCB detection in CSF is a hallmark of MS diagnosis as defined by the McDonald criteria. The methodology used to detect OCBs is well established and has 95% accuracy in the diagnosis and differential diagnosis of MS (77). However, the current method of CSF sampling is a lumbar puncture which has drawbacks in accessibility and invasiveness, thus cannot be performed frequently (78). This study is an evaluation of OCB and FLC detection in non-invasive and easily accessible secretions: tear fluid and saliva as an alternative to CSF.

Lumbar punctures, although generally safe, carry a risk of physical and emotional side effects (79). The most common adverse effects are headaches (80), nausea and anxiety leading up to and surrounding the extraction. In extremely rare circumstances, the procedure can cause trauma to the spine which may result in loss in sensation to paralysis (79). Furthermore, some patients are unsuitable for the procedure, such as those with skin infections, platelet disorders, scoliosis, and idiopathic cranial pressure (81). By contrast, the methods used to extract tear fluid and saliva are far less invasive, carry no risks other than eye irritation, and are suitable for the majority of patients.

A lumbar puncture is costly in both time and resources to the NHS. Owed to its complex nature, the procedure demands highly trained clinicians and specialist equipment and takes between 30 minutes to an hour. In the case of an X-ray guided lumbar puncture, additional time, staff, and equipment are required. In this study, the method of saliva sampling costs 20p in equipment and takes 5 minutes. Tear fluid extraction costs 60p in equipment and takes 10 minutes. Both tear fluid and saliva sample collection can be carried out by any appropriately trained healthcare worker, in primary care, hospitals or in the community.

A less invasive and more cost-effective method of testing has vast clinical implications for MS patients (82). Currently, an MS patient cannot expect to undergo frequent lumbar punctures, whereas there is no clinical reason to discourage regular saliva and tear fluid extraction. This allows for more frequent thus more accurate longitudinal monitoring. Even in the UK, where MS is well recognised, it can take around months to be diagnosed and longer for disease to be categorised. Frequent testing can be used for clinicians to acquire a precise picture of the disease, thus diagnose, and categorise the disease faster and with

more accuracy. As a result of this, patients can be prescribed the most appropriate personalised treatments, and faster. Ultimately, faster intervention means better prognosis.

Frequent monitoring can additionally help clinicians understand the underlying disease better. With more data points, a clinician may be able to predict the conversion to MS from clinically isolated syndrome (CIS), or to predict and identify a relapse, therefore allowing for quick intervention. Frequent monitoring may also be used in the predisposed, to gather more data on the immunopathology of MS. Furthermore, monitoring can be run in parallel to medication prescriptions and alterations to better understand the condition. Turning from OCB positive to OCB negative is a good prognostic marker (83) but is not currently used as lumbar punctures are not performed frequently. Frequent monitoring via other secretions could make use of this prognostic marker.

The exploration of using non-invasive tear and saliva secretions to diagnose and monitor MS is promising not only for their practicality and non-invasive collection, but also for their biochemical properties. Saliva, secreted by the salivary glands, contains water, electrolytes, digestive enzymes, proteins, mucins, as well as several immune components such as immunoglobulins (predominantly IgA) and antimicrobials (such as lactoferrin and lysozymes). Amongst digestive and oral health functions, saliva is one of the first lines of defence in mucosal immunity (84). Tear fluid, produced by lacrimal glands, has a similar composition to saliva and is mostly made up of water, electrolytes, proteins, lipids, mucins, as well as several immune components such as immunoglobulins (predominantly IgA) and antimicrobials (such as lactoferrin and lysozymes). The role of tear fluid is to lubricate the eye surface as well as protect the eye from infection (85). CSF is produced by the choroid plexus in the brain and is composed of water, electrolytes, glucose, proteins, immunoglobulins (predominantly IgG) and a small number of cells. As previously

mentioned, CSF serves to physically support and protect the brain and spinal cord and allow for substance exchange (53).

Biochemical differences and compartmentalisation between the different sample matrices present challenges in comparing biomarkers for MS. Despite these challenges, recent research has explored saliva and tear biomarkers for MS with variable degrees of success. The detection of IgG OCBs in CSF is a diagnostic hallmark of MS. Coyle reported the presence of OCBs in the tears of 67% MS patients tested (86), though further research is needed to understand the origin of the tear OCBs. Furthermore, other studies were unable to replicate this (87). To date, there is no published research on the presence of OCBs in saliva in the context of MS diagnosis. Additionally, elevated Kappa-FLCs in CSF has also shown high diagnostic accuracy. Whilst FLCs are detectable in all three matrices, their concentrations vary. Studies by Lotan (88) and Kaplan (89) quantified salivary FLCs, finding that the ratio of FLC monomers to dimers correlates with disease state in MS patients. Currently there is no research on FLC detection in tears in the context of MS.

The IEF method used to detect OCBs has been thoroughly optimised by the Clinical Immunology service and has demonstrated sensitivity of around 0.05 mg/L. Kappa and Lambda FLC ELISAs, also pre-established for saliva analysis in the Clinical Immunology Service using in-house antibodies, has demonstrated sensitivity of around 0.01 mg/L. The combined utility of these methods holds a promising approach in capturing any differences in saliva and tear OCB presence and FLC profiles in MS patients, provided they exist.

2. HYPOTHESES AND AIMS

2.1 Hypotheses

- Using IEF methods, pre-established for clinical diagnostics and reoptimized for tears and saliva, OCBs will be detectable in the tear and saliva fluid of MS patients and individuals with OCB-positive CSF.

- Using an ELISA, pre-established for saliva and reoptimized for tears, Kappa FLCs will be elevated in the tears and saliva of individuals with MS.

- Using Binding site Optilite analyser, Kappa FLC will be elevated in the serum of MS patients.

2.2 Aims

- Recruit 40 healthy donors, 40 MS patients and 40 NCCs with other neurological conditions. Collect serum, saliva, and tears from healthy donors. Collect CSF, serum, saliva, and tears from the MS patients and NCCs.

- Reoptimize current IEF methods for tear and saliva testing, then test presence of OCB in matched CSF (if available), serum, saliva, and tears.

- Reoptimize FLC ELISA for use in tears, then generate reference ranges of Kappa and Lambda FLC parameters in saliva and tears between healthy donors and patient cohorts.

3. METHODOLOGY

3.1 Ethical approval and consent

Ethical approval for the collection and analysis of saliva, tear fluid, blood and CSF was granted by the Health Research Authority (HRA) and Health Care Research Wales (HCRW) – Integrated Research Application System (IRAS) Project ID: 62053. The research passport was authorised by the University of Birmingham, which sponsored the study, and letter of access was issued by University Hospitals Birmingham (UHB) research and development department – UHB reference: RRK4750.

All eligible participants received a study information pack (see Appendix 1) and those who agreed provided full written informed consent prior to sample collection. For UHB participants, consenting was conducted by neurologist Professor Saiju Jacobs, and for healthy donors, Miss Chloe Tanner conducted consenting. Signed and dated consent forms were stored securely.

3.2 Participant recruitment and cohorts

3.2.1 Healthy donors

40 healthy donors were recruited from University of Birmingham staff and students between October 2022 and April 2023. Inclusion criteria was defined as having no known immune or neurological disorders. This cohort donated blood, saliva, and tears.

3.2.2 Patient cohorts

80 participants were recruited from ambulatory care and the neurology ward at the Queen Elizabeth hospital, Birmingham between July 2023, and January 2024. These participants were undergoing lumbar puncture investigations for MS amongst other neurological

diagnoses but had not yet received intervention. At the point of sample collection, diagnosis was unknown. However, later in the process, the group was sub-categorised into MS patients and non-MS neurological conditions, named neurological condition controls (NCCs). These combined cohorts donated blood, saliva, tears, and CSF.

3.3 Sample retrieval, processing, and storage

All samples were collected using the same methods for healthy donors and patient cohorts. All samples were processed on the day of collection.

3.3.1 Pre-sampling checks

Participants were requested to not eat, drink (other than water), smoke or brush their teeth within 30 minutes of the sampling appointment to avoid saliva contamination. Deviations from this were recorded but sample collection was still possible.

Participants were also asked whether they wear contact lenses or glasses. Neither impact the collection of tears, however, gathering this information may inform barriers to tear collection, as well as the potential impact of contact lenses on tear constituents, further assessing the feasibility and reliability of using tears for biomarker analysis.

3.3.2 Intravenous blood collection and processing

10ml of blood were collected in a serum tube (BD Vacutainer® #367895) by a trained phlebotomist or clinical staff. The filled tube was inverted 5-10 times then left for at least 30 minutes at room temperature to allow the distribution of silica coagulation activators.

The blood tube was then centrifuged at 3500RPM for 5 minutes. Serum was separated from blood cells with a pipette, aliquoted into 2ml microcentrifuge tubes (Thermo Scientific #11519934) then immediately stored at -80°C until analysis. Aliquot volumes were recorded.

3.3.3 Saliva collection and processing

Saliva was collected by a passive drool method by which the participant was asked to rinse any existing saliva out of their mouth, then sit with their head down and empty any new saliva into pre-weighed 50ml conical centrifuge tubes (Falcon #352070) over four minutes. They were given the option to empty their mouth continuously or once after the four minutes had passed.

When sampling was complete, saliva was stored at 4-8°C in the fridge until processing began (maximum of six hours) to maintain protein stability. The filled saliva tube was weighed, and salivary rate was calculated as ml/minute by subtracting the post-sampling weights from pre-sampling weights of tubes, then dividing the result by the minutes of collection according to the equation below. Density of the saliva was assumed to be 1g/ml. The saliva was then centrifuged at 4000RPM for 10 minutes to remove cells and contaminants. Supernatant was extracted with a pipette, aliquoted into 2ml tubes, then immediately stored at -80°C until analysis. Aliquot volumes and salivary rates were recorded.

3.3.4 Saliva flow rate equation

$$Salivary\ rate\ (g/minute) = \frac{(Pre\ weight\ (g) - Post\ weight\ (g))}{Time\ (minutes)}$$

3.3.5 Tear fluid collection and processing

Tear fluid was collected using Schirmer tear strips (Contacare Ophthalmics and Diagnostics) according to manufacturer's instructions (See Figure 3.1). Microcapillary tubes are another commonly used tear collection device (90). However, ease of application in a clinical setting and tear flow insight was prioritised when deciding the method of collection (91). The end of the strip was folded to 90° and placed beneath the lower eyelid. The strip was left for five minutes unless it became fully saturated in less than five minutes. In either case, the strip was removed, and the saturation length of the strip and time of strip usage was recorded.

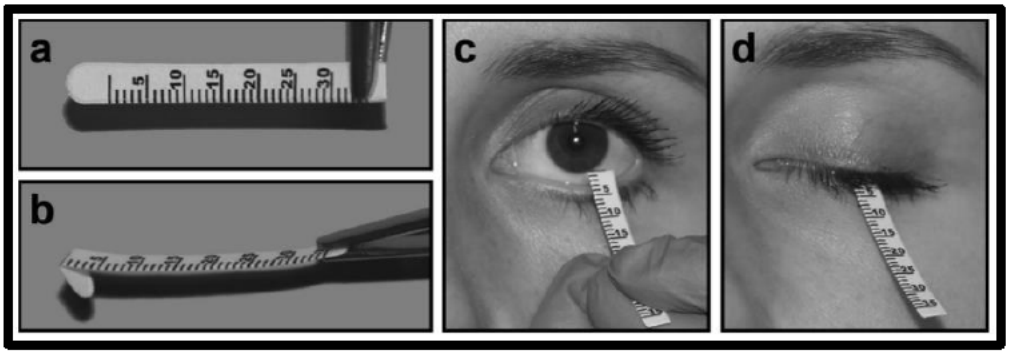


Figure 3.1 Tear fluid extraction via Schirmer strip method.
This Figure was sourced from (91). A: Schirmer strip with printed mm increments. B: End of Schirmer strip is folded 90° in a hook shape. C: Strip is placed beneath the lower eyelid and D: Participant is asked to close their eyes and leave the strip for 5 minutes.

The unsaturated end of the strip was cut off and discarded. The saturated end of the strip was submerged in 500µl Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco #14190144) in a 2ml tube. This process was performed in both eyes. Tear flow was calculated by dividing the saturated length of the strip (mm) by the minutes taken for

collection as shown in the equation below. The tubes containing the Schirmer strips were stored immediately at -80°C until analysis.

3.3.6 Tear flow rate equation

$$Tear\ flow\ (mm/minute) = \frac{Saturated\ length\ of\ Schirmer\ strip\ (mm)}{Time\ (minutes)}$$

3.3.7 CSF collection and processing

Within the terms of the ethical approval, CSF was only taken from the patient cohort of participants, who were pre-scheduled to have an investigative lumbar puncture.

The procedure was conducted by a specialised clinician, who administered local anaesthetic and extracted the CSF from between the L3-L4 or L4-L5 vertebral interspace (57) (see Figure 3.2). Sometimes, for patients with conditions such as obesity, scoliosis, or spinal damage, the lumbar puncture was x-ray assisted. A small sub-aliquot of approximately 1ml was taken for the purpose of this study (92).

Post collection, the CSF sample was centrifuged for 3 minutes at 3000RPM. Supernatant was extracted with a pipette, aliquoted into 2ml tubes, then immediately stored at -80°C until analysis. Aliquot volumes were recorded.

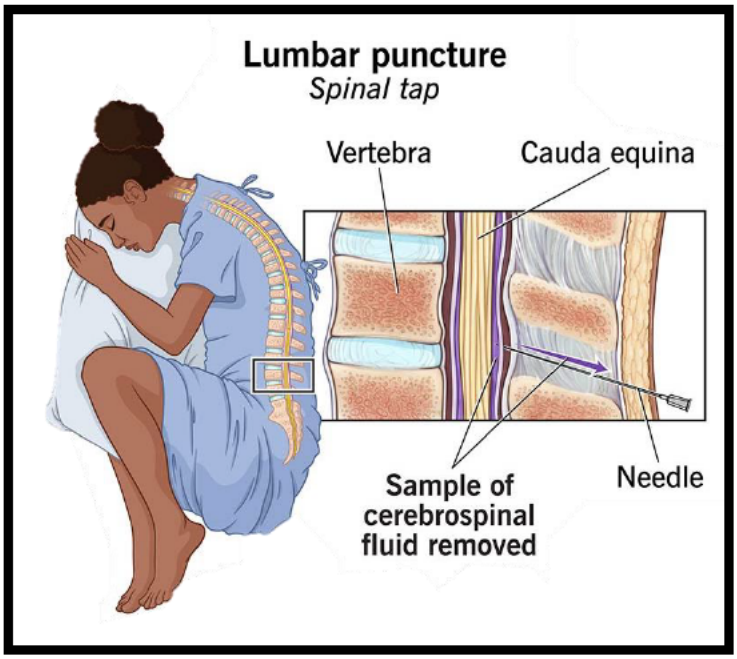


Figure 3.2 Lumbar puncture procedure.
This Figure was sourced from my.clevelandclinic.org (93). CSF is extracted by a lumbar puncture, whereby a specialist doctor administers local anaesthetic and inserts a needle between the L3-L4 or L4-L5 interspace.

3.3.8 Sample tracking and quality control

To monitor the consistency of sample collection, quality and storage, a sample processing form was implemented. This allowed the recording of details such as timings of collection, aliquoting information and any sample abnormalities (see Appendix 2). This information was collated into a corresponding excel sheet. Both physical and digital trackers were anonymised and stored securely.

Due to individual circumstances, some samples could not be collected according to protocol. For example, where some patients were in discomfort after their lumbar puncture, it was not appropriate to ask them to sit upright for saliva collection. Therefore, saliva was collected over shorter period, or single spit volume was obtained. Where participants

experienced eye irritation in one or both eyes during tear collection, the Schirmer strip was optionally removed prematurely.

Incidences of these abnormalities were recorded in detail in the sample quality section of the sample trackers, and where appropriate, the affected data were excluded from the analysis (See Appendix 3). It is worth noting that these occurrences are still useful insights into the study, as they allow for the investigation of factors such as ease of application, side effects and patient collaboration in the reliability and feasibility of collecting and analysing saliva and tear samples.

3.6 Healthy donor screening

In contrast to patient cohort recruitment, which was decided by clinical information and neurologist, Professor Saiju Jacobs, the eligibility of healthy donors was reported by the individual. Thus, a general screening of the healthy donor cohort was implemented in the case of any unknown immune-related abnormalities. Healthy donor serum was screened for IgG, IgA, IgM and Kappa and Lambda FLC concentrations using the Binding site Optilite analyser. Disproportionate levels of these may indicate a malignancy, infection, or immune disorders. All but two healthy participants yielded results within normal reference ranges established by NHS guidelines (displayed in Appendix 4). Fortunately, after a further investigation by capillary zone electrophoresis (CZE), no further action was required, and the participants were not excluded. Serum screening and CZE analysis was conducted by Biomedical Scientist Mr Mohammed Afzal at the Clinical Immunology Service.

3.7 Tear volume-Schirmer strip saturation assays

3.7.1 Assay protocol

To quantify tear fluid volumes collected using Schirmer strips, a volume-to-saturation validation assay was performed. This experiment aimed to evaluate the hypothesis that the saturated length of the strip is proportional to the volume of tear fluid collected.

Due to the Schirmer strip method of tear collection yielding very low sample volumes, pure tear fluid was not available for this validation. Instead, Bovine Serum Albumin (BSA) (Sigma-Aldrich #A4503) dissolved in PBS (Phosphate Buffered Saline- Oxoid #BR0014G) was used to model the tear fluid. Four different concentrations of the solution were prepared to assess whether variability in tear concentration affects the rate of absorbance onto the strip.

1. The following solutions of BSA in PBS solution were prepared: 1µg/ml, 5µg/ml, 10µg/ml, and 15µg/ml.
2. Exact volumes of each BSA solution from 1µl to 35µl were pipetted on to separate Schirmer strips and left for five minutes.
3. After five minutes, the saturated lengths of the strips, indicated by printed millimetre increments, were measured and recorded.

3.7.2 Tear parameter adjustments

Data from the volume: saturation assays were used to normalise the observed FLC concentrations based on the volume of tears collected. However, the precise tear volume eluted from the strip into the PBS when the sample was collected was unknown. Therefore, tear volumes were not readjusted from the observed saturation of the Schirmer

strips. Due to the linear relationship between volume and saturation, any re-adjustment would be relative rather than absolute. The equation used to correct the raw FLC concentrations is outlined below.

3.7.3 Tear dilution factor equation

An equation to derive the dilution factor of tear fluid from the saturated length of the strip and the volume of PBS used to elute the tear fluid (controlled at 500µL).

$$Dilution\ factor = \frac{500}{Saturated\ length\ of\ strip\ (mm)}$$

3.7.4 Tear FLC corrected concentration equation

An equation to adjust the observed concentration of FLCs based on the dilution factor and saturated length of the Schirmer strip.

$$Corrected\ concentration\ (mg/L) = \frac{(Observed\ concentration\ (mg/L)\ x\ dilution\ factor)}{Saturated\ length\ of\ strip\ (mm)}$$

3.8 FLC ELISAs

An enzyme-linked immunosorbent assay (ELISA) is an immunological tool used to quantify a specific protein or molecule in a sample. The technique uses antibodies to bind to a target antibody or antigen. Then a secondary antibody (conjugated to an enzyme) is introduced and binds to the first antibody. Next, the corresponding substrate to the enzyme is added, which induces a colour change. The depth of colour change, measured as optical density (OD) by a colorimeter, can be quantified, and the concentration of the target

can be approximated by interpolating results on a standard curve. This curve is generated using a serial dilution of a sample with a known concentration of the target protein or molecule. For the purpose of this study, the target molecules were Kappa and Lambda FLCs.

3.8.1 Secondary antibody HRP conjugation

Secondary antibodies were conjugated to HRP (horseradish peroxidase) enzyme using Abcam (Lightning-Link #ab102890) HRP conjugation kit according to the manufacturers recommended protocol. For Kappa FLC quantification, BUCIS14 (Prepared by Dr Margaret Goodall #6e1.1.200119/pc) was used. For Lambda FLC quantification, BUCIS19 (Prepared by Dr Margaret Goodall #150619/P) was used.

