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The Dual Role of Bruton's Tyrosine Kinase Inhibitors in Multiple Sclerosis: From Clinical Efficacy to Molecular Modulation of EBV-Host Interactions

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INDEX

目录

Abstract 6
CHAPTER 1. 12
Introduction: Multiple sclerosis and Epstein-Barr virus and Bruton's tyrosine kinase inhibitors 12
1.1. Disease Background of Multiple Sclerosis 12
1.1.1 Pathophysiology and Clinical Phenotypes 12
1.1.2. Imaging 14
1.1.3 Immunopathology 15
1.1.4 Neurodegenerative Components and Disability Accumulation 16
1.1.5 Epidemiology and Disease Burden 16
1.1.6 Diagnosis and Early Intervention 17
1.2 Epstein–Barr Virus and Multiple Sclerosis 18
1.2.1 Biological Characteristics of Epstein–Barr Virus and Host Persistence 18
1.2.2 EBV's Role in B Cell Activation and Immune Reprogramming 19
1.3 Bruton's Tyrosine Kinase Inhibitors (BTKis) 20
1.3.1 Mechanism of Action of BTKis 20
1.3.2 Clinical Applications of BTKis 21
1.4 Limitations of Current Treatments and Scientific Gaps 22
1.4.1 Evolution of Treatment Goals for Multiple Sclerosis 22
1.4.2 Limitations of Current Treatment Strategies 23
1.4.3 Gaps in Immunological Target Selection 24
1.5 Research Objectives and Hypotheses 24
1.5.1 Overall Objective 24
1.5.2 Specific Aims 25
1.5.3 Hypotheses 26
CHAPTER 2. 27
The effects and side effects of Bruton's tyrosine kinase inhibitors for the treatment of multiple sclerosis patients: a systematic review and meta-analysis of clinical trials 27
2.1 Introduction 27

2.2 Research Question Definition 28
2.3 Methods 28
2.3.1 Eligibility Criteria 29
2.3.2 Data Synthesis and Statistical Analysis 30
2.3.3 Literature Search Strategy 33
2.4 Results 37
2.4.1 Overview of Included Studies 37
2.4.2 Efficacy Results for BTKis 39
2.4.3 Heterogeneity Test and Analysis for the Number of New or Enlarging T2 Lesions 47
2.4.4 Effect of BTKi Treatment on Relapse Rate 52
2.4 Discussion 59
2.4.1 Summary of Findings 59
2.4.2 The Key Role of Treatment Dosage 59
2.4.3 Side Effects and Safety 59
2.4.4 Heterogeneity and Sensitivity Analysis 59
2.4.5 Publication Bias and Study Quality 60
2.4.6 Limitations of the Study 60
2.4.7 Future Research Directions 60
2.4.8 Clinical Application Prospects 60
CHAPTER 3. 62
The Bruton Tyrosine Kinase (BTK) inhibitors on Epstein-Barr Virus (EBV)-host interactions in Multiple Sclerosis 62
3.1. Introduction 62
3.1.1 Pathogenic Mechanism of EBV in Multiple Sclerosis 64
3.1.2 BTK Inhibitors: Pharmacology and Evidence for Multiple Sclerosis (MS) 66
3.2 Project Objectives and Research Design 68
3.3 Relevance and Innovation 68
3.4 Methods 68
3.4.1 In Vitro Testing 69
3.4.2 Participant Selection and Blood Sample Collection 69
3.4.3 PBMC Isolation and EBV Infection 69
3.4.4 Grouping 70
3.4.4 BTK Inhibitor Dose-Response Analysis 70
3.4.5 Cell Treatment with BTKi and Extraction 71
3.4.6 Western Blot Experimental Procedure 72
3.4.7 RT-PCR Experimental Method 75

3.4.8 Statistical Analysis 76
3.5 Results 77
3.5.1 Dose-Response Analysis: 24-hour and 48-hour Treatment Results 77
3.5.2 Regulatory Effects of Evobrutinib on BTK, LMP1, and EBNA2 Expression in MS and HD Group Cells 78
3.5.3 Regulatory Effects of Tolebrutinib on BTK and LMP1 Expression in MS and HD Group Cells 79
3.5.4 Effects of Orelabrutinib on BTK and LMP1 Expression in MS and HD Group Cells 81
3.5.5 Effects of Fenebrutinib on BTK and LMP1 Expression in MS and HD Group Cells 82
3.6 Effects of Different BTKi Drugs on Target Gene Expression in MS Group Cells 83
3.6.1 Evobrutinib 83
3.6.2 Tolebrutinib 84
3.6.3 Orelabrutinib 85
3.6.4 Fenebrutinib 86
3.7 Discussion 87
3.7.1 Summary of Findings 87
3.7.2 The Role of EBV in MS Pathogenesis 87
3.7.3 Dose-Response Analysis and Its Significance in MS Treatment 88
3.7.4 Western Blot Results Analysis 89
3.7.5 qPCR Results Analysis 90
3.7.6 WB and qPCR Results Analysis 91
3.7.7 Evobrutinib's Broad Inhibitory Effect in Immune Regulation 93
3.7.8 Comparison with Existing Treatment Regimens 94
3.7.9 Outlook 95
CHAPTER 4. 96
GENERAL DISCUSSION 96
4.1 Overall Framework Review and Key Conclusions 96
4.2 Clinical Translation and Significance of Drug Stratification 99
4.3 Research Limitations 100
4.4 Future Research Directions 102
REFERENCES 104

Abstract

Background

Multiple sclerosis (MS) is an immune-mediated central nervous system disorder characterized by recurrent local neurological deficits and progressively accumulating neurodegenerative damage. As the disease progresses, patients experience varying degrees of motor, sensory, and cognitive dysfunction. Although current treatments have made progress in alleviating symptoms, reducing relapses, and slowing disease progression, they remain ineffective in controlling the accumulation of neurological damage, particularly in chronic progressive MS, where further control of neurodegenerative damage remains a therapeutic challenge.