1. Antibodies were diluted in PBS to a 1ml 1mg/ml solution.
2. 100µl of modifier reagent was added to each antibody and mixed.
3. Antibody mixtures were pipetted onto the lyophilised HRP label, gently resuspended, then incubated overnight at room temperature, protected from light.
4. The following day, 100µl quencher reagent was added to each mixture, and left for at least 30 minutes before use.
5. The antibody conjugates were stored in the fridge at 4-8°C.

According to manufacturer's guidance, these conjugations were stable 18 months from the day of preparation.

3.8.2 FLC ELISA optimisation

The FLC ELISA method is established in the Clinical Immunology Service and was a useful starting point for establishing the protocol. However, with changes in reagents, operators, sample types and cohort, it was important to re-optimise the assays in the context of this study. Serial dilution ranges for the standard curve, sample dilutions, secondary antibody concentrations, substrate concentration and incubation times were evaluated simultaneously in “matrix” style assays in order to decipher the most appropriate conditions. The basis for these validation assays is outlined below:

A. Range of detection:

Appropriate assay thresholds and dilution factors of the standard curve were investigated to ensure that the assays range of detection accurately covered the anticipated range of results. This is particularly important for the upper and lower extremes of results. The range of detection was 2mg/l-0.05mg/l for both Kappa and Lambda which was decided based on reference ranges previously published by the department (94). Serial dilution factors for the standard curved were trialled between 1:2 and 1:10 and was derived from a mixed pool of healthy control standard serum (TCS Biosciences).

B. Sample dilution:

Appropriate sample dilution ensures results fall within accurate range of the assay, whilst also conserving maximal sample volume. Optimal saliva dilution could be tested based on previous saliva ELISAs (94). Using tears for this assay was novel to the department, therefore initial optimisation assays trialled the same dilutions as saliva. For both sample types, multiple dilutions were trialled, from neat to 1:8, with the aim to obtain results in the

middle-lower region of the curve, where interpolation of FLC concentrations is most accurate as this should be the most linear section of the curve.

C. Secondary antibody dilution:

The secondary antibodies used in previous studies in the department were also used for this study. However, the antibodies were newly conjugated to HRP for this study and therefore needed slightly different dilutions to meet sensitivity and specificity requirements. Also, for consistency, it was important to establish a secondary antibody dilution which worked for both saliva and tears. Multiple secondary antibody dilutions were tested between 1:1000 and 1:10,000 dilution, with the aim to obtain maximal signal in known positive controls and minimal background in known negative controls.

D. Substrate concentration and incubation time:

Appropriate substrate conditions were optimised to ensure maximal signal within detectable range of plate reader's absorbance settings. This must be balanced by minimising background noise. Different dilutions of substrate (neat and 1:2) as well as incubation times between five and ten minutes were trialled. The aim was to obtain strong signals, whilst ensuring the upper limit of the curve could be detected by the plate reader's absorbance settings, and blank wells yielded minimal signal.

E. Inter-assay variability

The function of the antibodies may degrade over time, and due to delays in recruitment, there were significant time gaps between assays. In order to monitor the inter-assay variability, I ran the same three samples across all plates and calculated the coefficient of

variability (CV) which in best practise should be 10% or less (see equation below). These chosen samples were from the healthy donor cohort and had a high volume. The aliquot was thawed and re-aliquoted into a number of smaller volumes to avoid inconsistencies associated with multiple freeze-thaw cycles.

$$CV = \frac{\sigma}{\mu}$$

CV = Coefficient of variation

σ = Standard deviation

μ = Mean

F. Intra-assay variability

Pipetting errors, cross-contamination, evaporation, and handling inconsistencies can play a significant role in result variability within an assay. To monitor intra-assay variability, the standard curves and controls were always tested in duplicate, and CVs were calculated as above. To conserve sample volume, samples were tested in singlicate.

3.8.3 FLC ELISA protocol

Following the optimisation assays, the following protocol was deduced.

1- Coating of Capture Antibody

96-well flat-bottom high-binding plates (Corning #9018) were coated with 100µl per well of 1µg/ml capture antibody in PBS. For Kappa FLC quantification, BUCIS04 (Prepared by Dr Margaret Goodall #141210/3b) was used. For Lambda FLC quantification, BUCIS09

(Prepared by Dr Margaret Goodall #9230614/p) was used. The plates were sealed with adhesive seals (Thermo Fisher Scientific #AB0558), then incubated overnight at 4-8°C.

2- Blocking

The following day, the plates were washed four times with 200µl per well of wash buffer. The wash buffer consisted of 0.1% Tween 20 (Sigma-Aldrich #P2287) in PBS made up according to manufacturer's guidance. The plates were then blocked with 150µl per well of blocking buffer. The blocking buffer consisted of 2% BSA in PBS. The plates were blocked for an hour at room temperature. During this incubation period, the samples and controls were prepared.

3- Sample Preparation

All samples, standards and controls were pre-prepared in dilution plates. Tear samples were thawed and prepared neat. Saliva samples were thawed and centrifuged at 10,000RPM for 10 minutes and prepared at a 1:2 dilution in 1% BSA in PBS. A 12-point 1:2 standard curve from a starting concentration of 2mg/L FLC concentration was prepared using the mixed pool of human standard serum. Three saliva samples were placed on each plate to assess inter-assay variability. The standards and controls were tested in duplicate to measure for intra-assay variability. Once the blocking was complete, the plates were washed four times with 200µl per well of wash buffer, before adding the contents of the diluted samples. For saliva, 100µl per well was added, for tears, 70µl per well was added to conserve volume. The plates were left to incubate for one hour at room temperature.

4- Secondary Antibody

Plates were washed four times with 200µl per well of wash buffer, then secondary antibody was added. For Kappa FLCs, HRP-conjugated BUCIS14 was diluted 1:10,000 in PBS and for Lambda FLCs, HRP-conjugated BUCIS19 was diluted 1:20,000 in PBS. The antibodies were added to the plate at 100µl per well, then the plates were covered from the light and incubated at room temperature for one hour.

5- Development

Plates were washed four times with 200µl per well of wash buffer. Tetramethylbenzidine (TMB) substrate (Binding Site #EA003) was diluted 1:2 in PBS for both Kappa and Lambda FLC plates, 100µl per well of substrate was added. The plates were left uncovered for 10 minutes at room temperature.

6- Stop and Plate Reading

Finally, once the plates had been developing for 10 minutes, they were stopped using Orthophosphoric Acid Stop Solution (Binding Site #EA004) at 100µl per well. Absorbance was measured at 450nm using a plate reader within 5 minutes.

3.9 Interpreting FLC parameters

For saliva and tears, FLC concentrations were interpolated from the serum standard curve.

The following calculations were used to derive FLC parameters in all sample types.

3.9.1 Kappa and Lambda FLC sum equation

This equation was the same for sum concentration and secretion rates.

$$Kappa\ and\ Lambda\ sum = Kappa\ value + Lambda\ value$$

3.9.2 Kappa and Lambda FLC difference equation

This equation was the same for difference in FLC concentration and secretion rates.

$$Kappa\ and\ Lambda\ \ difference = \ \ Lambda\ value - Kappa\ value$$

3.9.3 Kappa: Lambda ratio equation

This equation was the same for concentration and secretion ratios.

$$Kappa{:}\ Lambda\ ratio = \frac{Kappa\ value}{Lambda\ value}$$

3.9.4 Saliva FLC secretion rate equation

$$Saliva\ secretion\ rate\ (mg/minute)$$
$$= FLC\ concentration\ (mg/L) \times Flow\ rate\ (mL/minute)$$

3.9.5 Tear FLC secretion rate equation

$$Secretion\ rate\ (mg/minute)$$
$$= Corrected\ concentration\ (mg/L) \times Flow\ rate\ (mm/minute)$$

3.10 Isoelectric focussing (IEF)

Isoelectric focussing (IEF) is the gold standard method used to detect oligoclonal banding in serum and CSF. The method uses gel electrophoresis to separate proteins by charge. Then, the gel is transferred onto a nitrocellulose membrane, which is incubated in a number of antibody staining steps to visualise the protein migration pattern. Finally, the trace is developed and analysed by eye.

3.10.1 IEF optimisation

The method for isoelectric focussing has been comprehensively refined for sensitivity by clinical scientists at the Clinical Immunology Service. Therefore, the established standard operating procedure (SOP) was used: Oligoclonal bands in CSF. Document code: IEF. Version 5.3 October 2021. Author: Abid Karim. Training and support for the IEF method was provided by Biomedical Scientists: Mr Bilal Jeewa and Mrs Beena Emmanuel, at the Clinical Immunology Service. For the purpose of this study, the method was re-optimised for OCB detection in saliva and tears.

A. Sample dilution

In order to test samples at approximately the same protein concentration, CSF was tested neat. Serum, with a typical protein content of 80mg/ml was diluted 1:400. Saliva has a typical protein concentration of 0.5-3mg/ml so was trialled at a 1:4 dilution. Tears have a typical protein concentration of 3-5mg/ml, however as they were diluted in PBS, they were trialled neat.

B. Antibody target

IgG oligoclonal band detection is the gold standard method for diagnosing MS. Given the evidence that Kappa FLCs are elevated in MS patients, total free and unbound immunoglobulin were trialled additionally.

3.10.2 IEF method

Following the optimisation assays, the following protocol was deduced.

1. Gel casting

3.6g of d-sorbitol (Sigma-Aldrich #240850) and 0.3g of agarose (GE Healthcare #17055402) were added to 27ml of 10% v/v glycerol (Sigma-Aldrich #G9012). The mixture was heated in short intervals until fully dissolved, then placed in a 75°C water bath to equilibrate for 10 minutes.

Following this, 2ml of pH 3-10 and 0.5ml pH 8-10.5 Pharmalyte (GE Healthcare #GE17-0456-01, #GE17-0455-01) were added to the gel mixture and incubated for a further 5 minutes at 75°C.

The gel was poured onto a hydrophilic gel bond film (LONZA Ltd #54733) in a pre-warmed cast and spread evenly. This was left to set for 10 minutes, then stored in damp chamber for up to 3 days at 2-8°C until use.

2. Sample preparation

All samples were centrifuged at 3500RPM for 3 minutes to pellet contaminants and cells. Following this, the following sample dilutions were prepared: CSF was tested neat; serum

was diluted 1:400, saliva was diluted 1:4 and tear fluid was tested neat. A positive control was diluted 1:400.

3. Gel electrophoresis

Once samples were prepared, the gel was placed into the IEF chamber, and the cooling unit was set to 10°C.

The anode was prepared by saturating a strip of filter paper in 0.05M sulphuric acid. The cathode was prepared by saturating another strip of filter paper in 1M sodium hydroxide. Both electrodes were placed on the anodal and cathodal side of the gel respectively, 7cm apart.

The diluted samples were vortexed, then 5µl of each sample were pipetted onto the sample applicator, placed 0.5cm from the anode.

The electrophoresis power unit was programmed to the following settings: 1250V, 100mA and 20W. Throughout the run, the power supply is paused incrementally to ensure condensation within the chamber did not interfere with the migration. The migration stopped automatically at 1000VH.

4. Gel transfer

Following electrophoresis, the gel was removed from the chamber. Firstly, a single sheet of nitrocellulose membrane (Ultra-Cruz/Santa Cruz Biotech #201698) was applied for 10 seconds and promptly removed. The purpose of this step was to remove surface proteins. A second sheet of NCM was applied to the surface of the gel, on which the proteins would

be transferred. Layered above the NCM were five sheets of filter paper to absorb excess moisture and a 2.5kg weight. This was left to transfer for 30 minutes.

5. Blocking

Block was prepared as a 50ml solution of 5% dried milk powder (Marvel) in PBS. The filter paper and weights were carefully removed from the gel. The NCM was heat fixed then placed into a container and the prepared block solution was added. The NCM was blocked for 30 minutes on a rocking platform at room temperature. After the incubation, the NCM was washed three times with tap water.

6. Antibody incubation

The antibody staining steps differed between the separate IgG and total free and bound immunoglobulin assays.

For IgG staining (the gold standard clinical method), the NCM was placed into a 0.2% solution of milk powder. The secondary antibody was added in a 1:800 dilution. This was incubated on the rocking platform for 1 hour at room temperature. After the incubation, the NCM was washed thoroughly, 20 times with tap water, then immersed in PBS for 5 minutes, on the rocking platform.

For immunoglobulin staining, two antibody staining steps are required. Firstly, the NCM was placed into a 0.2% solution of milk powder. The primary antibodies, BUCIS14 and BUCIS19 were added at a 1:800 dilution. This was incubated on the rocking platform for 1 hour at room temperature. After the incubation, the NCM was washed thoroughly, 20 times with tap water, then immersed in PBS for 5 minutes. Next, the NCM was transferred into

another container of a 0.2% solution of milk powder. The secondary antibody, goat anti-mouse IgG Human-ads HRP conjugate (Southern Biotech #1030-05) was added at a 1:500 dilution. This was incubated on the rocking platform for 1 hour at room temperature. After the incubation, the NCM was washed thoroughly, 20 times with tap water, then immersed in PBS.

7. Development

Development solution was prepared by dissolving one Tablet of 3, amino-9-ethylcarbazole (Sigma-Aldrich # A5754) in 2.5ml 100% methanol. In a separate tube, 835µl of sodium acetate (Sigma-Aldrich #567422) and 33ml 30% hydrogen peroxidase (Sigma-Aldrich #H1009) was added to 50ml deionised water. Just before development the two mixtures were combined and poured onto the NCM. This was covered from light and incubated on the rocking platform for 10-15 minutes.

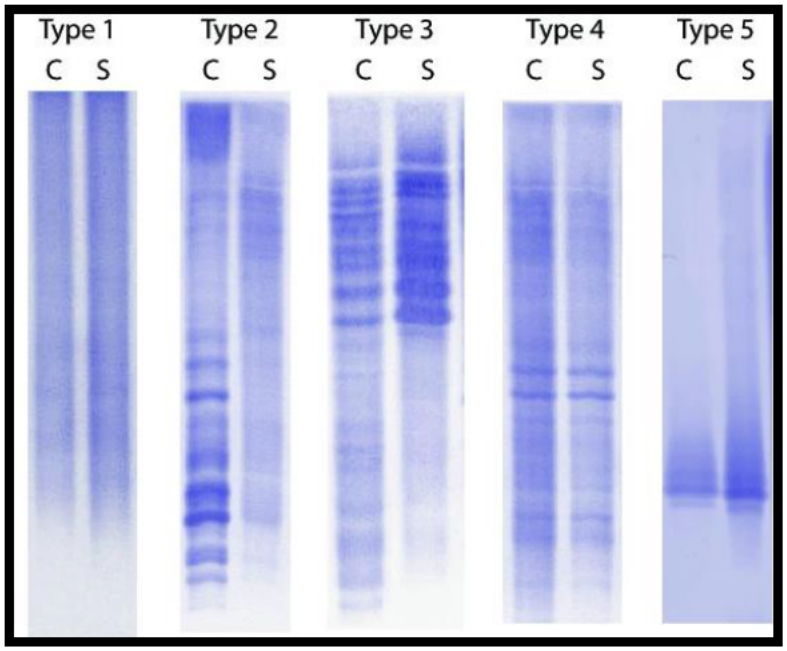
The development was stopped by washing the NCM 20 times in tap water then in deionised water 5 times.

When development was finished, the NCM was removed from the container and left to dry overnight on filter paper, covered from light. Alternatively, for same day analysis, the NCM was dried in a warm room at 37°C for 5 minutes.

3.10.3 Interpretation of Result

The IEF result can be categorised into five standard patterns (95) (see Figure 3.3). Two or more bands are considered as a positive result.

A



B

Pattern code	Meaning
Type 1	No oligoclonal banding seen in CSF or serum.
Type 2	Oligoclonal bands in CSF only. Suggests intrathecal IgG synthesis which may be seen in MS.
Type 3	Identical oligoclonal band pattern in serum and CSF with extra bands in CSF. Suggests intrathecal and systemic IgG synthesis.
Type 4	Identical oligoclonal band pattern in serum and CSF. Suggests systemic IgG synthesis
Type 5	Monoclonal "ladder" IgG bands in serum and CSF. Suggests paraproteinemia

Figure 3.3 IEF result interpretation
A: Examples of OCB patterns were sourced from (95). C: CSF, S: Serum. B: Table displaying the interpretation of pattern types 1-5. For each participant, the result is categorised by the operator. The interpretation is given a second opinion by a senior member of staff. Two or more bands are considered as a positive result.

The result of IEF is qualitative thus subjective to the operator of the assay. Therefore, standard practise is initial interpretation, followed by a second opinion by a senior member of staff from the Clinical Immunology Service. Second opinion was provided by the Clinical Immunology Service Biomedical Scientists: Mrs Beena Emmanuel and Mr Bilal Jeewa.

3.11 Statistical analysis

All data was analysed using GraphPad prism software (Version 10.1.0). A paired T-test was used to compare right and left tear parameters. ANOVA was used to compare all parameters between healthy donor, MS patients and NCC cohorts. All data presented in tables were reported as median (range).

4. RESULTS

4.1 Cohort characteristics

40 healthy donors and 80 patients were recruited for this study. The demographic profiles of the cohorts are summarised in Table 4.1 and presented as median and range.

4.1.1 Healthy donors

The healthy donor group were aged between 20 and 67 years old with a median age of 37. Among them, 29 were female and 11 were male. No participants in the healthy donor cohort were excluded or withdrew and no data was excluded.

4.1.2 Patient cohorts

Of the 80 patients recruited from QEHB, 20 were subsequently diagnosed with MS while 60 were NCCs. The ages of the MS patient group NCCs were significantly higher than healthy donors, however there was a non-significant age difference between MS patients and NCCs. For the non-MS NCC cohort, specific diagnosis details were not disclosed but included a range of neurological, immune, chronic, or other medical conditions.

Seven patients had at least one dataset excluded due to difficulties in sample collection or sample quality. An additional two patients declined to provide some sample sets but did not withdraw completely. A full breakdown of this excluded data is outlined in Appendix 3.

Table 4.1 Cohort age and gender demographics.
Values are reported as median (range). Age was significantly higher in MS patients and NCCs compared to healthy donors. *p*-values reported as 0.0017 and 0.0057 respectively.

	Healthy donors N=40	MS patients N=20	NCCs N=60
Age (years)	37 (20-67)	56 (17-74)	48 (19-76)
Gender ratio (%)	72.5% (29) Female 27.5% (11) Male	75% (15) Female 25% (5) Male	63.3% (38) Female 36.7 (22) Male

4.2 Saliva and tear flow rates

Saliva and tear flow parameters are displayed in Table 4.3, presented as median and range, whilst graphical representations are shown in Figure 4.1.

When compared with healthy donors, saliva flow rate is significantly lower in both MS patients and NCCs. However, the difference between MS patients and NCCs is non-significant.

A similar pattern is observed in tear flow rate, in which tear flow rate is also significantly lower in MS patients and NCCs compared to healthy donors. Although the tear flow rate in MS patients appears slightly lower than the NCCs, the difference is again non-significant.

It is worth noting that a Schirmer strip saturation of 5mm or less, equating to a tear flow rate of 1mm/minute or less indicates severely dry eyes. None of the healthy donors were classed as having severely dry eyes, yet both the MS and NCC cohort had 37% and 27% of their respective cohort falling into this category.