In recent years, the relationship between Epstein-Barr virus (EBV) and MS has garnered significant attention. EBV is a widely prevalent human virus, with approximately 95% of the global population being infected at some point in their lives. Studies suggest that EBV infection may be a critical trigger for MS development, particularly the immune dysregulation following EBV infection that may drive the immune pathogenesis of MS. EBV activates immune cells such as B cells and T cells, affecting immune regulation and promoting immune system attacks on the nervous system, leading to demyelination. Therefore, modulating EBV-related immune responses may provide a new therapeutic strategy for MS.

Bruton’s tyrosine kinase (BTK) is a key tyrosine kinase widely present in immune cells such as B cells, T cells, dendritic cells, and microglia. It plays a central role in regulating

multiple immune signaling pathways, particularly in B cell activation, antibody production, and T cell co-activation. In recent years, Bruton's tyrosine kinase inhibitors (BTKis) have emerged as novel immunomodulatory drugs, demonstrating significant therapeutic potential in various immune-mediated diseases, especially in autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. Due to the central role of BTK in immune responses, BTKis are also considered potential candidates for the treatment of MS.

Several BTKis, including Evobrutinib, Tolebrutinib, Fenebrutinib, and Orelabrutinib, have been developed for MS treatment. These drugs not only inhibit immune cell activation and reduce central nervous system inflammation but also possess good blood-brain barrier permeability, allowing them to directly target the central nervous system, thus providing new therapeutic targets for MS. The regulation of EBV-related immune responses by BTKis is of particular interest. Studies have shown that EBV activates immune responses through proteins such as LMP1 (latent membrane protein 1) and EBNA2 (EBV nuclear antigen 2), which may promote the development of MS. Therefore, BTKis may slow or halt the progression of MS by modulating EBV-related immune responses.

This study systematically reviews and conducts a meta-analysis to evaluate the efficacy and safety of Evobrutinib, Tolebrutinib, Fenebrutinib, and Orelabrutinib in MS treatment. *We also investigate two main objectives: 1) whether BTKi compounds affect molecules important in the EBV lifecycle in B cells, either directly through their viral origin or indirectly through exploitation by the virus for survival; and 2) whether there are differences in the therapeutic efficacy of various BTKi compounds. This research not only advances our understanding of how BTKis modulate EBV-related immune responses in MS but also provides insights into potential personalized treatment strategies, particularly for EBV-driven immunopathology, where BTKis may offer an effective treatment option.*

Methods

In this study, we evaluated the efficacy and safety of BTKis in MS treatment through systematic review and meta-analysis. We conducted a literature search in the PubMed, Embase, Web of Science, and Cochrane Library databases from the inception of the databases to January 2025. According to the inclusion criteria, only randomized controlled

trials (RCTs) involving MS patients with both treatment and control groups using BTKis and placebo were included. Studies with incomplete data or missing necessary information were excluded, resulting in 9 eligible studies. In statistical analysis, both random-effects and fixed-effects models were applied using R Studio and Stata 15.1 software. The primary effect size was the standard mean difference (SMD) risk ratio (RR), and 95% confidence intervals (CIs) were calculated. All studies were grouped according to the different dosages of BTKis (ranging from 5mg to 200mg) for subgroup analysis. Subgroup analyses were conducted based on drug concentration, treatment duration, and drug type to explore their potential effects on efficacy. The focus of data analysis was to assess the impact of BTKis on MS patients' imaging endpoints, including new T1 Gd+ lesions, new or enlarged T2 lesions, relapse rates, and side effects. Additionally, in vitro experiments were conducted using peripheral blood mononuclear cells (PBMCs) from MS patients to isolate B cells for further processing. IC50 analysis was used to assess the inhibitory effects of the four BTKis on cell proliferation at different concentrations to determine the minimum effective concentration. Western blot (WB) and quantitative PCR (qPCR) analyses were used to evaluate the effects of Evobrutinib, Tolebrutinib, Fenebrutinib, and Orelabrutinib on the expression of MS-related proteins (such as BTK, LMP1, EBNA2, NF-κB, STAT3, ADAR1) and mRNA (such as EBNA2, LMP1, CD40, AICDA).