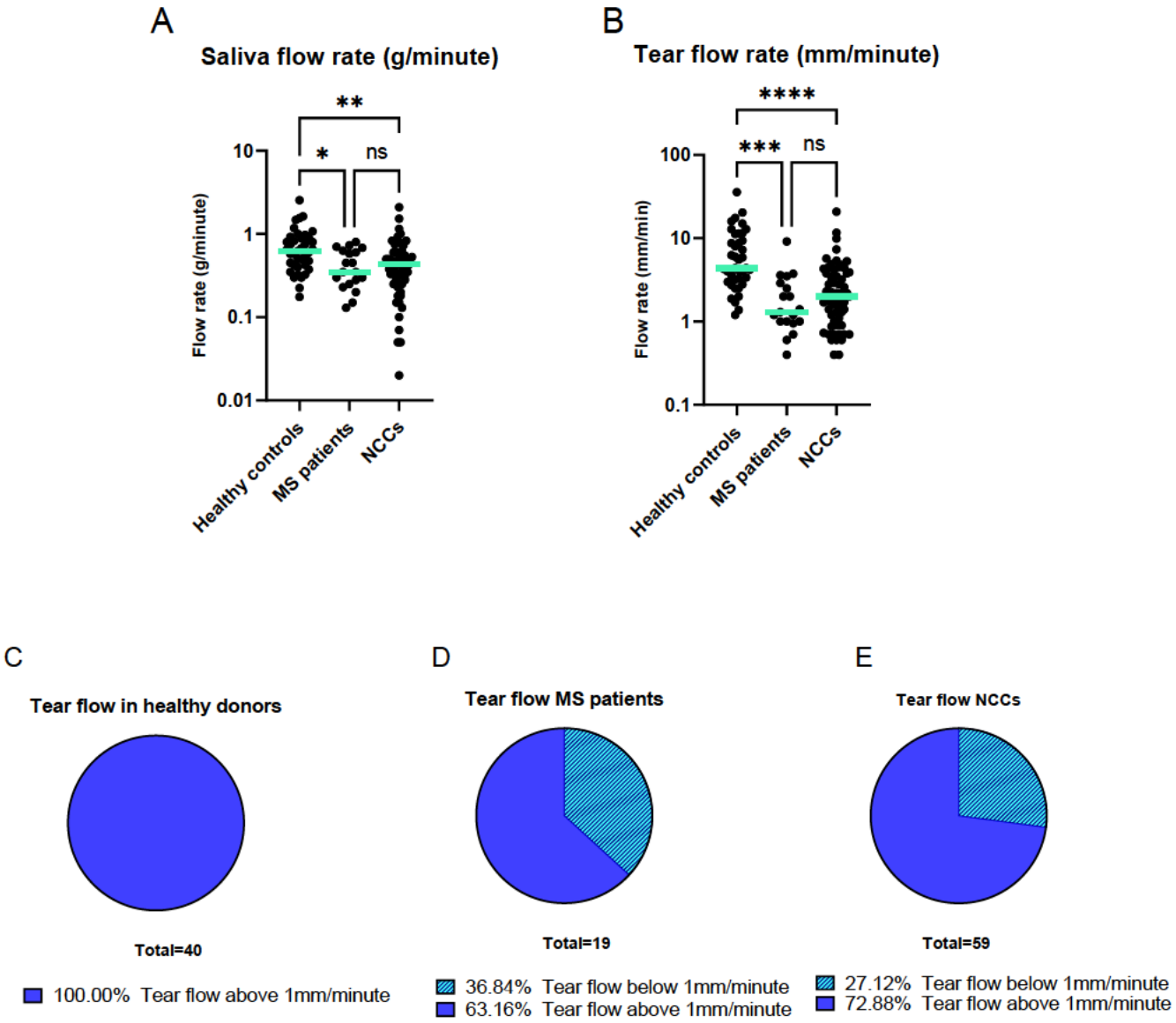


Figure 4.1 Saliva and tear flow rates.

A: Saliva flow rates. B: Tear flow rates. Each point represents an individual participant. Tear flow values are taken as an average between left and right eyes. Median line shown in blue. C: Proportion of healthy donors with severely low tear flow rates. D: Proportion of MS patients with severely low tear flow rates. C: Proportion of NCCs with severely low tear flow rates. Severe dry eyes are defined as having a tear flow rate of less that 1mm/minute (pale blue striped). Values above 1mm/ml are shown in dark blue. (ns= $p>0.05$, * = $p\leq 0.05$, ** = $p\leq 0.01$, *** = $p\leq 0.01$, **** = $p\leq 0.0001$).

4.3 Schirmer strip volume: saturation assays

As shown in Figure 4.2, there is a linear relationship between the volume of BSA solution added to the Schirmer strip and the resulting saturated length of the strip. 25µL is the maximum volume of fluid that can be added before the strip becomes completely saturated at 35mm.

There is a non-significant impact of BSA concentration on this relationship across the tested concentrations of 1µg/mL, 5µg/mL, 10µg/mL, and 15µg/mL. Linear regression analysis (generated by GraphPad prism 10.1.0) showed a strong linearity, and R^2 values were reported as 0.96, 0.97, 0.98 and 0.98 respectively.

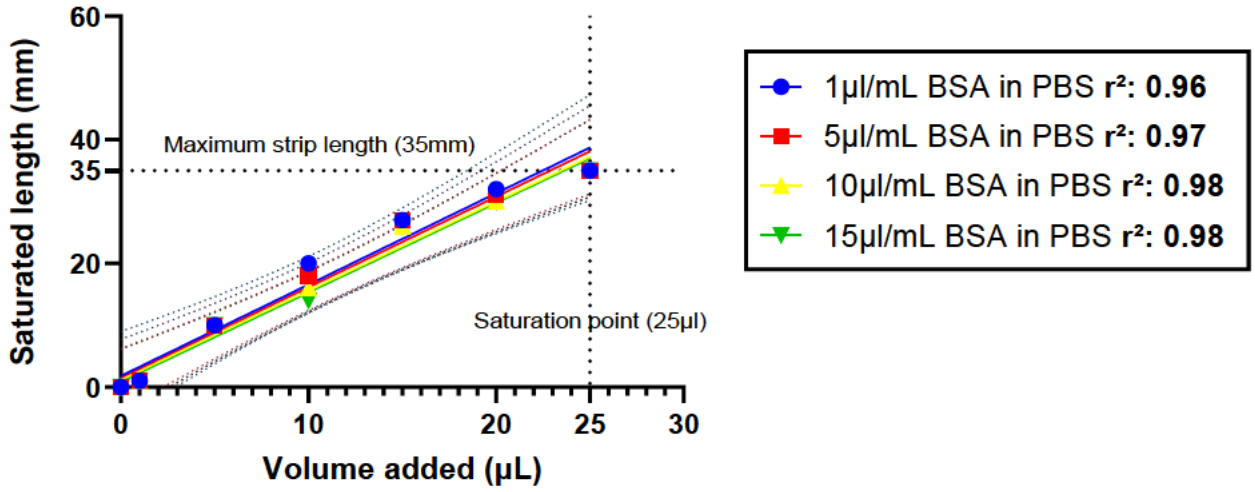


Figure 4.2 Relationship between the volume of BSA solution added to a Schirmer strip vs saturated length of the Schirmer strip.
Four different concentrations of BSA in PBS solution were tested: 1µg/mL (blue circle), 5µg/mL (red square), 10µg/mL (yellow triangle) and 15µg/mL (green upside-down triangle). R^2 values were reported as 0.96, 0.97, 0.98 and 0.98 respectively. The Schirmer strip becomes completely saturated (at 35mm mark) when 25µL of BSA is added.

4.4 Comparison of left and right eye tear parameters

Whilst individual differences in tear parameters between the right and left eyes were observed within our dataset, there were no statistically significant differences overall, as shown in Table 4.2 and graphically in Figure 4.3. This was true for all three cohorts, but for clarity, only healthy donor values are displayed. Averaged values between left and right eye parameters were used in subsequent data presentations. This aimed to decrease the impact of inter-eye variability whilst retaining the overall trend.

Table 4.2 Comparison of left and right eye tear parameters in healthy donors
Values are reported as median (range).

	Left eye	Right eye
Tear flow rate (mm/minute)	3.75 (0.5-60.034)	6.38 (0.75-23.33)
Tear FLC concentrations (mg/L)		
Kappa	0.55 (0.04-9.14)	0.33 (0.02-8.28)
Lambda	0.17 (0.02-2.91)	0.11 (0.00-1.13)
Tear FLC secretion rates (mg/minute)		
Kappa	1.99 (0.28-22.85)	1.92 (0.09-21.54)
Lambda	0.59 (0.11-11.75)	0.69 (0.02-3.46)

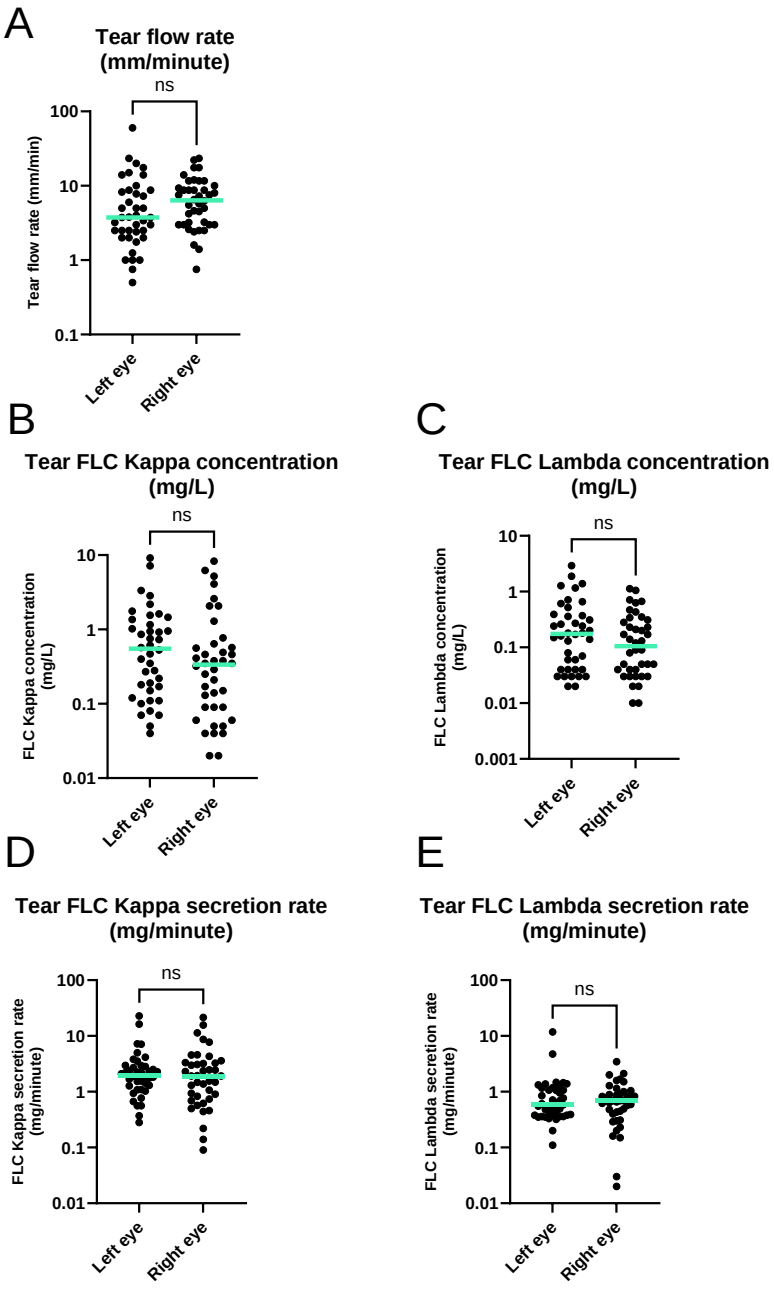


Figure 4.3 Comparison of left and right eye tear parameters.
A: Tear flow rate. B: FLC Kappa concentration. C: FLC Lambda concentration. D: FLC Kappa secretion rate. E: FLC Lambda secretion rate. Median line shown in blue. (ns= $p>0.05$, * = $p\leq 0.05$, ** = $p\leq 0.01$, *** = $p\leq 0.01$, **** = $p\leq 0.0001$).

4.5 IgGAM and FLC parameters

IgGAM and FLC parameters are displayed in Table 4.3 as median (range).

Table 4.3 Summary of serum, saliva, and tear parameters
All values are reported as median (range).

	Healthy donors N=40	MS patients N=20	NCCs N=60
Serum immunoglobulin concentrations (g/L)			
IgG	10.12 (6.67-15.03)	10.32 (5.24-15.31)	9.38 (<0.17-42.01)
IgA	1.90 (0.55-5.58)	2.52 (1.12-4.89)	1.94 (0.08-7.96)
IgM	1.23 (0.40-3.19)	0.92 (0.60-3.07)	1.07 (<0.10-7.54)
Serum FLC concentrations (mg/L)			
Kappa	8.87 (4.84-18.40)	18.74 (7.11-43.72)	17.47 (1.30-181.27)
Lambda	11.98 (5.43-18.81)	14.18 (6.83-24.60)	16.50 (2.09-89.52)
Kappa and Lambda sum	20.70 (10.27-34.45)	32.84 (13.94-66.38)	32.82 (4.78-270.79)
Kappa and Lambda difference	2.54 (-2.35-10.72)	-4.44 (-21.06-1.16)	-2.10 (-91.75-7.14)
Kappa: Lambda ratio	0.78 (0.38-1.23)	1.35 (0.95-1.93)	1.12 (0.37-3.54)
Saliva flow rate (ml/minute)	0.63 (0.175-2.55)	0.35 (0.13-0.80)	0.43 (0.02-2.10)
Saliva FLC concentrations (mg/L)			
Kappa	0.92 (0.06-7.42)	0.38 (0.02-2.27)	0.25 (0.00-9.34)
Lambda	0.14 (0.01-1.30)	0.34 (0.06-0.97)	0.28 (0.00-4.36)
Kappa and Lambda sum	1.10 (0.08-8.72)	0.74 (0.08-2.85)	0.50 (0.01-10.60)
Kappa and Lambda difference	-0.67 (-6.12-0.12)	-0.07 (-1.70-0.44)	0.01 (-8.08-1.27)
Kappa: Lambda ratio	7.17 (0.44-21.84)	1.26 (0.23-6.76)	0.87 (0.25-37.56)
Saliva FLC secretion rates (mg/minute)			
Kappa	0.55 (0.03-5.56)	0.12 (0.01-0.57)	0.10 (0.00-2.20)
Lambda	0.09 (0.00-0.98)	0.12 (0.03-0.44)	0.11 (0.00-0.62)
Kappa and Lambda sum	0.64 (0.03-6.54)	0.25 (0.05-1.01)	0.26 (0.00-2.40)
Kappa and Lambda difference	-0.44 (-4.59-0.07)	-0.03 (-0.38-0.31)	0.01 (-2.00-0.46)
Kappa: Lambda ratio	7.17 (0.44-21.84)	1.26 (0.23-6.76)	0.85 (0.25-37.56)
Tear flow rate (mm/minute)	4.38 (1.20-35.85)	1.30 (0.40-9.13)	2.00 (0.00-20.83)
Tear FLC concentrations (mg/L)			
Kappa	0.43 (0.05-6.70)	2.96 (0.36-54.75)	1.60 (0.01-21.16)
Lambda	0.17 (0.02-1.46)	0.75 (0.05-8.00)	0.53 (0.00-9.16)
Kappa and Lambda sum	0.59 (0.06-7.96)	3.55 (0.41-57.68)	2.12 (0.02-28.36)
Kappa and Lambda difference	-0.24 (-5.44-0.37)	-2.36 (-51.81--0.23)	-0.89 (-16.94-0.08)
Kappa: Lambda ratio	2.86 (0.63-19.03)	3.24 (1.37-18.64)	2.88 (0.66-12.43)
Tear FLC secretion rates (mg/minute)			
Kappa	1.84 (0.18-17.11)	3.99 (1.06-42.87)	2.72 (0.03-67.08)
Lambda	0.65 (0.06-6.19)	1.02 (0.44-3.81)	0.94 (0.01-6.09)
Kappa and Lambda sum	2.52 (0.25-21.20)	6.51 (1.79-46.68)	3.66 (0.05-71.88)
Kappa and Lambda difference	-1.28 (-13.02-0.46)	-2.66 (-39.07--0.32)	-1.75 (-62.28-0.33)
Kappa: Lambda ratio	2.99 (0.66-18.31)	3.38 (1.37-17.91)	2.92 (0.65-13.98)

4.5.1 Serum

Serum parameters are displayed in Table 4.3 and Figure 4.4. There are no significant differences in serum IgG, IgA, or IgM concentrations between the cohorts.

An elevation of Kappa and Lambda FLC concentrations was noted in both MS and NCC groups compared to healthy donors, meaning the FLC sum was slightly higher and the FLC difference was slightly lower in these groups. However, these trends were only significant in the NCC group. Notably, 40% of MS patients 38% of the NCC cohort exceeded the healthy range of serum Kappa FLC concentration as defined by NHS serology parameters (see Appendix 4), whilst none of the healthy donors had out-of-range Kappa concentrations.

Additionally, Kappa: Lambda ratios are significantly higher in both MS patients and NCCs, although this difference is more profound in the MS group, the difference between MS patients and NCCs was not statistically significant.

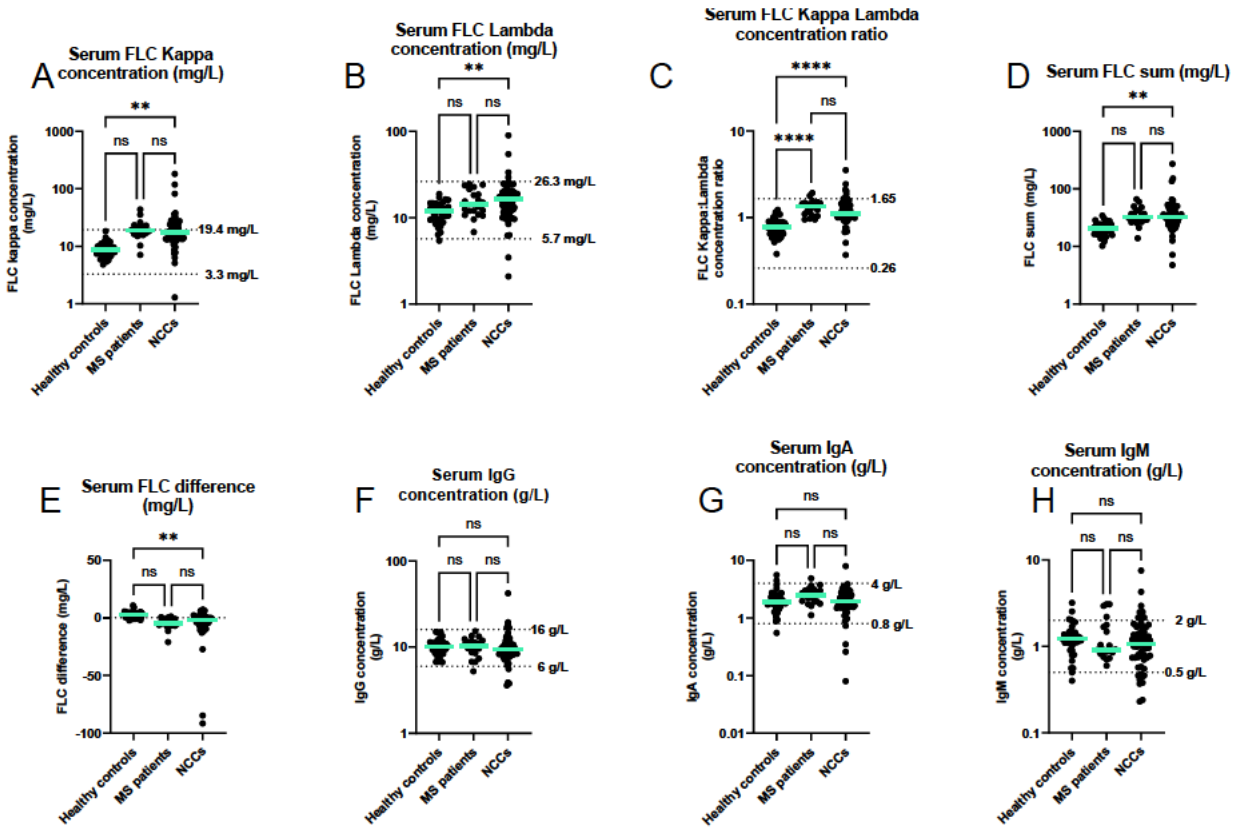


Figure 4.4 Serum IgGAM and FLC parameters.