Results

The meta-analysis results showed that BTKi treatment significantly reduced the number of new T1 Gd+ lesions, with an SMD of 0.340, 95% CI of 0.108 to 0.573, and a p-value of less than 0.05. Additionally, BTKi treatment significantly decreased the number of new or enlarged T2 lesions, with an SMD of 0.349, 95% CI of 0.192 to 0.506, and a p-value of less than 0.001. These results indicate that BTKi treatment effectively reduces active lesions in the central nervous system of MS patients and slows disease progression. Furthermore, BTKi treatment significantly reduced relapse rates, with an effect size (ES) of 0.134, 95% CI of 0.096 to 0.173, and a p-value of less than 0.001, further validating its potential in improving clinical function. Regarding side effects, no significant differences were observed between the BTKi treatment and control groups (RR = 0.99, p = 0.759). Additionally, common adverse reactions, including headache (RR = 0.648, p = 0.942), elevated AST/ALT (RR = 0.91, p = 0.161), and rhinitis (RR = 1.39, p = 0.337), did not

show significant differences in incidence. This suggests that BTKi has a favorable safety profile with no significant adverse effects in the treatment of MS.

IC50 analysis revealed that Evobrutinib had IC50 values of 42.49 μM (24 hours) and 17.68 μM (48 hours), Tolebrutinib had IC50 values of 25.13 μM (24 hours) and 9.77 μM (48 hours), Fenebrutinib had IC50 values of 20.87 μM (24 hours) and 20.63 μM (48 hours), and Orelabrutinib had IC50 values of 20.63 μM (24 hours) and 15.01 μM (48 hours). These results indicate that Tolebrutinib significantly inhibits cell proliferation at lower concentrations, whereas Evobrutinib requires higher concentrations for significant inhibitory effects. In vitro WB analysis showed that Evobrutinib, Tolebrutinib, Fenebrutinib, and Orelabrutinib significantly downregulated the expression of BTK and LMP1 proteins. In qPCR analysis, Evobrutinib and Tolebrutinib significantly downregulated the expression of EBNA2, LMP1, and CD40 genes, while Fenebrutinib had a significant effect on LMP1 expression.

Discussion:

This study addressed two key objectives: 1) to verify whether BTKi compounds affect molecules that are important in the EBV lifecycle in B cells, either directly because of viral origin or indirectly through their exploitation by the virus for survival; and 2) to assess whether there are differences between various BTKi compounds in their therapeutic effects on MS. Our results indicate that BTKis, including Evobrutinib, Tolebrutinib, Fenebrutinib, and Orelabrutinib, modulate the expression of EBV-related proteins such as LMP1 and EBNA2, which are involved in MS pathogenesis. These findings suggest that BTKis effectively suppress EBV-related immune activation, potentially slowing excessive immune responses and neurodegeneration in MS patients. Dose-response analysis aimed at assessing differences between the various BTKi compounds showed that Tolebrutinib significantly inhibited cell proliferation at lower concentrations, exhibiting strong immunomodulatory effects. In contrast, Evobrutinib required higher concentrations to achieve significant inhibitory effects. Despite the weaker inhibitory effect of Evobrutinib, its immunological and clinical effects may remain relevant.

Systematic review and meta-analysis demonstrated that BTKis significantly reduced the number of new T1 Gd+ lesions and new/enlarged T2 lesions, indicating their effectiveness in suppressing central nervous system activity-related inflammation. BTKi treatment also significantly reduced relapse rates, further proving its potential in improving clinical function. The study further highlighted the critical role of treatment dosage in BTKi efficacy. Higher-dose groups (≥60mg QD) showed significantly better results than lower-dose groups (<60mg QD), suggesting that increasing the dosage could significantly improve treatment outcomes. Future clinical treatments should adjust drug doses based on the individual patient to achieve optimal efficacy. However, the low-dose group showed no statistical significance, indicating limited effectiveness for MS treatment, especially in reducing central inflammation and neurodegeneration. Regarding side effects and safety, no significant differences were found between the BTKi treatment and control groups. Common side effects such as headache, liver enzyme elevation, and upper respiratory symptoms showed no significant difference between the two groups, providing a solid safety foundation for BTKi clinical applications. However, the long-term side effects and different patient responses across MS subtypes require further investigation. Sensitivity analysis confirmed the overall consistency of treatment effects, and although heterogeneity was present, most studies indicated stable efficacy, particularly in the high-dose group. Future research should further evaluate the long-term effects of BTKi treatment, particularly in large-sample populations with different MS subtypes.

CHAPTER 1.

Introduction: Multiple sclerosis and Epstein-Barr virus and Bruton's tyrosine kinase inhibitors

1.1. Disease Background of Multiple Sclerosis

1.1.1 Pathophysiology and Clinical Phenotypes

Multiple sclerosis (MS) is the most common autoimmune disease of the central nervous system (CNS), primarily characterized by neurodegenerative lesions(Chaudhuri, 2013). Late 19th and early 20th-century histological studies revealed that MS is caused by demyelination, astrocytic gliosis, and axonal degeneration in the CNS(Mix et al., 2010). The pathological features of MS include focal and diffuse inflammatory infiltration, damage and death of oligodendrocytes, demyelination, axonal transection, reactive astrogliosis, and persistent activation of microglia and infiltrating myeloid cells(Lassmann, 2018). Significant progress has been made in controlling the inflammatory factors of MS, but focus has now shifted towards researching and treating the progressive and degenerative aspects of the disease(Ontaneda et al., 2017). The pathological core of MS is no longer understood simply as a transient inflammatory response caused by autoimmune attacks on the myelin sheath. These abnormalities are not limited to local lesions during acute relapses but reflect a persistent abnormal immune activation that maintains a focal inflammatory state in the CNS. Over time, this leads to progressive demyelination, axonal damage, and neurodegeneration, causing irreversible damage and neurological deficits in various regions,

including white matter, spinal cord tracts, optic nerves, submeningeal areas, and peri-cortical regions(Lapides & McDonald, 2020). This suggests that MS pathogenesis is characterized by persistence and multi-focal distribution of lesions.

The classic clinical manifestations of MS include optic neuritis, acute myelitis, brainstem, and cerebellar syndromes, with significant heterogeneity depending on the anatomical sites affected by inflammatory demyelination(Burda & Sofroniew, 2014). Visual involvement in MS is usually unilateral, characterized by gradual onset of monocular vision loss, visual field defects, central scotoma, and eye movement pain(Dhanapalaratnam et al., 2022). Spinal cord involvement is typically localized, presenting as weakness in the limbs, sensory loss, or motor deficits, spasticity, gait stiffness, and sphincter dysfunction(Dobkin, 2021). Brainstem and cerebellar involvement results in symptoms such as facial numbness or weakness, dysarthria, dizziness, diplopia, nystagmus, ataxia, postural instability, dysphagia, and dysphonia(Freiha et al., 2020). In addition to these "focal neurological deficits," there is a category of subtle symptoms that, while not necessarily corresponding directly to a single focal lesion, are closely associated with the patient's daily living, work capacity, and social participation. These may occur several years before the classic MS symptoms appear, reflecting an undiagnosed pre-disease stage(Demin et al., 2019). Non-specific MS symptoms may include pathological fatigue, decreased information processing speed, poor concentration, impaired executive function, diminished emotional regulation, and anxiety/depression-like mood changes.

MS is characterized by CNS inflammation and degeneration, which dominate different stages of the natural disease course. Clinical subtypes of MS were classified in 1996 based on the extent of inflammatory and degenerative activity into relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS)(Antel et al., 2012). The most common form of onset is RRMS(McKay et al., 2015), which presents as newly occurring or significantly worsening neurological deficits, with discrete acute or subacute relapsing events followed by partial or complete remission. As our understanding of MS disease mechanisms continues to evolve, it is recognized that patients experience a continuum throughout the disease course. For example, over 80% of RRMS patients develop SPMS within 10-20 years, characterized by the absence of multiple acute relapse events and instead ongoing deterioration of neurological function even in the absence of obvious relapses, such as difficulty walking, impaired balance, reduced fine motor skills, cognitive impairment, and sphincter control issues(Koch et al., 2013). In some patients, chronic progressive neurodegeneration occurs and accumulates without frequent acute

relapses, as seen in PPMS(Rocca et al., 2002), where patients experience slow and continuous neurofunctional decline without clear relapse-remission fluctuations. This suggests that MS is a progressive neurodegenerative condition, not simply an inflammatory disease caused by recurrent acute demyelination events.

MS is marked by demyelination, but it is not a myelin-specific disease. It is an acute immune-driven, relapsing disease primarily characterized by demyelination, but it is also a chronic immune-driven central inflammatory state that may gradually evolve into a progressive neurodegenerative process in some patients. The accumulation of clinical disability is closely related to axonal degeneration and neural network disruption caused by the inflammatory microenvironment, which is a continuous, low-grade, but long-lasting inflammatory state that is not entirely dependent on relapse frequency(Muzio et al., 2021). Based on this understanding, treatment should not be limited to controlling acute inflammatory events and reducing relapse frequency but should also aim to delay the possible development of structural damage to permanent disability.

1.1.2. Imaging

Magnetic resonance imaging (MRI) is the imaging standard for the diagnosis, disease activity assessment, and treatment monitoring of MS(Wattjes et al., 2015). MRI can sensitively detect current inflammatory activity in the CNS and quantitatively assess the cumulative trend of structural damage over time(Stoll & Bendszus, 2009). Two key MRI indicators are worth noting: gadolinium-enhanced T1-weighted imaging, which detects new gadolinium-enhancing lesions (new T1 lesions), and T2-weighted imaging, which identifies new or enlarging high-signal lesions (new or enlarging T2 lesions)(Rovira et al., 2024). T1-weighted imaging is commonly used to detect active lesions and typically indicates recent blood-brain barrier disruption with immune cell infiltration and localized acute inflammation(Candelario-Jalil et al., 2022). These lesions reflect significant immune responses in the CNS, so T1 gadolinium-enhanced imaging is widely used to assess disease activity. T2-weighted imaging is primarily used to observe the accumulation of structural damage related to inflammation over weeks to months, reflecting new demyelination or the continued expansion of old lesions(Brück et al., 1997). Therefore, they are key indicators for determining the long-term disease progression. These two indicators can be used to assess whether a particular intervention has objectively inhibited the formation of new CNS lesions.

However, MRI also has limitations, as it cannot entirely exclude CNS inflammatory activity based on the absence of new or enhancing lesions(Stoll & Bendszus, 2009). In particular, cortical and deep gray matter lesions often do not appear well on routine MRI sequences(Hulst & Geurts, 2011), although newer techniques such as double inversion recovery, phase-sensitive inversion recovery (PSIR), and higher magnetic field 7T MRI have somewhat improved this issue(Fechner et al., 2019). Traditional MRI methods also struggle to capture subtle changes such as atrophy and slow lesion expansion without volume analysis, and these minor changes are often difficult to detect(Nistri et al., 2024; Rocca et al., 2017). While volumetric analysis has not been widely applied in clinical practice, it is gradually gaining traction in MS research with technological advancements.