A: FLC Kappa concentration. B: FLC Lambda concentration. C: FLC Kappa: Lambda ratio. D: FLC sum. E: FLC difference. F: IgG concentration. G: IgA concentration. H: IgM concentration. Each point represents an individual participant. Dotted lines indicate NHS adult reference ranges. Median line shown in blue. (ns= $p>0.05$, * = $p\leq 0.05$, ** = $p\leq 0.01$, *** = $p\leq 0.01$, **** = $p\leq 0.0001$).

4.5.2 Saliva

Saliva parameters are displayed in Table 4.3 and Figure 4.5. A non-significant trend decrease in Kappa FLC concentration was observed in both MS patient and NCC cohorts when compared to healthy donors. However, when normalised to secretion rate, this difference became significant. The difference between MS patients and NCCs was not statistically significant.

No significant differences were observed in Lambda FLC concentration or secretion rates between the cohorts. However, decreased sum secretion rates were observed in both MS and NCC groups and the difference between Kappa and Lambda secretion rates were increased when compared with healthy donors. Again, differences between MS and NCC were non-significant.

Furthermore, the Kappa: Lambda concentration and secretion ratios were found to be significantly lower in both MS patient and NCC groups compared with healthy donors. Once again, the difference between MS patients and the NCC cohort was non-significant.

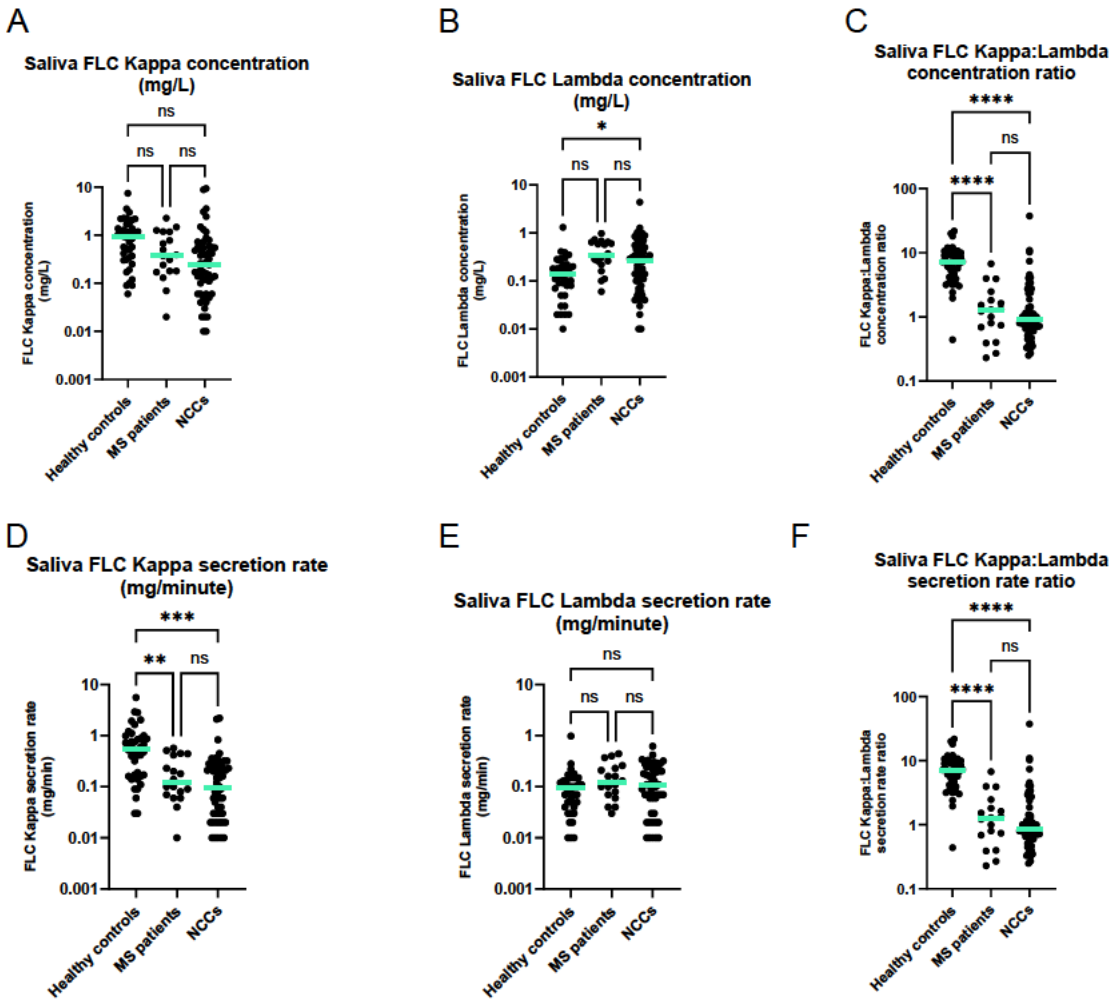
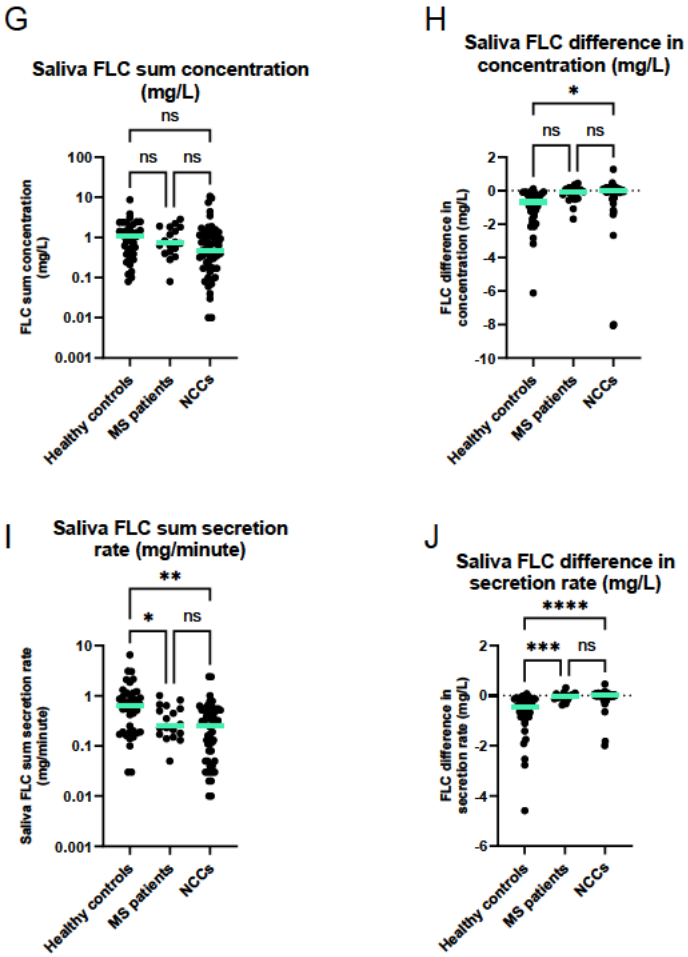


Figure 4.5 Salivary FLC parameters.
A: FLC Kappa concentration. B: FLC Lambda concentration. C: FLC Kappa: Lambda ratio of concentration. D: FLC Kappa secretion rate. E: FLC Lambda secretion rate F: FLC Kappa: Lambda ratio of secretion. Each point represents an individual participant. Median line shown in blue. (ns= $p>0.05$, * = $p\leq 0.05$, ** = $p\leq 0.01$, *** = $p\leq 0.01$, **** = $p\leq 0.0001$).

Figure 4.5 is continued on following page.



(Figure 4.5 continued) Salivary FLC parameters.

G: FLC sum of concentration. H: FLC difference in concentration. I: FLC sum of secretion rate. J: FLC difference in secretion rate. Each point represents an individual participant. Median line shown in blue. (ns= $p>0.05$, * = $p\leq 0.05$, ** = $p\leq 0.01$, *** = $p\leq 0.01$, **** = $p\leq 0.0001$).

4.5.3 Tears

Tear parameters are displayed in Table 4.3 and Figure 4.6. Kappa FLC concentration in MS patients, Lambda FLC concentrations in MS patients and NCCs and sum FLC concentrations in MS patients were significantly higher compared to healthy donor values.

Despite the differences in FLC concentrations, when normalised to secretion rates, levelled out and were not statistically significant.

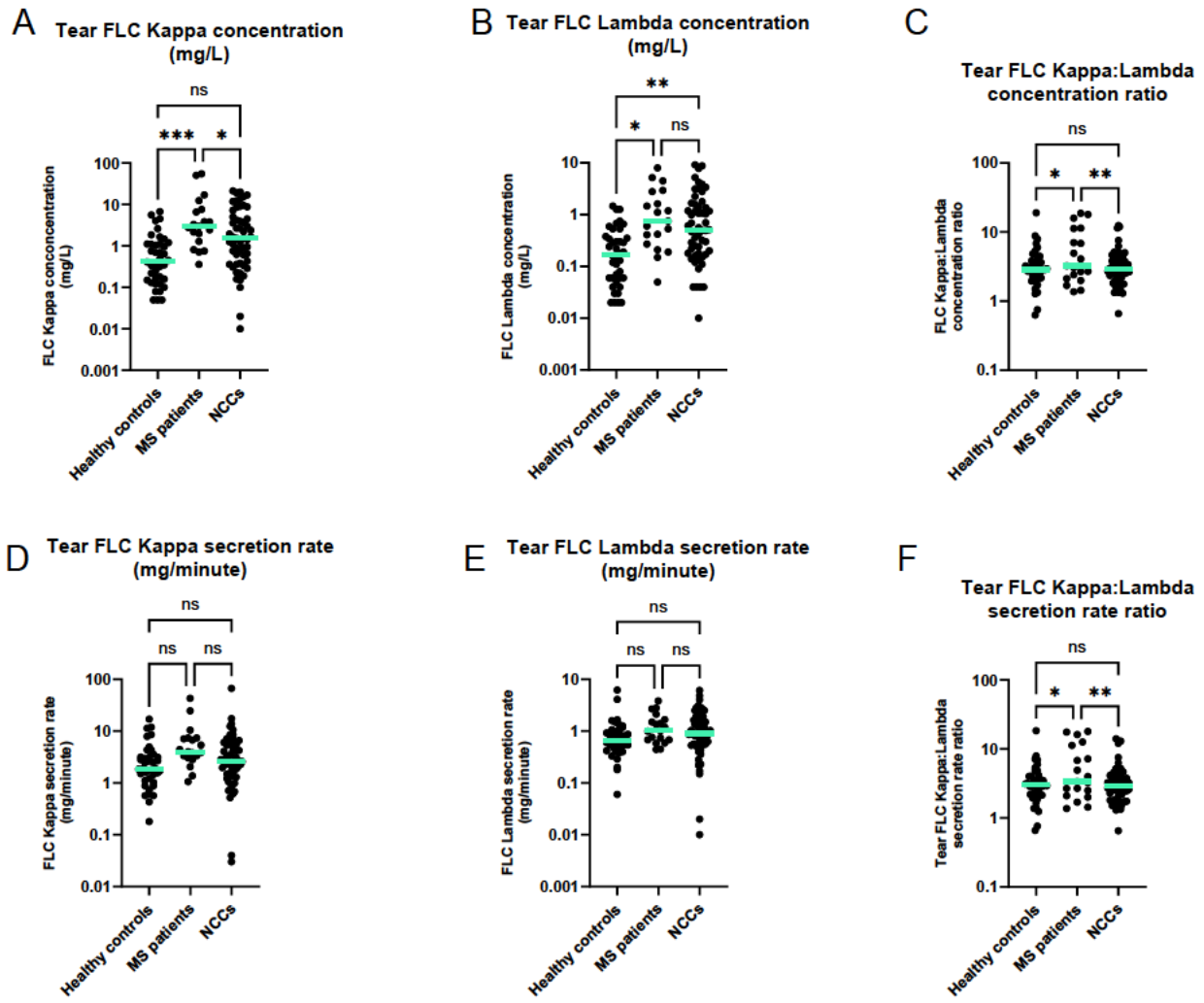
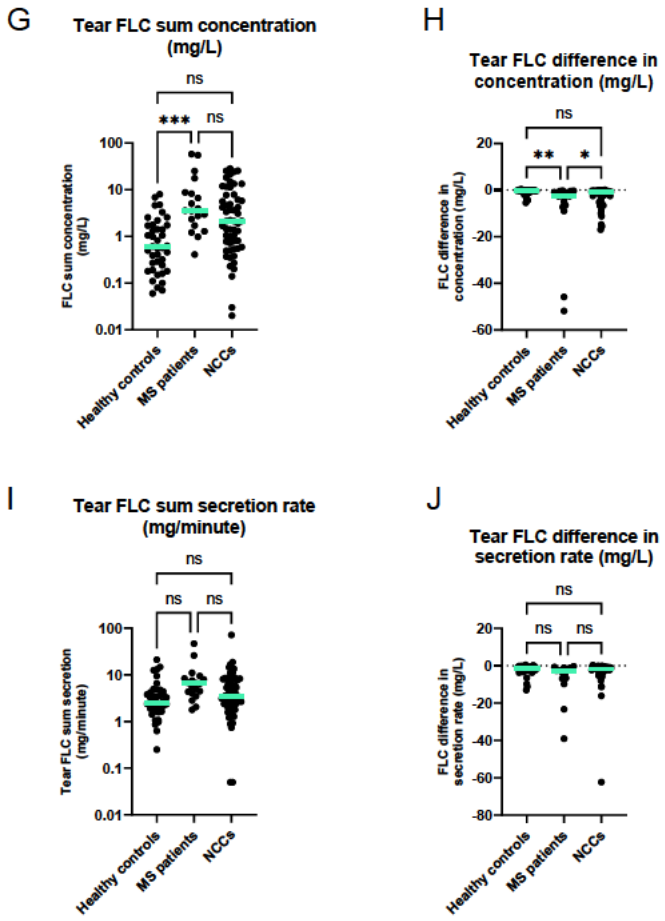


Figure 4.6 Tear fluid FLC parameters.

A: FLC Kappa concentration. B: FLC Lambda concentration. C: FLC Kappa: Lambda ratio of concentration. D: FLC Kappa secretion rate. E: FLC Lambda secretion rate F: FLC Kappa: Lambda ratio of secretion. Each point represents an individual participant, the value being an average between right and left eye. Median line shown in blue. (ns= $p>0.05$, * = $p\leq 0.05$, ** = $p\leq 0.01$, *** = $p\leq 0.01$, **** = $p\leq 0.0001$).

Figure 4.6 is continued on following page.



(Figure 4.6 continued) Tear fluid FLC parameters.

G: FLC sum of concentration. H: FLC difference in concentration. I: FLC sum of secretion rate. J: FLC difference in secretion rate. Each point represents an individual participant, the value being an average between right and left eye. Median line shown in blue.
(ns= $p>0.05$, * = $p\leq 0.05$, ** = $p\leq 0.01$, *** = $p\leq 0.01$, **** = $p\leq 0.0001$).

4.6 IEF results

4.6.1 IEF resolution optimisation

All samples were tested on IEF twice. Firstly, using IgG immunostaining (standard in clinical diagnostics) and secondly using total immunoglobulin staining. Contrast between OCB bands and background signal were sometimes slightly higher when staining for total immunoglobulin compared with IgG immunostaining (see Figure 4.7).

4.6.2 IEF results

A summary of IEF results is displayed in Table 4.4. Of the five healthy donors tested for OCBs via the IEF method, all five were OCB negative in the collected serum, saliva, and tear samples. CSF was not collected for this cohort. Of the 20 MS patients, 18 were OCB positive and two were OCB negative in CSF. All of the NCC patients were OCB negative.

Three participants, CIC 031 (MS patient), CIC 006 (NCC) and CIC 011 (NCC) displayed a paraproteinemia pattern of bands in all four sample types: CSF, serum, saliva, and tears Example immunoblots for these participants are shown in Figure 4.8.

As shown in Table 4.4, with examples of immunoblots shown in Figure 4.9, OCB presence in saliva and tears is faint and variable. 25% MS patients exhibited bands in saliva and 20% in tears. Additionally, results were not always consistent between using IgG and total immunoglobulin staining. For two participants (CIC 016 and CIC 022), OCBs could be visualised in tears when stained for IgG but not when stained for total immunoglobulin, (see Figure 4.9). Examples of a healthy donor (MSS 003) and NCC (CIC 044) and OCB negative MS patients (CIC 018 and CIC 035) are additionally displayed in Figure 4.9.

Table 4.4 Summary of IEF results

		IgG		Total immunoglobulin	
Sample ID	Cohort	OCB+ CSF	OCB+ saliva / tears	OCB+ CSF	OCB+ saliva / tears
HD 001	Healthy donor	-	Negative	-	Negative
HD 002	Healthy donor	-	Negative	-	Negative
HD 003	Healthy donor	-	Negative	-	Negative
HD 004	Healthy donor	-	Negative	-	Negative
HD 011	Healthy donor	-	Negative	-	Negative
CIC 001	MS	Positive	Negative	Positive	Negative
CIC 003	MS	Positive	Negative	Positive	Negative
CIC 012	MS	Positive	Negative	Positive	Negative
CIC 016	MS	Positive	Tear	Positive	Negative
CIC 022	MS	Positive	Tear	Positive	Negative
CIC 023	MS	Positive	Negative	Positive	Negative
CIC 030	MS	Positive	Saliva	Positive	Saliva
CIC 031	MS	Positive + paraprotein	Saliva, tears + paraprotein	Positive + paraprotein	Saliva, tears + paraprotein
CIC 047	MS	Positive	Negative	Positive	Negative
CIC 054	MS	Positive	Negative	Positive	Negative
CIC 057	MS	Positive	Negative	Positive	Negative
CIC 064	MS	Positive	Saliva	Positive	Saliva
CIC 067	MS	Positive	Saliva	Positive	Saliva
CIC 069	MS	Positive	Negative	Positive	Negative
CIC 072	MS	Positive	Saliva	Positive	Saliva
CIC 075	MS	Positive	Tears	Positive	Negative
CIC 077	MS	Positive	Negative	Positive	Negative
CIC 018	MS (OCB-)	Negative	Saliva	Positive	Saliva
CIC 035	MS (OCB-)	Negative	Negative	Negative	Negative
CIC 002	NCC	Negative	Negative	Negative	Negative
CIC 004	NCC	Negative	Negative	Negative	Negative
CIC 006	NCC	Paraprotein	Paraprotein	Paraprotein	Paraprotein
CIC 007	NCC	Negative	Negative	Negative	Negative
CIC 008	NCC	Negative	Negative	Negative	Negative
CIC 009	NCC	Negative	Negative	Negative	Negative
CIC 010	NCC	Negative	Negative	Negative	Negative
CIC 011	NCC	Paraprotein	Paraprotein	Paraprotein	Paraprotein
CIC 013	NCC	Negative	Negative	Negative	Negative
CIC 019	NCC	Negative	Negative	Negative	Negative
CIC 026	NCC	Negative	Negative	Negative	Negative
CIC 028	NCC	Negative	Negative	Negative	Negative
CIC 044	NCC	Negative	Negative	Negative	Negative

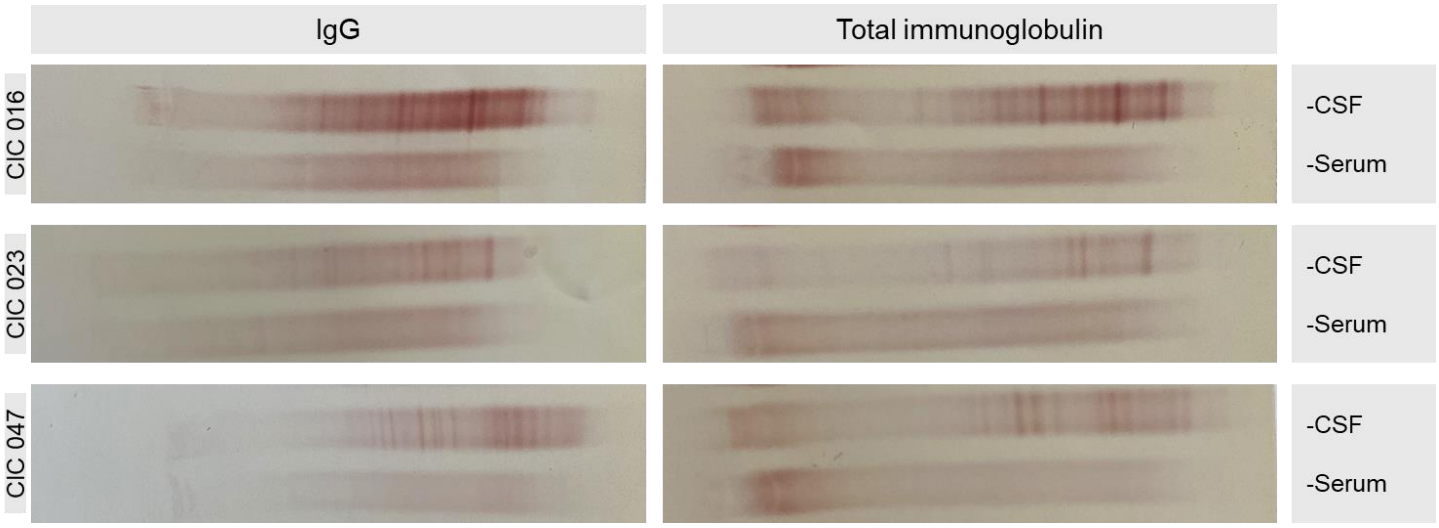


Figure 4.7 Example of OCB IgG versus OCB total free and bound immunoglobulin.
IEF immunoblots stained with IgG and total immunoglobulin. All three participants shown are MS patients. IEF immunoblots are interpreted by eye according to the SOPs. A slightly deeper contrast between oligoclonal banding and polyclonal banding can be observed when staining for total immunoglobulin instead of IgG (standard clinical method). The process of scanning reduces the resolution of the staining. Images were captured with a smartphone camera as this produced the highest quality images.