1.1.3 Immunopathology

Early research on MS viewed it as an autoimmune demyelinating disease model dominated by myelin-specific T lymphocytes(Huseby et al., 2001). Peripheral autoreactive T cells cross the blood-brain barrier and trigger local immune responses that lead to inflammatory and demyelinating damage during acute relapses(Huang et al., 2021). However, it was later recognized that the T cell-driven model could not fully explain the chronic progression and sustained activity of MS(Lückel et al., 2019). In chronic and sustained MS lesions(Absinta et al., 2019), infiltration of B cells, plasma cells, follicular-like structures, microglia, and myeloid cells can be observed in submeningeal spaces, near-cortical regions, white matter-gray matter junctions, and spinal cord longitudinal tracts, accompanied by local pro-inflammatory cytokine deposition and immunoglobulin accumulation. This represents a long-lasting structure resembling peripheral lymphoid tissues, rather than short-term acute inflammatory responses. The main feature of this immune ecosystem is that, even in the absence of obvious clinical relapses, many immune cell populations continuously exert low but stable inflammatory pressure, with local self-amplification of immune cells(Zhou et al., 2025). MS is not driven by the short-term activation of a single immune cell population but is dominated by an immune ecosystem maintained by persistent B cells, T cells, and myeloid cells, which can remain activated in specific regions of the CNS, generating sustained inflammation.

This immune ecosystem depends on B cell involvement. B cells serve as antigen-presenting cells (APCs) and present myelin-related antigens to T cells, further activating pathogenic T cells(Sabatino Jr et al., 2019). B cells and plasma cells secrete various pro-

inflammatory factors that maintain the persistence of a local pro-inflammatory microenvironment. In some MS patients, submeningeal structures resembling germinal centers can form, potentially associated with cortical demyelination, cortical thinning, and subcortical network reorganization(Magliozzi et al., 2023). This theory has also been validated clinically; for example, removing specific B cell subpopulations can significantly reduce relapse rates and the formation of new lesions, indicating that B cells play a key role in the central inflammatory activity of MS and are core participants in CNS inflammation(Cencioni et al., 2021).

1.1.4 Neurodegenerative Components and Disability Accumulation

Acute relapses do not necessarily lead to disability in MS. The neurological deficits during acute relapses are reversible to some extent in some patients(Berkovich, 2016), particularly in cases of conduction block and transient myelin damage, where partial recovery can occur after inflammation subsides and some remyelination takes place. In the chronic progressive phase, sustained low-grade inflammatory pressure and glial cell activation can lead to axonal transection, degeneration, and irreversible loss(Correale et al., 2017). Once axons undergo structural damage, functional recovery is unlikely, even if the inflammation levels decrease(Hirschberg et al., 1994). A common phenomenon is that some patients, despite not experiencing frequent relapses in recent years, still exhibit reduced walking ability, balance instability, loss of fine motor skills in the upper limbs, sphincter dysfunction, and cognitive and emotional regulation impairments.

1.1.5 Epidemiology and Disease Burden

The global prevalence of MS is gradually increasing(Walton et al., 2020), with the number of MS patients reaching 2.3 million in 2013, 2.8 million in 2020, and 2.9 million in 2023(Kapica-Topczewska et al., 2025). The incidence of MS varies by region, with the highest prevalence in North America and Europe and lower rates in Asia and Africa(Pugliatti et al., 2002). In the UK, it is estimated that over 150,000 people are affected by MS, with a prevalence rate of 211 per 100,000 residents(Bull, 2015). The prevalence of MS in Scotland may be the highest in the world, with 402 people per 100,000 residents affected(Barnes, 2020). The median age of diagnosis is 32 years, and women are typically affected 2-5 years earlier than men(Nick et al., 2010). Overall, the average age of MS

patients is increasing, with the highest prevalence seen in the 60th decade of life(Solaro et al., 2015). According to the MS Atlas, the global average incidence rate of MS is 2.1 per 100,000 residents annually, although this number varies by region(Walton et al., 2020). This means that someone in the world is diagnosed with MS every five minutes, with 300 new cases diagnosed each day.

MS not only has significant epidemiological implications but also has a considerable impact on the burden of daily living. MS is not only associated with significant motor, sensory, and balance impairments but also closely linked to persistent fatigue, decreased cognitive processing speed, and impaired executive function(Chiaravalloti & DeLuca, 2008). Even if patients have not yet reached the traditional "severe disability" standard, they may experience significant functional limitations in jobs that require high cognitive load, physical exertion, or sustained concentration, leading to career path adjustments. The potential progressive motor disabilities, bladder or bowel control issues, and cognitive-behavioral changes create long-term caregiving burdens, which may be passively assumed by families as patients enter mid-life or even early adulthood, prior to hospital care. Therefore, the structural characteristics of MS-related social resource consumption differ from traditional elderly care models and are more akin to "middle-aged long-term family care." MS management should aim to maintain social participation and delay the onset of lifestyle dependency, rather than simply focusing on short-term symptom control. Patients with MS face a potential, irreversible decline in neurological function early in the disease course, which itself can lead to significant emotional distress, helplessness, and social withdrawal(Kalb, 2007). The disability burden of MS is not limited to the original neurological function score but may also include individual psychological instability caused by the anticipated decline in function.

1.1.6 Diagnosis and Early Intervention

MS lacks a confirmatory diagnostic test and is a diagnosis of exclusion. The diagnostic criteria for MS aim to identify whether a patient has entered the MS disease course as early as possible while ensuring specificity(McDonald et al., 2001). This allows intervention before structural damage occurs. Clinical radiological features are relied upon to define the disease(Poser & Brinar, 2001). The McDonald diagnostic criteria, first developed in 2001 and revised several times, with the latest update in 2024, are awaiting publication(Samara & Ontaneda, 2025). With the maturity of MRI technology, imaging evidence can confirm the

timing of the disease. For example, even if a patient experiences only a single significant clinical event, if MRI shows the presence of pre-existing demyelinating lesions and newly active gadolinium-enhancing lesions, it suggests that the patient has entered the MS disease course(Miller et al., 1993). MRI is no longer just an auxiliary diagnostic tool but has become a core basis for identifying the disease at an early stage. Due to its temporal advantage, the earlier new lesions are suppressed, the more likely it is to reduce subsequent axonal loss and irreversible functional changes. Early intervention goes beyond the old concept of waiting until clear progression occurs before intervening.

1.2 Epstein–Barr Virus and Multiple Sclerosis

1.2.1 Biological Characteristics of Epstein–Barr Virus and Host Persistence

EBV is a member of the γ-herpesvirus subfamily in the Herpesviridae family(Backovic et al., 2009). Unlike many respiratory viruses that spread via mucosal transmission and replicate for short periods, EBV operates in both a lytic phase and a latent phase(Tsurumi et al., 2005). In the lytic phase, the virus replicates and spreads through high-level viral gene expression and release(Kenney, 2007). In the latent phase, the virus remains within host cells, expressing a limited set of latent-associated genes to maintain infection while avoiding detection and clearance by immune cells(Chen et al., 2022). EBV is not just an environmental exposure factor but manifests as a long-duration, low-level yet persistent chronic inflammatory microenvironment in certain patients(Thorley-Lawson, 2015). For example, EBV can establish long-term latent infection in host B cell populations. Upon entering B cells, it induces a state of continuous survival and proliferative advantage, allowing these cells to escape the normal immune system's removal of abnormal B cells. The pathology of MS is associated with a chronic inflammatory microenvironment formed at the boundaries of the CNS, characterized by sustained presence, not transient dissipative inflammatory responses. Studies have observed "germinal center-like aggregates," which are follicular structures enriched with B cells and plasma cells(Roszkowska et al., 2021). These structures can provide antigen presentation locally and maintain continuous cytokine signaling, further supporting the existence of a persistent inflammatory environment within the CNS.

1.2.2 EBV's Role in B Cell Activation and Immune Reprogramming

During its latent phase, EBV is not passively dormant but maintains infection through a set of latent-associated genes while simultaneously reprogramming B cell signaling and immune functions(Jin et al., 2023). Several key molecules play vital roles in the interaction between the virus and the host during EBV-mediated B cell reprogramming(Cao et al., 2021). LMP1 is a downstream signal mimicking the host CD40 receptor(Brown et al., 2001). The CD40-CD40L axis in normal immunity mediates T cell help, promoting B cell activation and antibody maturation(Elgueta et al., 2009). However, sustained expression of LMP1 can regulate B cells to remain in an activated state even in the absence of physiological T cell co-stimulation(Chen & Flies, 2013). LMP1 stimulation leads to an abnormal survival advantage for B cells, enhancing their antigen presentation capabilities and causing a tendency for continuous pro-inflammatory cytokine release(Wu et al., 2021). LMP1 also upregulates several host pro-inflammatory signaling pathways, such as NF-κB and STAT3(Huang et al., 2024). In MS, B cells are not merely passive participants in the immune response but actively drive inflammation progression and expansion.

EBNA1 and EBNA2 regulate the gene expression profiles of host B cells at the transcriptional level(Hertle et al., 2009). EBNA2 is a viral replication maintenance factor, and it also directly intervenes in the host's transcriptional network, allowing B cells to remain immunologically active even in the absence of external antigenic stimulation(Di Pietro, 2020). BZLF1 is a regulatory molecule during EBV's lytic phase, involved in the transition of the virus from latency to the lytic phase(Murata et al., 2021). Expression of BZLF1 marks the shift from a latent to an active replicative state, where the virus begins extensive replication and prepares to spread(Murata & Tsurumi, 2014). Even without fully entering the lytic replication cycle, BZLF1 can push infected B cells toward a more aggressive immune phenotype(Cheng, 2025). EBV-infected B cells are no longer entirely subject to the original immune regulation, and by providing B cells with a continuously activated phenotype, EBV enhances their ability to sustain inflammation in the CNS boundary regions.

ADAR1 (adenosine deaminase acting on RNA 1) is an RNA-editing enzyme that plays a regulatory role in innate immune recognition and amplification of intracellular inflammatory signals(Wang et al., 2017). Upregulation of AICDA (activation-induced cytidine deaminase) is a marker of B cells in a highly immunologically active reprogramming state(Cantaert et al., 2015). AICDA is required for B cells during maturation

and class switching, further exacerbating the local immune response(Xu et al., 2007). By molecularly reprogramming B cells, EBV maintains their activity and interaction with the host immune system even in the absence of external antigenic stimuli, thus sustaining chronic local immune responses and leading to structural damage and functional loss within the CNS.