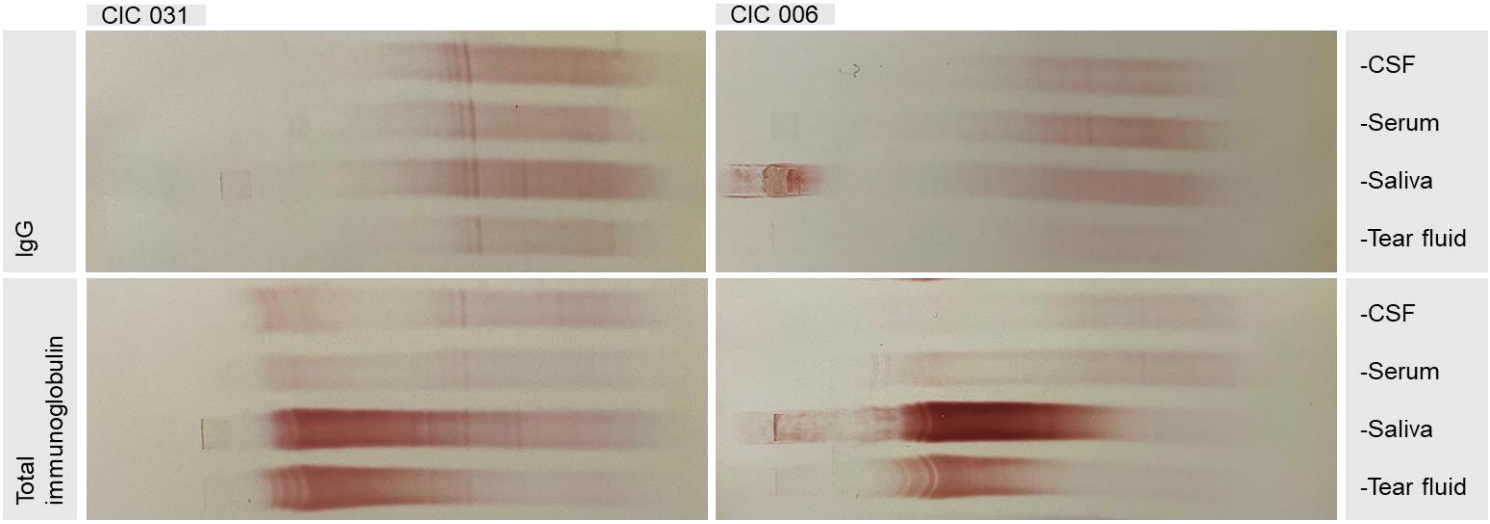


Figure 4.8 Example of paraproteinemia in CSF, serum, saliva, and tears.
IEF immunoblots stained with IgG (top) and total immunoglobulin (bottom). CIC 031(left) was an MS patient, CIC 006 (right) was an NCC but for both patients, paraproteinemia ladder pattern was observed. IEF immunoblots are interpreted by eye according to the SOPs. The process of scanning reduces the resolution of the staining. Images were captured with a smartphone camera as this produced the highest quality images.

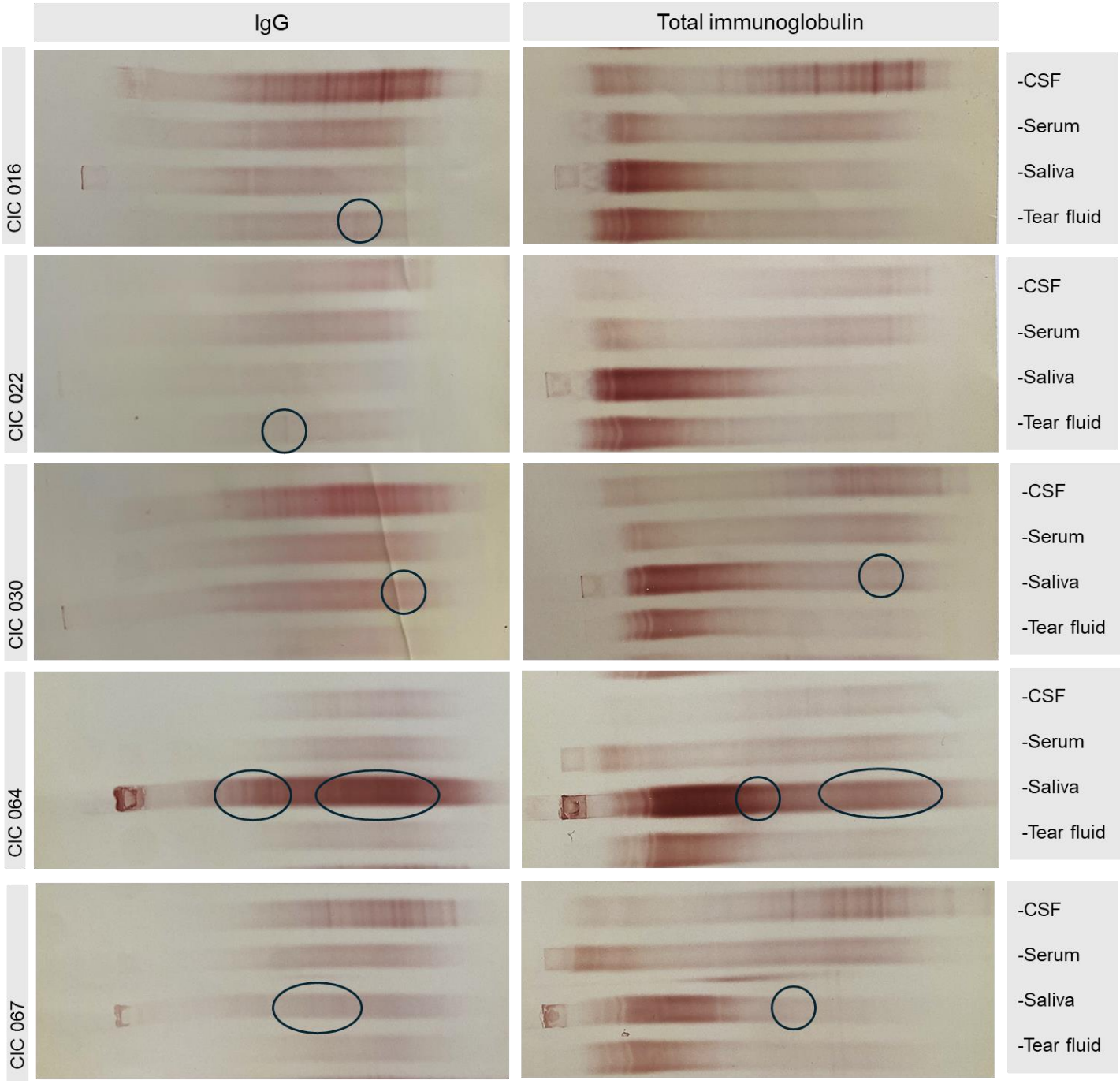


Figure 4.9 OCB in tears and saliva of MS patients
IEF immunoblots stained with IgG (left) and total immunoglobulin (right). OCB in saliva and tears encircled. IEF immunoblots are interpreted by eye according to the SOPs. The process of scanning reduces the resolution of the staining. Images were captured with a smartphone camera as this produced the highest quality images.

Figure 4.9 continues on the following page.

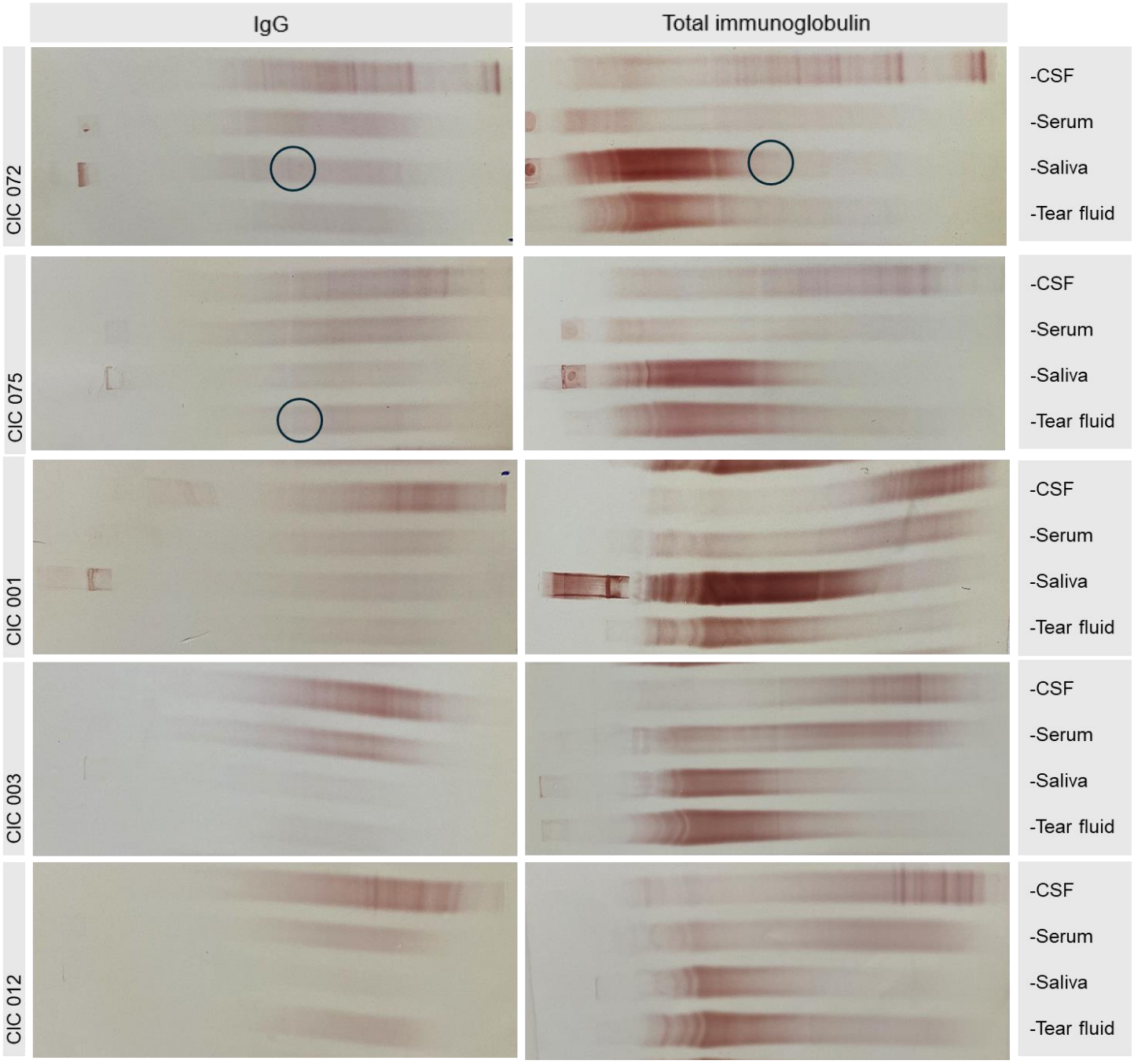


Figure 4.9 (continued) OCB in tears and saliva of MS patients
IEF immunoblots stained with IgG (left) and total immunoglobulin (right). OCB in saliva and tears encircled. IEF immunoblots are interpreted by eye according to the SOPs. The process of scanning reduces the resolution of the staining. Images were captured with a smartphone camera as this produced the highest quality images.

Figure 4.9 continues on the following page.

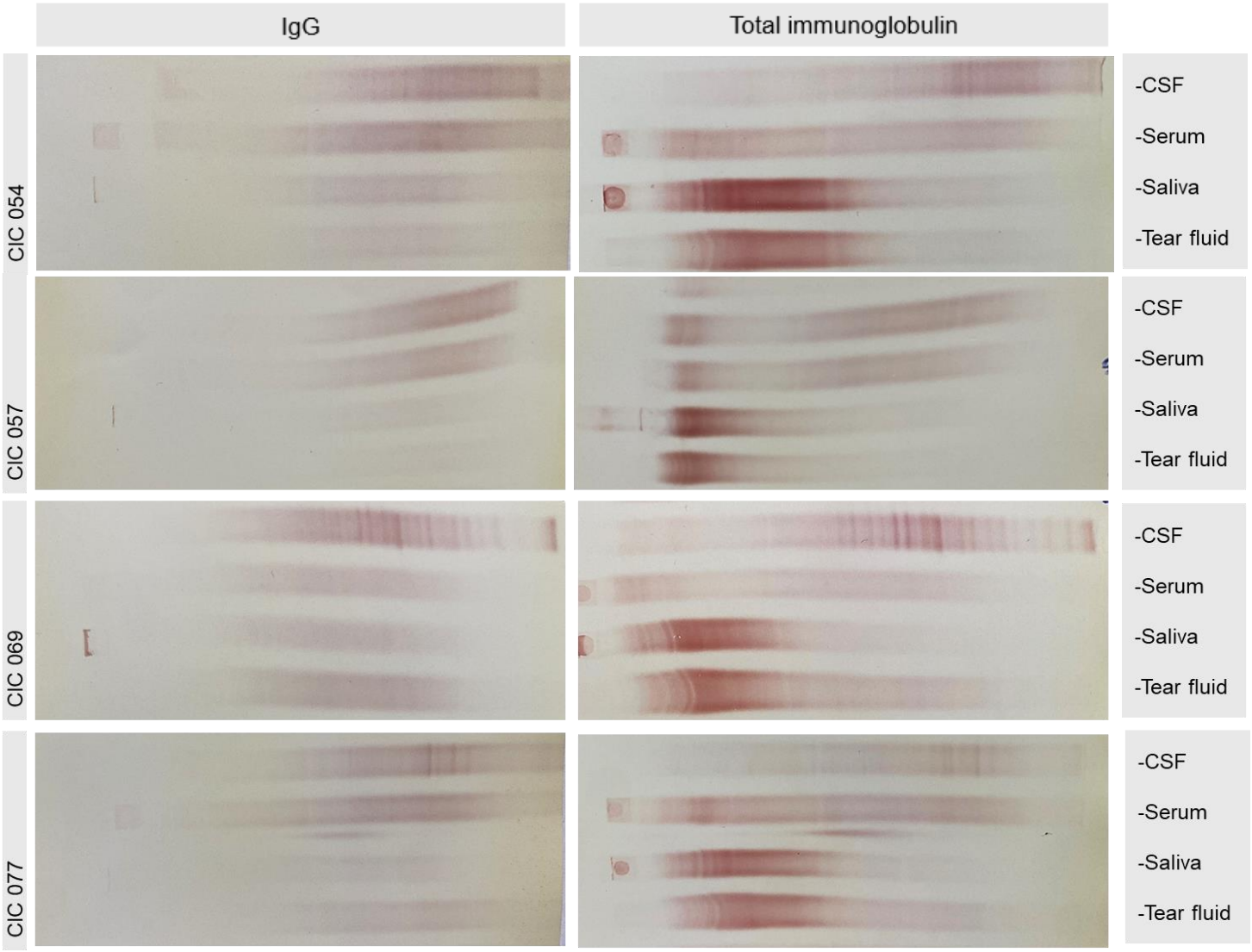


Figure 4.9 (continued) OCB in tears and saliva of MS patients
IEF immunoblots stained with IgG (left) and total immunoglobulin (right). OCB in saliva and tears encircled. IEF immunoblots are interpreted by eye according to the SOPs. The process of scanning reduces the resolution of the staining. Images were captured with a smartphone camera as this produced the highest quality images.

Figure 4.9 continues on the following page.

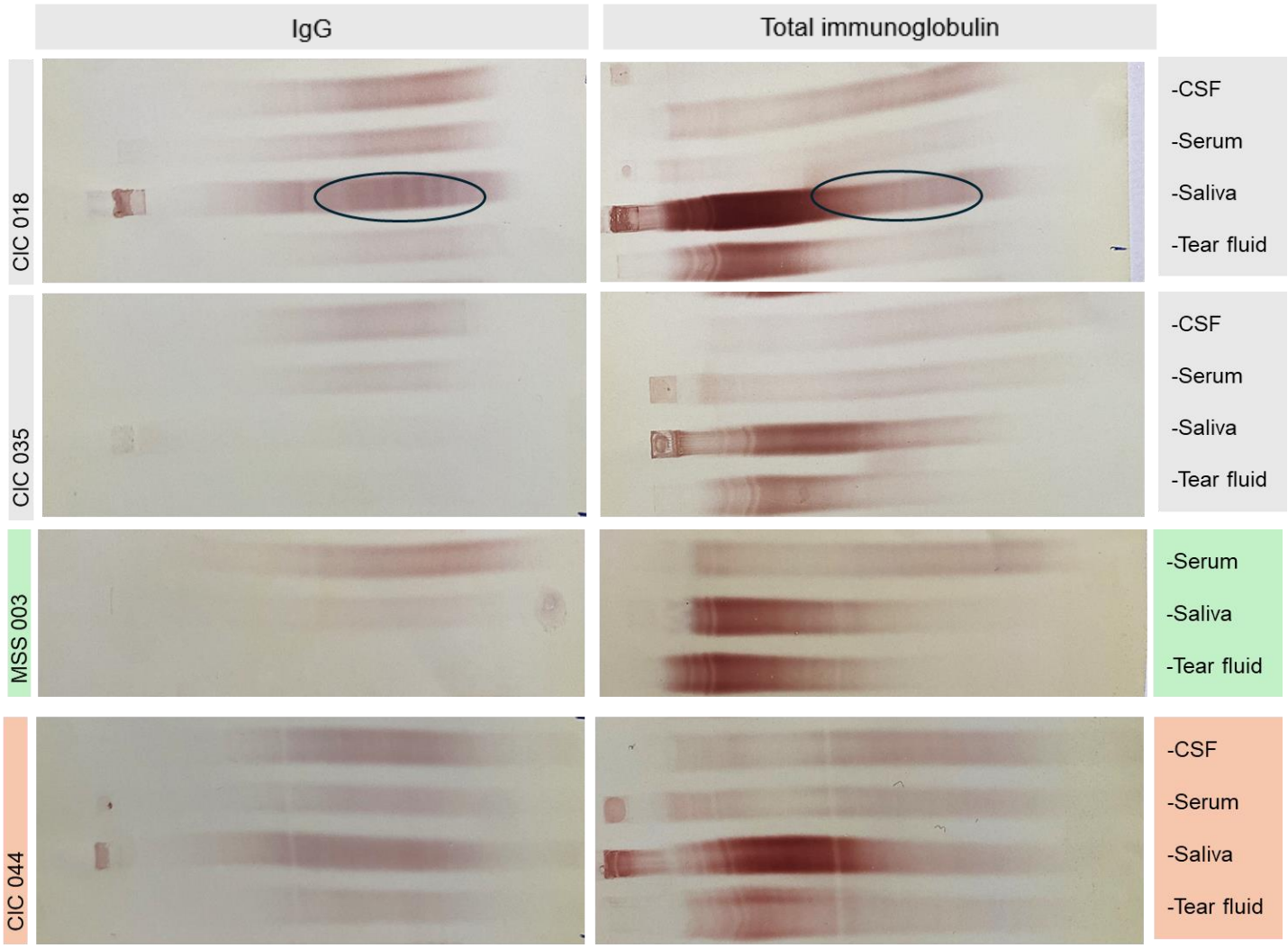


Figure 4.9 (continued) OCB in tears and saliva of MS patients
IEF immunoblots stained with IgG (left) and total immunoglobulin (right). OCB in saliva and tears encircled. IEF immunoblots are interpreted by eye according to the SOPs. The process of scanning reduces the resolution of the staining. Images were captured with a smartphone camera as this produced the highest quality images.

5. DISCUSSION

5.1 Study outcomes

This study investigated the detection of OCBs and elevated FLCs in saliva and tear fluid to explore potential implementation in MS diagnosis, monitoring, and prognosis. Evidence in the literature regarding the utility of saliva and tear biomarkers was variable. However, the minimally invasive, simple, and cost-effective methods of collection highlighted them as attractive candidates for applications in MS. Additionally IEF and FLC ELISAs have undergone thorough optimisation by the Clinical Immunology Service, ensuring high levels of sensitivity to enable the accurate analysis of these specimens.

5.1.1 IEF findings

IEF analysis was conducted on all 20 MS patients, along with a matched number of non-MS NCCs and healthy donors. OCB presence in the tears and saliva was rare and inconsistent in the MS group and absent in the NCC and healthy donor cohorts. Furthermore, OCB presence in immunoblots applying IgG or total free and bound immunoglobulin staining could be inconsistent for the same sample. Inconsistencies within the MS cohort may reflect the characteristic heterogeneity of the condition, making the presence of OCB in tears and saliva an unsuitable replacement for CSF. Additionally for the few OCBs observed in saliva and tears, the banding pattern was distinct to that in the CSF, suggesting there is no crossover of OCBs into the saliva and tear compartments from the CSF and any banding is a result of local inflammation.

The earliest investigations into OCB in tears using IEF was conducted by Coyle who reported their presence in 67% MS patients who were tested (86). Subsequent works by

Forzy (96), Devos (97), Calais (98) and Lebrun (99) agreed concordance between MS diagnosis and the presence of OCB in tears. However, studies carried out by Mavra (100), Liedke (101), Martino (102), and Hummet (87) were unable to replicate comparable results thus could not recommend it as a suitable biomarker. Variations between the studies in cohorts, sample collection methods and analysis made direct comparisons challenging. Notably, the publications arguing for the presence of tear OCBs were produced by the same lab group (96–99) and have already been criticised for lacking clear evidence of banding, often providing only one example per report (103). Furthermore, these papers did not publish examples traces of healthy donors. Although IEF immunoblots can be difficult to capture clearly, completely omitting qualitative data from a paper employing qualitative techniques made it difficult to examine the literature. Understanding the authors definition of what constitutes a band would help decipher the differences in OCB detection between studies, whether due to methodological or interpretive techniques. The fact that four further studies, carried out by separate lab groups, could not advocate for OCB detection in tears adds to scepticism regarding the findings.

An interesting find was the presence of a monoclonal gammopathy of undetermined significance (MGUS) banding pattern in CSF, serum, saliva, and tears. MGUS, a precursor condition to multiple myeloma (104), is characterised by the presence of a monoclonal “ladder” banding pattern and is found in 3% of the over-50 population in the UK (105). Internal investigations within the Clinical Immunology Service have detected MGUS to a very high sensitivity. Consequently, this incidental finding may demonstrate that the absence of OCBs found in the tears and saliva is not due to method sensitivity, but pure absence of the markers in the sample types.

Improved resolution could be observed in some of the immunoblots when employing total immunoglobulin staining instead of IgG (diagnostic standard). In some cases, immunoblots may appear unclear, particularly in specimens with high background polyclonal banding. This can lead to discrepancies among users and prolonged result turnaround times, as interpretation of isoelectric focusing (IEF) is subject to individual perception. Consequently, any enhancements in immunoblot resolution could alleviate this problem. Exact protein concentrations of samples applied to IEF were not standardised, resulting in cautious interpretation. However, employing this in a future study would aid a more comprehensive investigation into the immunoblotting method optimisation.

5.1.2 FLC findings

Serum IgGAM and FLC parameters were measured using the Optilite analyser, the Kappa and Lambda FLC ELISA was optimized for detection in saliva and tear fluid. Reference ranges were generated in serum saliva and tears in all three cohorts.

In agreement with the hypothesis, MS patients and NCCs demonstrated elevated Kappa FLCs in serum compared to healthy donors. Although this difference was only statistically significant in the NCC group, substantial proportions of both cohorts demonstrated Kappa values above the NHS-defined healthy threshold which would warrant further clinical investigations. As a result, Kappa: Lambda FLC ratios were significantly higher in MS patients and NCCs, although there is no significant difference between MS patients and NCCs. These trends in serum were expected, as Kappa FLCs are synthesised by B-cells during an immune response, thus may reflect the heightened B-cell activity in MS and any other auto-immune or inflammatory conditions observed in the NCC group (106).

Surprisingly, normalising saliva FLC concentrations to secretory rates revealed the opposite trend to serum. Saliva secretion rates of Kappa and Lambda FLCs were significantly lower in MS patients than in healthy donors. As a result, sum secretory rates and Kappa: Lambda ratios were also lower. Again, these differences were not statistically significant between MS patients and NCCs. This could be explained by the dysregulated nature of the immune system observed both in MS patients as well as the varied neurological, immune, or other conditions exhibited by the NCC group. Although MS patients had not yet begun targeted interventions, both the MS group and NCC group may be prescribed symptom-specific medications, such as anti-inflammatories, painkillers, muscle relaxants, anti-depressants, and digestion-related medication as well as non-specific immunosuppressants and steroids. Medication is just one factor that may explain a decrease in mucosal inflammation. However, data on individual medication use was not collected during this study and therefore cannot be used to investigate the trend further.

In tear fluid, Kappa FLC concentration in MS patients, Lambda FLC concentrations in MS patients and NCCs and sum FLC concentrations in MS patients were significantly higher compared to healthy donor values. Despite this, the differences in FLC concentrations, when normalised to secretion rates, levelled-out and were not statistically significant. Further investigations could be designed to understand the underlying mechanisms behind this trend. As tear flow rates were typically lower in MS patients and NCC cohorts, perhaps a protective compensatory mechanism exists to maintain stable secretion of inflammatory FLCs, particularly as the eye exists as an immune privileged site (107).

Notably, many of the trends seen in FLC parameters are significant compared to healthy donors, but non-significant between MS and NCC groups. This prompts the question of

whether the patterns observed in FLC parameters are indicative of general auto-immune, inflammatory, or neurological conditions, rather than distinct markers of MS.

The utility of serum and CSF FLC quantification in MS has been well documented and displays a similar diagnostic sensitivity to OCBs (55,108). The kappa FLC index is a measure of intrathecal synthesis of kappa FLCs by taking albumin levels (a measure of BBB function) and serum FLCs into account (109). The Kappa FLC index can be quantified using Optilite or similar serology analysers as potential replacements for the more complex IEF technique. Future studies could apply the FLC ELISA to CSF and investigate concordance with the Optilite analyser. It would additionally be helpful to measure FLC parameters in CSF, to quantify the concordance of FLC between saliva and tear compartments.

5.1.3 Saliva and tear flow rates

Both saliva and tear flow rates were substantially lower in the MS patient and NCC groups when compared with the healthy donors, and participants with severely dry eyes were only observed in the MS and NCC groups. It must be noted that dry eye disease is a condition of its own but given its complete lack of incidence in the healthy donor group, this could be a significant “red flag” symptom when coupled with other signs of neurodegeneration. This may indicate a decline in glandular function associated with the neurodegeneration seen in MS patients. Again, additional factors such as age (significantly higher in MS and NCC groups) and medication use in the MS and NCC cohorts may be contributing to this trend and should be investigated further (110).

5.1.4 Saliva and tear fluid as accessible sample types

The Clinical Immunology Service Kappa and Lambda FLC ELISAs and IEF method to detect OCBs are capable of high sensitivity detection required for non-invasive tear and saliva specimens. Therefore, the combined utility of these methods offered a promising approach in capturing any differences in saliva and tear OCB and FLC profiles in MS patients, provided they exist. While the present study was unable to prove their clinical utility for applications in MS, both saliva and tear fluid proved to be accessible sample types, exhibiting low rates of sample collection abnormalities or withdrawals.

In the case of tear collection, two participants withdrew from donating tears, with one requesting for tears to be extracted from one eye only. Three participants did not secrete enough tear fluid to be analysed. Collection time was not extended further to prioritise patient comfort, however the protocol for tear collection could be revisited, given the low tear secretion in the patient cohorts.

In terms of saliva collection, one patient withdrew from donation and two participants experienced significant discomfort following the lumbar puncture, preventing their ability to sit upright for sample collection according to the protocol. This highlighted the discomfort associated with lumbar punctures and the subsequent demand for less invasive sample collection.

Typically, the entire appointment to collect serum, saliva, and tears ranged from 20 to 30 minutes. In contrast, lumbar punctures typically require 30 minutes to an hour alone, excluding extra appointments and occasional arrangements for x-ray guided procedures. Additionally, the process of collecting the samples proved to be straightforward, and any

clinical staff member could be trained. This demonstrated the efficiency and simplicity of saliva and tear collection in comparison to CSF.

Although qualitative data regarding patient opinions and comfort ratings were beyond the terms of ethical approval, there was an extremely high overall willingness to participate in the study and low discomfort levels during the saliva and tear collection were observed. Outside the context of MS, measuring alternative biomarkers in saliva and tears could be valuable for other clinical applications, to simplify testing and improve patient experience.

5.2 Study limitations

In terms of meeting the aims of the study, the project was successful in recruiting the target of 40 healthy donors, and 80 patients. Serum, saliva, tears, and CSF (in the MS patients and NCC group only) were collected and analysed by ELISA and IEF methods. Whilst this study has provided additional insights into saliva and tear analysis, the following limitations and improvements should be acknowledged for future investigations.

The proportion of MS patients was lower than the anticipated 40, lowering the statistical significance and reproducibility of the results. An extended recruitment period could not be granted within the terms of the ethical approval for this study but could be expanded in subsequent studies.

The healthy donor cohort was recruited from a narrow demographic, who were primarily students and staff at the Medical School, Institute of Immunology and Immunotherapy and Clinical Immunology Service. The likelihood of similar lifestyles, schedules and occupations between healthy donors may have introduced a bias. This was due to existing

consent and ethical approval for University of Birmingham staff. To avoid this in future studies, a more diverse recruitment plan should be implemented.

Additionally, the NCC cohort was a highly heterogenous population of participants with a range of different health conditions, which calls for cautious interpretation of results. Future studies should aim for a more homogenous cohort to strengthen reliability of the results or a far more meticulous stratification of the cohorts, which would require a much larger sample size.

Furthermore, age, gender, time of day and medication may have an impact on FLC parameters (111) but was not controlled for in this study. Future studies could investigate these factors in more detail to enhance the precision of FLC measurements and may help to explain the variety of results between participants within cohorts.

To enhance the reliability of tear collection and analysis for other studies, a number of validation studies on tear fluid could be performed. For example, the exact volume of tear fluid eluted from Schirmer strips was not measured in this study, therefore reported Kappa and Lambda FLC values were not absolute. Further protein quantification assays could be performed, or the collection of neat tear fluid by capillary tube collection could be trialled. Stability assays in the eluted tear samples could also be carried out to assess the shelf life of samples once collected and the impact of freeze-thaw cycles on the reproducibility of results.

5.3 Conclusions

In conclusion, though the findings of this study may not justify immediate expansion into broader clinical investigations, they provide valuable insights into the collection and analysis of saliva and tear samples in MS patients.

The observed high variability in results amongst MS patients, particularly concerning OCB presence in tears and saliva, highlight the vast heterogeneity of the disease. Future studies could investigate the underlying reasons for these differences.

Given the complex nature of MS diagnosis, prognosis, and monitoring, caused by diverse clinical presentations and variable disease courses, there is an evident need for the identification of new biomarkers. Biomarker research has the potential to aid accurate stratification of patients, paving the way for more personalised approaches to treatment and management of the disease as well as enhancing our understanding of MS pathophysiology for the benefit of patients worldwide.

6. LIST OF REFERENCES

1. Dobson R, Giovannoni G. Multiple sclerosis – a review. Eur J Neurol. 2019 Jan 18;26(1):27–40.
2. The Multiple Sclerosis International Federation Atlas of MS, 3rd ed [Internet]. 2020 Sep. Available from: https://www.atlasofms.org
3. Mackenzie IS, Morant S V., Bloomfield GA, MacDonald TM, O'Riordan J. Incidence and prevalence of multiple sclerosis in the UK 1990-2010: a descriptive study in the General Practice Research Database. J Neurol Neurosurg Psychiatry. 2014 Jan 1;85(1):76–84.
4. Kobelt G, Thompson A, Berg J, Gannedahl M, Eriksson J. New insights into the burden and costs of multiple sclerosis in Europe. Multiple Sclerosis Journal. 2017 Jul 1;23(8):1123–36.
5. Cerqueira JJ, Compston DAS, Geraldes R, Rosa MM, Schmierer K, Thompson A, et al. Time matters in multiple sclerosis: can early treatment and long-term follow-up ensure everyone benefits from the latest advances in multiple sclerosis? J Neurol Neurosurg Psychiatry. 2018 Aug;89(8):844–50.
6. Brownlee WJ, Hardy TA, Fazekas F, Miller DH. Diagnosis of multiple sclerosis: progress and challenges. The Lancet. 2017 Apr;389(10076):1336–46.
7. Koch-Henriksen N, Sørensen PS. The changing demographic pattern of multiple sclerosis epidemiology. Lancet Neurol. 2010 May;9(5):520–32.
8. Alfredsson L, Olsson T. Lifestyle and Environmental Factors in Multiple Sclerosis. Cold Spring Harb Perspect Med. 2019 Apr;9(4):a028944.
9. Ramien C, Taenzer A, Lupu A, Heckmann N, Engler JB, Patas K, et al. Sex effects on inflammatory and neurodegenerative processes in multiple sclerosis. Neurosci Biobehav Rev. 2016 Aug;67:137–46.
10. Willer CJ, Dyment DA, Risch NJ, Sadovnick AD, Ebers GC. Twin concordance and sibling recurrence rates in multiple sclerosis. Proceedings of the National Academy of Sciences. 2003 Oct 28;100(22):12877–82.
11. Hollenbach JA, Oksenberg JR. The immunogenetics of multiple sclerosis: A comprehensive review. J Autoimmun. 2015 Nov;64:13–25.
12. Betrams J, Kuwert E, Liedtke U. HL-A Antigens and Multiple Sclerosis. Tissue Antigens. 1972 Nov 9;2(6):405–8.
13. Simmonds M, Gough S. The HLA Region and Autoimmune Disease: Associations and Mechanisms of Action. Curr Genomics. 2007 Nov 1;8(7):453–65.
14. Muñiz-Castrillo S, Vogrig A, Honnorat J. Associations between HLA and autoimmune neurological diseases with autoantibodies. Autoimmunity Highlights. 2020 Dec 22;11(1):2.
15. International Multiple Sclerosis Genetics Consortium, Hafler D, Compston A. Risk Alleles for Multiple Sclerosis Identified by a Genomewide Study. New England Journal of Medicine. 2007 Aug 30;357(9):851–62.
16. Peerlings D, Mimpen M, Damoiseaux J. The IL-2 – IL-2 receptor pathway: Key to understanding multiple sclerosis. J Transl Autoimmun. 2021;4:100123.

17. Ysrraelit MC, Correale J. Impact of sex hormones on immune function and multiple sclerosis development. Immunology. 2019 Jan 11;156(1):9–22.

18. Bjornevik K, Cortese M, Healy BC, Kuhle J, Mina MJ, Leng Y, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. Science (1979). 2022 Jan 21;375(6578):296–301.

19. Lanz T V., Brewer RC, Ho PP, Moon JS, Jude KM, Fernandez D, et al. Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. Nature. 2022 Mar 10;603(7900):321–7.

20. Sintzel MB, Rametta M, Reder AT. Vitamin D and Multiple Sclerosis: A Comprehensive Review. Neurol Ther. 2018 Jun 14;7(1):59–85.

21. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol. 2008 Sep;8(9):685–98.

22. Bosch-Queralt M, Cantuti-Castelvetri L, Damkou A, Schifferer M, Schlepckow K, Alexopoulos I, et al. Diet-dependent regulation of TGFβ impairs reparative innate immune responses after demyelination. Nat Metab. 2021 Feb 18;3(2):211–27.

23. Hedström A, Olsson T, Alfredsson L. Smoking is a major preventable risk factor for multiple sclerosis. Multiple Sclerosis Journal. 2016 Jul 12;22(8):1021–6.

24. Hedström AK, Olsson T, Alfredsson L. High body mass index before age 20 is associated with increased risk for multiple sclerosis in both men and women. Multiple Sclerosis Journal. 2012 Sep 10;18(9):1334–6.

25. McLaughlin L, Clarke L, Khalilidehkordi E, Butzkueven H, Taylor B, Broadley SA. Vitamin D for the treatment of multiple sclerosis: a meta-analysis. J Neurol. 2018 Dec 3;265(12):2893–905.

26. Cassard SD, Fitzgerald KC, Qian P, Emrich SA, Azevedo CJ, Goodman AD, et al. High-dose vitamin D3 supplementation in relapsing-remitting multiple sclerosis: a randomised clinical trial. EClinicalMedicine. 2023 May;59:101957.

27. Maple PA, Ascherio A, Cohen JI, Cutter G, Giovannoni G, Shannon-Lowe C, et al. The Potential for EBV Vaccines to Prevent Multiple Sclerosis. Front Neurol. 2022 Jun 24;13.

28. National Statistics. National Child Measurement Programme, England 2020/21 School Year [Internet]. 2021 Nov. Available from: https://digital.nhs.uk/data-and-information/publications/statistical/national-child-measurement-programme/2020-21-school-year

29. National statistics. National Child Measurement Programme, England 2018/19 School Year [NS] [Internet]. 2019 Oct. Available from: https://digital.nhs.uk/data-and-information/publications/statistical/national-child-measurement-programme/2018-19-school-year

30. Office for National Statistics. Adult smoking habits in the UK: 2022 [Internet]. 2023 Sep. Available from: https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/drugusealcoholandsmoking/datasets/adultsmokinghabitsingreatbritain

31. Carson MJ, Doose JM, Melchior B, Schmid CD, Ploix CC. CNS immune privilege: hiding in plain sight. Immunol Rev. 2006 Oct;213(1):48–65.

32. Ransohoff RM, Engelhardt B. The anatomical and cellular basis of immune surveillance in the central nervous system. Nat Rev Immunol. 2012 Sep 20;12(9):623–35.

33. Stadelmann C, Timmler S, Barrantes-Freer A, Simons M. Myelin in the Central Nervous System: Structure, Function, and Pathology. Physiol Rev. 2019 Jul 1;99(3):1381–431.

34. Lubetzki C, Stankoff B. Demyelination in multiple sclerosis. In 2014. p. 89–99.

35. Attfield KE, Jensen LT, Kaufmann M, Friese MA, Fugger L. The immunology of multiple sclerosis. Nat Rev Immunol. 2022 Dec 4;22(12):734–50.