1.3 Bruton's Tyrosine Kinase Inhibitors (BTKis)

BTK is a core non-receptor tyrosine kinase in the B cell receptor (BCR) signaling pathway(Pal Singh et al., 2018). Upon antigen recognition, BTK helps immune cells respond by initiating a series of intracellular signaling processes(Ye et al., 2019). BTK regulates the activation of B cells and also modulates immune responses in microglia and macrophages(Gruber et al., 2024). As a key modulator of adaptive immunity, BTK also plays a role in innate immune responses, leading to the proposal of BTK inhibitors (BTKis) as potential disease-modifying therapeutic strategies for autoimmune diseases and hematologic malignancies.

1.3.1 Mechanism of Action of BTKis

BTKis are targeted drugs that regulate immune responses by inhibiting BTK activity(Palma et al., 2021). BTK, as a central tyrosine kinase in the BCR signaling pathway, is involved in immune cell activation and signal transduction, playing an essential role in B cell activation and proliferation(Pal Singh et al., 2018) BTKis inhibit the tyrosine kinase activity of BTK, blocking the upstream and downstream signaling of BCR in immune cells, thereby modulating immune responses and reducing B cell activation and proliferation(Wang et al., 2022).BTKis inhibit the tyrosine kinase activity of BTK, blocking the upstream and downstream signaling of BCR in immune cells, thereby modulating immune responses and reducing B cell activation and proliferation(Wang et al., 2022). This mechanism has been validated in diseases such as rheumatoid arthritis and systemic lupus erythematosus, where it effectively diminishes the function of aberrant B cells and reduces immune system attacks on self-tissues.

BTK plays a critical role in various immune cells, especially in the functional regulation of microglia, macrophages, and T cells(Bassani et al., 2025). In microglia and macrophages, BTK activity is involved in their activation and pro-inflammatory responses(Li, 2023). By inhibiting BTK activity, BTKis can effectively reduce cell activation, alleviating inflammation in the nervous system and reducing immune-mediated neurodegeneration.

BTK also plays a key role in immune cell chemotaxis and migration(Buggy & Elias, 2012). Immune cells rely on BTK-mediated signaling to accumulate in areas of inflammation. BTKis inhibit immune cell chemotaxis, reducing their accumulation at inflammatory sites and decreasing the intensity of local inflammation(Rip et al., 2019). In T cells, BTK primarily participates in immune responses by regulating activation and cytokine secretion(Corneth et al., 2015). BTKis reduce T cell activation levels and cytokine secretion, thus alleviating T cell-mediated immune responses and inhibiting the onset of autoimmune diseases. In natural killer (NK) cells and dendritic cells (DCs), BTKis inhibit BTK activity, lowering their contribution to immune responses and effectively modulating the immune system's response, reducing chronic inflammation and immune-mediated tissue damage(Palma et al., 2021).

1.3.2 Clinical Applications of BTKis

As targeted therapeutic agents, BTKis have demonstrated significant therapeutic potential in various immune-related diseases and hematologic malignancies in recent years(Olejarz & Basak, 2023). By selectively inhibiting BTK function, BTKis block the overactivation of B cells and other immune cells, thereby modulating immune responses and reducing diseases caused by immune system overreaction. Below are the clinical trial phases and applications of four BTKis:

Evobrutinib is a selective BTKi primarily used for treating MS(Prajjwal et al., 2025). In phase III clinical trials, Evobrutinib did not significantly reduce the annual relapse rate (ARR) in patients with relapsing forms of MS (RMS)(Papasouliotis et al., 2022), but it showed some efficacy in reducing new gadolinium-enhanced T1 lesions and improving imaging findings(Gkotsis et al., 2025). Despite not meeting the primary endpoint in phase III trials, Evobrutinib is still undergoing long-term follow-up studies to explore its role in controlling disease activity.

Tolebrutinib is a BTKi used primarily for MS treatment(Orlandi & Gajofatto, 2022). In the phase III HERCULES trial, it significantly delayed confirmed disability progression (CDP) in patients with non-relapsing secondary progressive MS (nrSPMS), becoming the first BTKi to achieve positive results in this MS subtype(Fox et al., 2025). Based on these trial results, Tolebrutinib has received breakthrough therapy designation from the U.S. FDA and is currently under regulatory review(Wen et al., 2021).

Fenebrutinib is a non-selective BTKi currently undergoing phase III clinical trials(Rozkiewicz et al., 2023). The FENhance 1 and FENhance 2 trials are assessing the

efficacy of Fenebrutinib versus Teriflunomide in patients with RMS, while the FENtrepid trial is evaluating its effects on primary progressive MS (PPMS)(Meglio, 2023). Fenebrutinib has demonstrated good efficacy in clinical trials, with preliminary results expected by the end of 2025(Bar-Or et al., 2025).

Orelabrutinib is an oral BTKi primarily used for treating hematologic malignancies, such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL)(Dhillon, 2021). In a phase II trial, Orelabrutinib showed significant efficacy in patients with RMS(Sheng et al., 2025), particularly in reducing new gadolinium-enhanced T1 lesions(Sheng et al., 2025).