36. Stys PK, Zamponi GW, van Minnen J, Geurts JJG. Will the real multiple sclerosis please stand up? Nat Rev Neurosci. 2012 Jul 20;13(7):507–14.

37. Libbey JE, McCoy LL, Fujinami RS. Molecular Mimicry in Multiple Sclerosis. In 2007. p. 127–47.

38. Lubetzki C, Zalc B, Williams A, Stadelmann C, Stankoff B. Remyelination in multiple sclerosis: from basic science to clinical translation. Lancet Neurol. 2020 Aug;19(8):678–88.

39. Lassmann H. Pathogenic Mechanisms Associated With Different Clinical Courses of Multiple Sclerosis. Front Immunol. 2019 Jan 10;9.

40. Cree BAC, Arnold DL, Chataway J, Chitnis T, Fox RJ, Pozo Ramajo A, et al. Secondary Progressive Multiple Sclerosis. Neurology. 2021 Aug 24;97(8):378–88.

41. Ontaneda D, Fox RJ. Progressive multiple sclerosis. Curr Opin Neurol. 2015 Jun;28(3):237–43.

42. NICE (National Institute for Health and Care Excellence) guidline. Multiple sclerosis in adults: Management [Internet]. 2022 Jun. Available from: www.nice.org.uk/guidance/ng220

43. Klineova S, Lublin FD. Clinical Course of Multiple Sclerosis. Cold Spring Harb Perspect Med. 2018 Sep;8(9):a028928.

44. Briggs FBS, Trapl ES, Mateen FJ, Nadai A De, Conway DS, Gunzler DD. Common Social and Health Disparities Contribute to Racial Differences in Ambulatory Impairment in Multiple Sclerosis. Int J MS Care. 2024 Jan 1;26(1):36–40.

45. Dobson R, Rice DR, D'hooghe M, Horne R, Learmonth Y, Mateen FJ, et al. Social determinants of health in multiple sclerosis. Nat Rev Neurol. 2022 Dec 11;18(12):723–34.

46. McDonald WI, Compston A, Edan G, Goodkin D, Hartung H, Lublin FD, et al. Recommended diagnostic criteria for multiple sclerosis: Guidelines from the international panel on the diagnosis of multiple sclerosis. Ann Neurol. 2001 Jul 26;50(1):121–7.

47. Polman CH, Reingold SC, Edan G, Filippi M, Hartung H, Kappos L, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Ann Neurol. 2005 Dec 10;58(6):840–6.

48. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. Ann Neurol. 2011 Feb 8;69(2):292–302.

49. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol. 2018 Feb;17(2):162–73.

50. Filippi M, Rocca MA, Ciccarelli O, De Stefano N, Evangelou N, Kappos L, et al. MRI criteria for the diagnosis of multiple sclerosis: MAGNIMS consensus guidelines. Lancet Neurol. 2016 Mar;15(3):292–303.

51. Zalc B. One hundred and fifty years ago Charcot reported multiple sclerosis as a new neurological disease. Brain. 2018 Dec 1;141(12):3482–8.

52. Tintore M, Rovira À, Río J, Otero-Romero S, Arrambide G, Tur C, et al. Defining high, medium and low impact prognostic factors for developing multiple sclerosis. Brain. 2015 Jul;138(7):1863–74.

53. Wichmann TO, Damkier HH, Pedersen M. A Brief Overview of the Cerebrospinal Fluid System and Its Implications for Brain and Spinal Cord Diseases. Front Hum Neurosci. 2022 Jan 21;15.

54. Arrambide G, Tintore M, Espejo C, Auger C, Castillo M, Río J, et al. The value of oligoclonal bands in the multiple sclerosis diagnostic criteria. Brain. 2018 Apr 1;141(4):1075–84.

55. Hegen H, Berek K, Deisenhammer F. Cerebrospinal fluid kappa free light chains as biomarker in multiple sclerosis—from diagnosis to prediction of disease activity. Wiener Medizinische Wochenschrift. 2022 Nov 8;172(15–16):337–45.

56. Arneth B, Kraus J. The Use of Kappa Free Light Chains to Diagnose Multiple Sclerosis. Medicina (B Aires). 2022 Oct 24;58(11):1512.

57. Doherty CM, Forbes RB. Diagnostic Lumbar Puncture. Ulster Med J. 2014 May;83(2):93–102.

58. Caillon H, Avet-Loiseau H, Attal M, Moreau P, Decaux O, Dejoie T. Comparison of Sebia Free Light Chain Assay With Freelite Assay for the Clinical Management of Diagnosis, Response, and Relapse Assessment in Multiple Myeloma. Clin Lymphoma Myeloma Leuk. 2019 May;19(5):e228–37.

59. Link H, Huang YM. Oligoclonal bands in multiple sclerosis cerebrospinal fluid: An update on methodology and clinical usefulness. J Neuroimmunol. 2006 Nov;180(1–2):17–28.

60. Kuhle J, Disanto G, Dobson R, Adiutori R, Bianchi L, Topping J, et al. Conversion from clinically isolated syndrome to multiple sclerosis: A large multicentre study. Multiple Sclerosis Journal. 2015 Jul 13;21(8):1013–24.

61. Myhr KM, Mellgren SI. Corticosteroids in the treatment of multiple sclerosis. Acta Neurol Scand. 2009 Aug;120:73–80.

62. Wingerchuk DM, Weinshenker BG. Disease modifying therapies for relapsing multiple sclerosis. BMJ. 2016 Aug 22;i3518.

63. NHS England. Treatment Algorithm for Multiple Sclerosis Disease-Modifying Therapies. NHS England Reference: 170079ALG [Internet]. 2023 [cited 2024 Feb 10]. Available from: https://www.england.nhs.uk/wp-content/uploads/2024/03/treatment-algorithm-for-multiple-sclerosis-disease-modifying-therapies-july-23.pdf

64. MS Society. Disease modifying therapies [Internet]. 2022 [cited 2024 Feb 9]. Available from: https://www.mssociety.org.uk/about-ms/treatments-and-therapies/disease-modifying-therapies

65. Havrdova E, Horakova D, Kovarova I. Alemtuzumab in the treatment of multiple sclerosis: key clinical trial results and considerations for use. Ther Adv Neurol Disord. 2015 Jan 6;8(1):31–45.

66. Filipi M, Jack S. Interferons in the Treatment of Multiple Sclerosis. Int J MS Care. 2020 Jul 1;22(4):165–72.

67. Giovannoni G, Mathews J. Cladribine Tablets for Relapsing–Remitting Multiple Sclerosis: A Clinician's Review. Neurol Ther. 2022 Jun 23;11(2):571–95.

68. Subei AM, Cohen JA. Sphingosine 1-Phosphate Receptor Modulators in Multiple Sclerosis. CNS Drugs. 2015 Jul 4;29(7):565–75.

69. Tobin WO, Weinshenker BG. Stopping immunomodulatory medications in MS: Frequency, reasons and consequences. Mult Scler Relat Disord. 2015 Sep;4(5):437–43.

70. Cameron E, Rog D, McDonnell G, Overell J, Pearson O, French DP. Factors influencing multiple sclerosis disease-modifying treatment prescribing decisions in the United Kingdom: A qualitative interview study. Mult Scler Relat Disord. 2019 Jan;27:378–82.

71. Muraro PA, Martin R, Mancardi GL, Nicholas R, Sormani MP, Saccardi R. Autologous haematopoietic stem cell transplantation for treatment of multiple sclerosis. Nat Rev Neurol. 2017 Jul 16;13(7):391–405.

72. Muraro PA, Pasquini M, Atkins HL, Bowen JD, Farge D, Fassas A, et al. Long-term Outcomes After Autologous Hematopoietic Stem Cell Transplantation for Multiple Sclerosis. JAMA Neurol. 2017 Apr 1;74(4):459.

73. Gromisch ES, Kerns RD, Czlapinski R, Beenken B, Otis J, Lo AC, et al. Cognitive Behavioral Therapy for the Management of Multiple Sclerosis–Related Pain. Int J MS Care. 2020 Jan 1;22(1):8–14.

74. Das J, Rog DJ, Middleton R, Rodgers JW, Fry R, Nicholas R. The association between deprivation and the access to disease modifying therapies for multiple sclerosis: An England wide community-based study in the UK MS Register. Mult Scler Relat Disord. 2022 Jan;57:103474.

75. Merkel B, Butzkueven H, Traboulsee AL, Havrdova E, Kalincik T. Timing of high-efficacy therapy in relapsing-remitting multiple sclerosis: A systematic review. Autoimmun Rev. 2017 Jun;16(6):658–65.

76. Brown JWL, Coles A, Horakova D, Havrdova E, Izquierdo G, Prat A, et al. Association of Initial Disease-Modifying Therapy With Later Conversion to Secondary Progressive Multiple Sclerosis. JAMA. 2019 Jan 15;321(2):175.

77. Deisenhammer F, Zetterberg H, Fitzner B, Zettl UK. The Cerebrospinal Fluid in Multiple Sclerosis. Front Immunol. 2019 Apr 12;10.

78. Sievänen H, Kari J, Eskola V, Huurre A, Soukka H, Palmu S. Incidence of traumatic lumbar punctures in adults: the impact of a patient's first procedure. Clinical Medicine. 2023 Jan;23(1):31–7.

79. Kim KT. Lumbar puncture: considerations, procedure, and complications. encephalitis. 2022 Oct 10;2(4):93–7.

80. Kuntz KM, MD EK, Stevens JC, RN PM, Offord KP, Ho MM. Post-lumbar puncture headaches. Neurology. 1992 Oct;42(10):1884–1884.

81. Engelborghs S, Niemantsverdriet E, Struyfs H, Blennow K, Brouns R, Comabella M, et al. Consensus guidelines for lumbar puncture in patients with neurological diseases. Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring. 2017 Jan 18;8(1):111–26.

82. Kodosaki E, Watkins WJ, Loveless S, Kreft KL, Richards A, Anderson V, et al. Combination protein biomarkers predict multiple sclerosis diagnosis and outcomes. J Neuroinflammation. 2024 Feb 17;21(1):52.

83. Joseph FG, Hirst CL, Pickersgill TP, Ben-Shlomo Y, Robertson NP, Scolding NJ. CSF oligoclonal band status informs prognosis in multiple sclerosis: a case control study of 100 patients. J Neurol Neurosurg Psychiatry. 2009 Mar 1;80(3):292–6.

84. Fábián TK, Hermann P, Beck A, Fejérdy P, Fábián G. Salivary Defense Proteins: Their Network and Role in Innate and Acquired Oral Immunity. Int J Mol Sci. 2012 Apr 2;13(4):4295–320.

85. de Paiva CS, St. Leger AJ, Caspi RR. Mucosal immunology of the ocular surface. Mucosal Immunol. 2022 Nov;15(6):1143–57.

86. Coyle PK, Sibony P, Johnson C. Oligoclonal IgG in tears. Neurology. 1987 May 1;37(5):853–853.

87. Hümmert MW, Wurster U, Bönig L, Schwenkenbecher P, Sühs KW, Alvermann S, et al. Investigation of Oligoclonal IgG Bands in Tear Fluid of Multiple Sclerosis Patients. Front Immunol. 2019 May 17;10.

88. Lotan I, Ganelin-Cohen E, Tartakovsky E, Khasminsky V, Hellmann MA, Steiner I, et al. Saliva immunoglobulin free light chain analysis for monitoring disease activity and response to treatment in multiple sclerosis. Mult Scler Relat Disord. 2020 Sep;44:102339.

89. Kaplan B, Golderman S, Ganelin-Cohen E, Miniovitch A, Korf E, Ben-Zvi I, et al. Immunoglobulin free light chains in saliva: a potential marker for disease activity in multiple sclerosis. Clin Exp Immunol. 2018 Mar 8;192(1):7–17.

90. Pieczyński J, Szulc U, Harazna J, Szulc A, Kiewisz J. Tear fluid collection methods: Review of current techniques. Eur J Ophthalmol. 2021 Sep 25;31(5):2245–51.

91. Bachhuber F, Huss A, Senel M, Tumani H. Diagnostic biomarkers in tear fluid: from sampling to preanalytical processing. Sci Rep. 2021 May 12;11(1):10064.

92. Cauley KA. Fluoroscopically Guided Lumbar Puncture. American Journal of Roentgenology. 2015 Oct;205(4):W442–50.

93. Cleveland Clinic [Internet]. 2023 [cited 2024 Feb 2]. Lumbar Puncture (Spinal Tap). Available from: https://my.clevelandclinic.org/health/diagnostics/12544-lumbar-puncture-spinal-tap

94. Heaney JLJ, Gleeson M, Phillips AC, Taylor IM, Drayson MT, Goodall M, et al. Salivary immunoglobulin free light chains: reference ranges and responses to exercise in young and older adults. Exerc Immunol Rev. 2016;22:28–41.

95. Jin H, Lu Q, Gao F, Hao H. Application of oligoclonal bands and other cerebrospinal fluid variables in multiple sclerosis and other neuroimmunological diseases: a narrative review. Ann Transl Med. 2023 Apr;11(7):282–282.

96. Forzy G, Gallois P, Hautecoeur P. Multiple sclerosis and oligoclonal bands in tears. Ann Biol Clin (Paris). 1999;57(2):240.

97. Devos D, Forzy G, de Seze J, Caillez S, Louchart P, Gallois Ph, et al. Silver stained isoelectrophoresis of tears and cerebrospinal fluid in multiple sclerosis. J Neurol. 2001 Aug;248(8):672–5.

98. Calais G, Forzy G, Crinquette C, Mackowiak A, de Seze J, Blanc F, et al. Tear analysis in clinically isolated syndrome as new multiple sclerosis criterion. Multiple Sclerosis Journal. 2010 Jan 22;16(1):87–92.

99. Lebrun C, Forzy G, Collongues N, Cohen M, de Seze J, Hautecoeur P. Tear analysis as a tool to detect oligoclonal bands in radiologically isolated syndrome. Rev Neurol (Paris). 2015 Apr;171(4):390–3.

100. Mavra M, Thompson EJ, Nikolic J, Krunic A, Ranin J, Levic Z, et al. The occurrence of oligoclonal IgG in tears from patients with MS and systemic immune disorders. Neurology. 1990 Aug;40(8):1259–1259.

101. Liedtke W, Weller M, Wiethölter H, Dichgans J. Immunological abnormalities in the tears of multiple sclerosis patients. Acta Neurol Scand. 2009 Jan 29;85(3):228–30.

102. Martino G, Servalli C, Filippi M, Maurizio B, Martinelli V, Furlan R, et al. Absence of oligoclonally restricted immunoglobulins in tears from multiple sclerosis patients. J Neuroimmunol. 1993 May;44(2):149–55.

103. Lolli F, Franciotta D. Oligoclonal bands in tears. Multiple Sclerosis Journal. 2010 Jun 17;16(6):760–760.

104. Atkin C, Richter A, Sapey E. What is the significance of monoclonal gammopathy of undetermined significance? Clinical Medicine. 2018 Oct;18(5):391–6.

105. Kyle RA, Therneau TM, Rajkumar SV, Larson DR, Plevak MF, Offord JR, et al. Prevalence of Monoclonal Gammopathy of Undetermined Significance. New England Journal of Medicine. 2006 Mar 30;354(13):1362–9.

106. Esparvarinha M, Nickho H, Mohammadi H, Aghebati-Maleki L, Abdolalizadeh J, Majidi J. The role of free kappa and lambda light chains in the pathogenesis and treatment of inflammatory diseases. Biomedicine & Pharmacotherapy. 2017 Jul;91:632–44.

107. Zhou R, Caspi RR. Ocular immune privilege. F1000 Biol Rep. 2010 Jan 18;2.

108. Berek K, Bsteh G, Auer M, Di Pauli F, Grams A, Milosavljevic D, et al. Kappa-Free Light Chains in CSF Predict Early Multiple Sclerosis Disease Activity. Neurol Neuroimmunol Neuroinflamm. 2021 Jul;8(4).

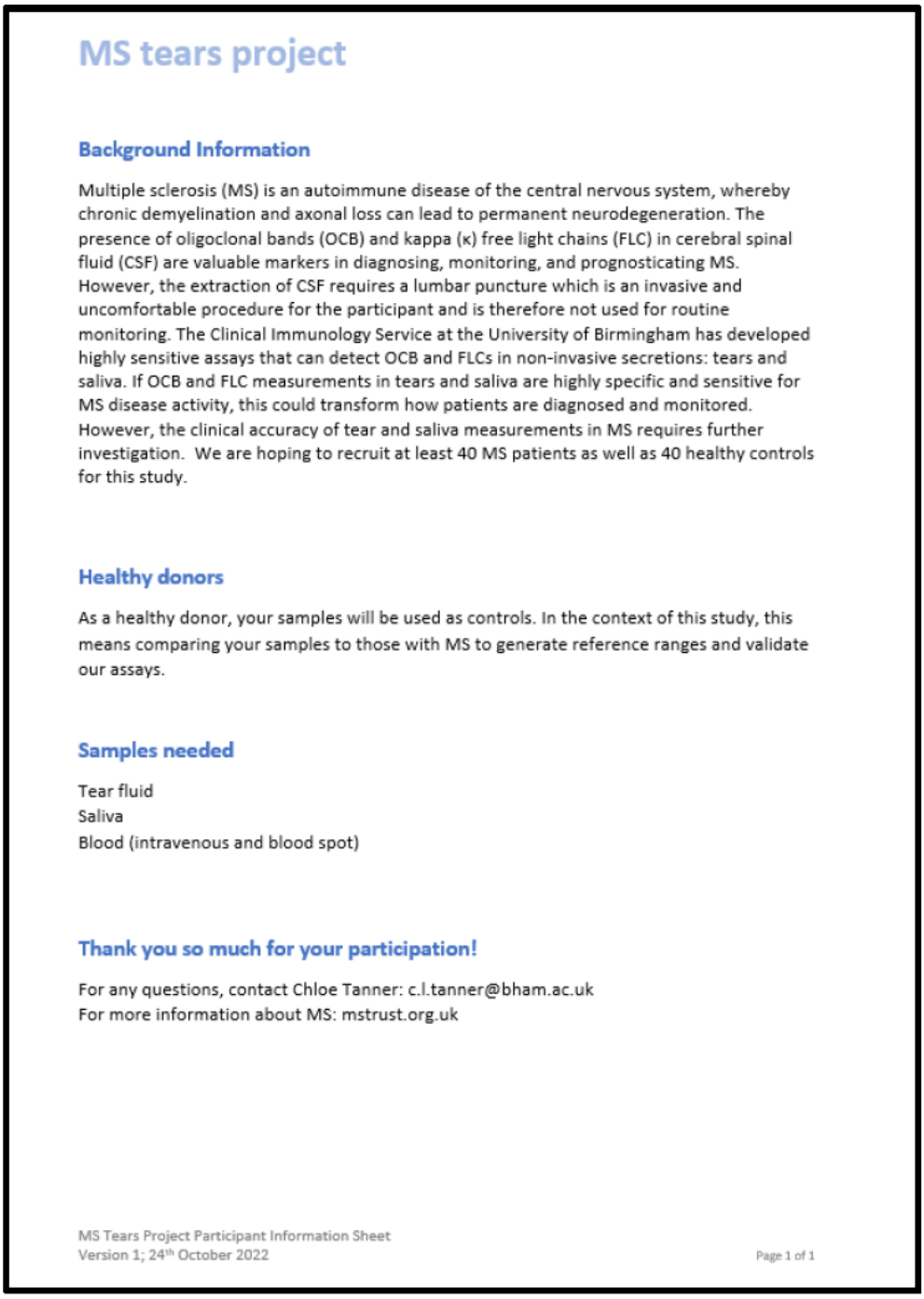
109. Hegen H, Berek K, Cavalla P, Christiansen M, Emeršič A, Di Filippo M, et al. Diagnostic value of kappa free light chain index in patients with primary progressive multiple sclerosis – a multicentre study. Front Immunol. 2023 Dec 19;14.

110. de Paiva CS. Effects of Aging in Dry Eye. Int Ophthalmol Clin. 2017;57(2):47–64.

111. Rapson A, Collman E, Faustini S, Yonel Z, Chapple IL, Drayson MT, et al. Free light chains as an emerging biomarker in saliva: Biological variability and comparisons with salivary IgA and steroid hormones. Brain Behav Immun. 2020 Jan;83:78–86.

7. APPENDICIES

7.1 Appendix 1: Participant information sheets



MS tears project

Background Information

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system, whereby chronic demyelination and axonal loss can lead to permanent neurodegeneration. The presence of oligoclonal bands (OCB) and kappa (κ) free light chains (FLC) in cerebral spinal fluid (CSF) are valuable markers in diagnosing, monitoring, and prognosticating MS. However, the extraction of CSF requires a lumbar puncture which is an invasive and uncomfortable procedure for the participant and is therefore not used for routine monitoring. The Clinical Immunology Service at the University of Birmingham has developed highly sensitive assays that can detect OCB and FLCs in non-invasive secretions: tears and saliva. If OCB and FLC measurements in tears and saliva are highly specific and sensitive for MS disease activity, this could transform how patients are diagnosed and monitored. However, the clinical accuracy of tear and saliva measurements in MS requires further investigation. We are hoping to recruit at least 40 MS patients as well as 40 healthy controls for this study.

Healthy donors

As a healthy donor, your samples will be used as controls. In the context of this study, this means comparing your samples to those with MS to generate reference ranges and validate our assays.

Samples needed

Tear fluid
Saliva
Blood (intravenous and blood spot)

Thank you so much for your participation!

For any questions, contact Chloe Tanner: c.l.tanner@bham.ac.uk
For more information about MS: mstrust.org.uk

MS Tears Project Participant Information Sheet
Version 1; 24th October 2022

Page 1 of 1

Figure 7.1 Participant information sheet for healthy donors.

Patient Information Sheet

Molecular and cellular studies on inflammation in Multiple Sclerosis

We would like to invite you to participate in this research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and ask a member of the research team if anything is not clear.

What is the purpose of the study?

Multiple Sclerosis has been shown to result from immune responses attacking tissues of the nervous system – particularly the brain and spinal cord. Many questions remain unanswered, including why some patients experience periods of relapses and remissions (relapsing remitting MS - RRMS), whereas some patients only experience slowly progressive disease (primary progressive MS - PPMS). Frequently patients start with RRMS and evolve into secondary progressive MS (SPMS). Understanding these processes better – and particularly the role for the immune system in causing them – would have important implications for the diagnosis, prognosis and treatment of MS. This study therefore aims to research how inflammation is involved in the development and progression of Multiple Sclerosis (MS).

Why have I been chosen?

In our research we want to compare immune responses between MS patients and other neurological conditions. Participants with other neurological conditions will be the "control" group for the MS group. You are being invited to take part in this study because you are being assessed and/or treated for MS or another relevant neurological condition.

Do I have to take part?

Your participation in this study is entirely voluntary. You do not have to take part, or give a reason if you choose not to. If you decide not to take part, this will not in any way affect the standard of care you receive.

What will I have to do if I take part?

If you decide to take part you will be asked to sign a consent form. You should only do this if you are happy that you understand the project and want to take part. For our research, we would like to take a sample of your blood, tears and saliva. If you are undergoing a lumbar puncture as part of your diagnosis, we would like to collect a sample of cerebrospinal fluid (CSF) as well. The taking of blood samples, tears and saliva are a routine procedure and is likely to be one of the tests that your doctor will be planning as part of your diagnosis or management. We will take an extra 50mls (about three tablespoonfuls). If CSF is collected as part of your lumbar puncture, we will collect 10mls of this fluid (less than one tablespoonful). This will not change the lumbar puncture procedure in any other way.

What will happen to my blood and CSF samples?

Figure 7.2 Participant information sheet for donors recruited from the Queen Elizabeth Hospital.

This information sheet was composed by Professor Saiju Jacobs and the research team at the Queen Elizabeth hospital. Form is continued on the proceeding pages.

If you agree to take part in the study, your blood and/or CSF samples will be used in experiments by researchers within Prof Douglas's or Prof Jacob's laboratories in the University of Birmingham. This research has been approved by a Research Ethics Committee.

The blood (and CSF sample when collected) will be spun in a centrifuge to separate the different components and used to prepare plasma and/or white cells for DNA extraction. The samples will then be stimulated by specific reagents and we will analyse the immune responses – for example the types of T cells and the chemicals (cytokines) that they produce. Tear and saliva samples will be collected for similar experiments. All samples, or derivatives, will be stored at the University of Birmingham and will only be analysed by members of the research group involved with this project. If you agree to donate your blood/CSF samples for this project, the samples will become the property of the University of Birmingham and will not be used for commercial gain.

If there are samples, or derivatives, remaining at the end of this study, these may be stored for use in future research projects on immune responses in MS and related aspects of immune responsiveness. However, before any additional studies are performed, approval would again be sought from a Research Ethics Committee.

What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any point and you do not have to give a reason why you wish to do so. If you do decide that you no longer wish to participate, all samples in storage will be destroyed, and all personal information will be deleted so that it can never be used again. If you change your mind after a long period of time the samples may already have been used to generate research data, so this may not be possible at this stage.

What are the possible disadvantages and side effects of taking part?

Your hospital appointment may take a few minutes longer than usual, but all other treatment and follow-up arrangements are unchanged. Complications from donating a blood sample are extremely rare, other than the brief discomfort of the needle. Some people may suffer bruising of the skin around where the sample was taken. Complications from saliva and/or tear collection have not been reported, Complications from a lumbar puncture are again rare and will have been discussed with you separately by your doctor. The most common complication consists of a headache, which is usually treated by bed rest, drinking fluids and simple pain killers.

What are the possible benefits of taking part?

This research will not benefit you directly. However, we hope that it will help us to understand why some people develop MS and why the condition progresses so that, in the future, this may lead to improvements in diagnosis and treatment.

Will my taking part in this study be kept confidential?

Yes. Your samples will be assigned a unique code which will be used in all experiments. The principal clinical investigator (Prof Douglas or Jacob) will keep a confidential record that links the unique code for

(Figure 7.2 continued). Participant information sheet for donors recruited from the Queen Elizabeth Hospital.

your sample back to you. This means that we can link together different samples taken at different times from the same person. This is obviously scientifically useful.

We may obtain basic information from your medical notes to help us understand the scientific results, but any such data will be handled in an anonymous format. No one outside your healthcare team will see your medical records. In general, all information about you and any results will remain confidential within the research group and will be stored in accordance with the UK Data Protection Act 1998. Your General Practitioner will not be informed of your participation in the study unless you ask us to do so.

It is intended that the results of the research will be presented at scientific meetings and published in relevant clinical/scientific journals but the data will be completely anonymised. We will aim to follow national guidelines which suggest that research data is stored for up to 10 years after the end of the study or as long as samples are retained.

Can I find out my results?

The experiments that we perform with your sample will not affect your treatment. For this reason, we do not report back the results of your own tests to you. However, we are very happy to explain more generally about what we learn from the experiments or to provide a written summary of the main findings. Please use the contact details below.

What if there is a problem?

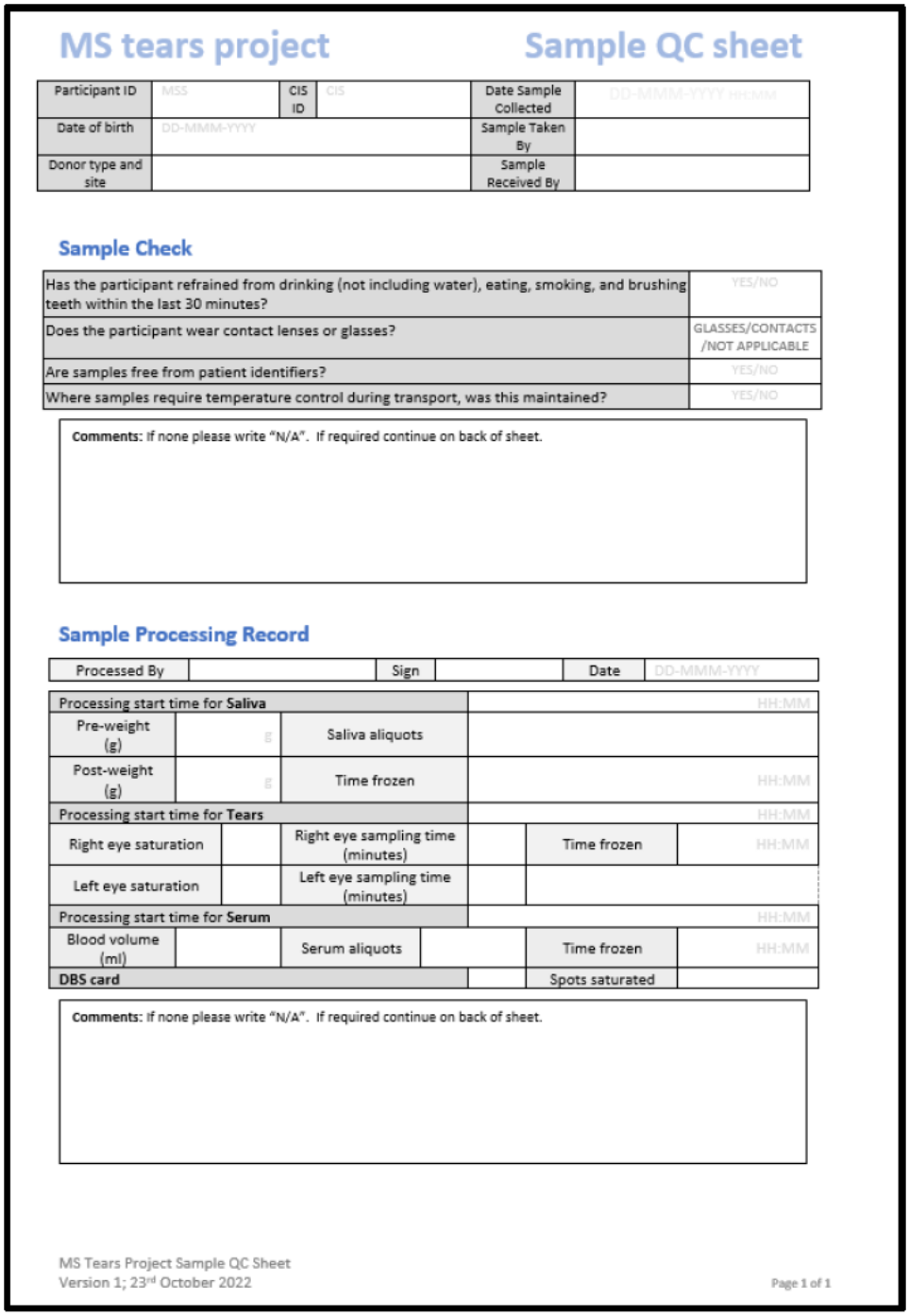
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (see contact details below). If you have a complaint and wish to contact someone who is not involved in the study you can contact the Patient Advice and Liaison Service (PALS), tel: 0800 073 0510. This is an independent service set up to help patients resolve any problems or concerns they may have.

If you have any questions, or do not understand anything, please contact

Prof Michael Douglas (Tel: 01384 244 046) or Prof Saiju Jacob (Tel: 0121 627 2000 ext 16844) or speak to the doctor taking consent for you today.

(Figure 7.2 continued). Participant information sheet for donors recruited from the Queen Elizabeth Hospital.

7.2 Appendix 2: Sample quality and processing forms



MS tears project **Sample QC sheet**

Participant ID	MSS	CIS ID	CIS	Date Sample Collected	DD-MMM-YYYY HH:MM
Date of birth	DD-MMM-YYYY			Sample Taken By	
Donor type and site				Sample Received By	

Sample Check

Has the participant refrained from drinking (not including water), eating, smoking, and brushing teeth within the last 30 minutes?	YES/NO
Does the participant wear contact lenses or glasses?	GLASSES/CONTACTS /NOT APPLICABLE
Are samples free from patient identifiers?	YES/NO
Where samples require temperature control during transport, was this maintained?	YES/NO

Comments: If none please write "N/A". If required continue on back of sheet.

Sample Processing Record

Processed By		Sign		Date	DD-MMM-YYYY

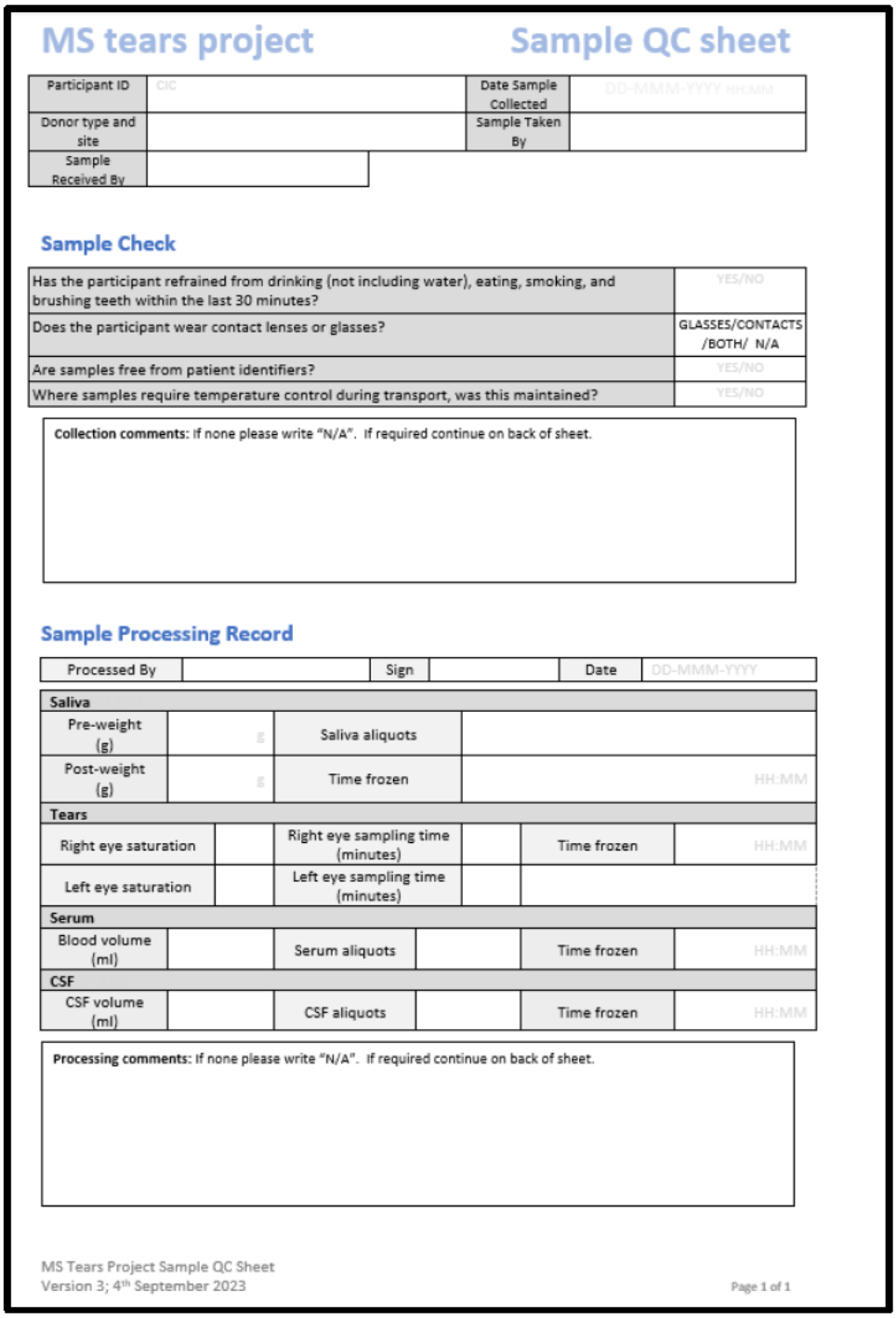
Processing start time for **Saliva**					HH:MM
Pre-weight (g)	g	Saliva aliquots			
Post-weight (g)	g	Time frozen			HH:MM
Processing start time for **Tears**					HH:MM
Right eye saturation		Right eye sampling time (minutes)		Time frozen	HH:MM
Left eye saturation		Left eye sampling time (minutes)			
Processing start time for **Serum**					HH:MM
Blood volume (ml)		Serum aliquots		Time frozen	HH:MM
DBS card				Spots saturated	

Comments: If none please write "N/A". If required continue on back of sheet.

MS Tears Project Sample QC Sheet
Version 1; 23rd October 2022

Page 1 of 1

Figure 7.3 Quality control sample processing form for healthy donors.
DBS card was taken from healthy donors but not processed or analysed for the purpose of this study.



MS tears project — Sample QC sheet

Participant ID	CIC	Date Sample Collected	DD-MMM-YYYY HH:MM
Donor type and site		Sample Taken By	
Sample Received By			

Sample Check

Has the participant refrained from drinking (not including water), eating, smoking, and brushing teeth within the last 30 minutes?	YES/NO
Does the participant wear contact lenses or glasses?	GLASSES/CONTACTS /BOTH/ N/A
Are samples free from patient identifiers?	YES/NO
Where samples require temperature control during transport, was this maintained?	YES/NO

Collection comments: If none please write "N/A". If required continue on back of sheet.

Sample Processing Record

Processed By		Sign		Date	DD-MMM-YYYY

Saliva					
Pre-weight (g)	g	Saliva aliquots			
Post-weight (g)	g	Time frozen	HH:MM		
Tears					
Right eye saturation		Right eye sampling time (minutes)		Time frozen	HH:MM
Left eye saturation		Left eye sampling time (minutes)			
Serum					
Blood volume (ml)		Serum aliquots		Time frozen	HH:MM
CSF					
CSF volume (ml)		CSF aliquots		Time frozen	HH:MM

Processing comments: If none please write "N/A". If required continue on back of sheet.

MS Tears Project Sample QC Sheet
Version 3; 4th September 2023

Page 1 of 1

Figure 7.4 Quality control sample processing form for participants recruited from the Queen Elizabeth Hospital.

7.3 Appendix 3: Data exclusions

Table 7.1 Summary of data excluded from study

Seven participants had data excluded due to difficulties in sample collection or sample quality. Two participants had data excluded because they withdrew from donating the full sample set.

Participant ID	Sample type	Data excluded	Reason for exclusion
CIC 011	Saliva	- Saliva flow rate - Saliva FLC secretion rates	Participant could not sit upright to complete 4-minute saliva collection. Instead, a single spit volume was obtained. FLC secretion rates could not be obtained.
CIC 035	Saliva	All saliva data	High viscosity of the saliva sample prevented it from being analysed in FLC ELISAs and IEF.
CIC 037	Saliva and tears	All saliva and tear data	Participant declined saliva and tear collection after consenting to the study. However, they agreed to serum and CSF samples to be used.
CIC 040	Saliva	- Saliva flow rate - Saliva FLC secretion rates	Participant could not sit upright to complete 4-minute saliva collection. Instead, a single spit volume was obtained. FLC secretion rates could not be obtained.
CIC 059	Tears	Right eye tear FLC data	After 5 minutes, the saturation of the right strip was less than 1mm. Accurate FLC concentrations could not be obtained.
CIC 061	Right eye tears	Right eye tear data	Participant requested for tears to be collected from the left eye only due to a dermatological condition affecting their right eye.
CIC 062	Tears	Right eye tear FLC data	After 5 minutes, the saturation of the right strip was less than 1mm. Accurate FLC concentrations could not be obtained.
CIC 063	Tears	All tear data	Participant declined tear collection after consenting to the study. However, they were agreed to the remainder of the samples to be used.
CIC 068	Tears	Tear FLC data	After 5 minutes, the saturations of both strips were less than the 1mm increment. Accurate FLC concentrations could not be obtained.

7.4 Appendix 4: NHS serology parameters for adult serum IgGAM and FLCs

Table 7.2 NHS serology parameters for adult serum IgGAM and FLCs

Parameter	Reference range (unit)
IgG	6-16 (g/L)
IgA	0.8-4 (g/L)
IgM	0.5-2 (g/L)
Kappa FLC	3.3-19.4 (mg/L)
Lambda FLC	5.7-26.3 (mg/L)
Kappa: Lambda ratio	0.26-1.65