1.4 Limitations of Current Treatments and Scientific Gaps

1.4.1 Evolution of Treatment Goals for Multiple Sclerosis

The treatment goals for MS have evolved from initially controlling acute relapses to now focusing on delaying the accumulation of long-term disability(Kuhlmann et al., 2023). Early treatment efforts primarily focused on controlling the frequency and severity of clinical relapses and reducing the formation of new lesions, especially those visible on MRI, such as new gadolinium-enhanced T1 lesions and new or enlarged T2 lesions. Since these indicators can be quantified within a short time frame and have a significant positive correlation with disease progression, they have long been considered core evidence for evaluating the biological activity of drugs.

With advances in research, the treatment goals have gradually shifted from preventing the next relapse to delaying irreversible structural and functional decline. In cases where clinical relapse rates decrease and the number of MRI activity lesions diminishes, some patients still experience sustained and irreversible neurological decline, manifested as reduced walking distance, loss of balance, fine motor impairment in the upper limbs, and persistent fatigue, all of which are core features of SPMS and PPMS disease courses. Therefore, merely suppressing acute inflammation is insufficient to prevent the accumulation of long-term functional disability. Medications must effectively suppress active inflammation in the CNS and demonstrate long-term safety and tolerability to ensure patients can continue treatment for many years, thus influencing the trajectory of long-term disability. This dual standard has become the key framework for assessing the efficacy of disease-modifying treatments (DMTs).

1.4.2 Limitations of Current Treatment Strategies

Current immunomodulatory and immunosuppressive treatment strategies have made some progress in improving the short- or medium-term prognosis of MS patients, especially those with RRMS(Lugaresi et al., 2013). However, they still have limitations, particularly in long-term management and application during the chronic stage. Current treatments mainly include broad-spectrum or relatively broad-spectrum immunosuppressive agents, drugs that regulate lymphocyte subpopulation migration, biologics targeting specific B cell populations, and other oral or injectable medications targeting specific molecular sites(Rossi, 2015). While these treatments can effectively reduce relapses and new lesion formation, some strategies rely on deep systemic immune suppression. Although this approach can reduce relapses and the number of new lesions, it often weakens the host's immune defense, increasing the risk of infections and potentially inducing severe adverse events, such as malignancies. For MS, particularly in patients diagnosed between the ages of 20 and 40, excessive systemic immunosuppression can affect their quality of life and may cause long-term sustainable treatment issues(Macaron et al., 2023). In SPMS and PPMS patients, local accumulation of immune cells and stable follicular-like structures make the immune microenvironment relatively independent, relying less on the recruitment of peripheral immune cells. In these cases, while the drug may show significant effects in suppressing acute relapses, its ability to modify long-term neurodegenerative changes may still be limited.

Some drugs inhibit the migration of immune cells, preventing them from entering the CNS from the periphery(Mrass & Weninger, 2006). While these strategies can significantly reduce the formation of active lesions and show efficacy from an imaging perspective, they mainly block immune cell arrival rather than directly intervening in the immune ecosystem that drives inflammation. Therefore, it remains unclear whether the current state will be maintained once drug exposure is reduced or discontinued. Depletion therapies targeting B cell populations, such as monoclonal antibody-based depletion of specific B cell subgroups, have proven that B cells play a crucial role in the active inflammation of MS(Lee et al., 2021). However, these treatments often involve comprehensive depletion, which, while effective, may impact immune reconstitution and long-term issues such as vaccine response. For patients requiring long-term treatment and maintaining social and professional functionality, balancing sufficient pathological suppression with acceptable immune system integrity remains an unresolved challenge.

1.4.3 Gaps in Immunological Target Selection

A core issue in MS treatment for many years has been the lack of a therapeutic approach that can precisely regulate the pathogenic B cell phenotype, rather than simply depleting B cell populations. Existing clinical strategies typically use bypass or depletion methods, either by inhibiting B cell migration or removing specific B cell subgroups(Lee et al., 2021). However, these approaches fail to finely modulate the pathological activation of B cells themselves.

The pathogenic B cell phenotype, especially in chronic inflammation, is considered a key driver of MS disease progression due to the long-term effects of EBV. EBV induces B cells to enter a hyperactivated state through several mechanisms, such as LMP1 mimicking CD40-like co-stimulation signals and EBNA2 modulating the host transcriptional network. This hyperactive B cell phenotype plays a crucial role in the chronic inflammatory ecosystem of MS, particularly within follicular-like structures in the submeningeal and near-cortical regions.

Current treatment strategies have failed to address this issue, as most rely on deep depletion of B cell subgroups. These approaches have not directly modulated the functional B cell phenotype. The emergence of BTKis provides a new solution. As an essential tyrosine kinase in the BCR signaling pathway, BTK not only regulates B cell survival and differentiation but also modulates its antigen presentation function and pro-inflammatory signal generation in immune responses. BTKis can directly intervene in the B cell phenotype activated by EBV, inhibiting its pro-inflammatory output, and may also regulate the function of myeloid cells, further suppressing chronic inflammation. This dual action mechanism makes BTKis a potential breakthrough target in MS treatment, offering new therapeutic ideas and filling existing gaps in treatment, especially for use in chronic and progressive disease stages.

1.5 Research Objectives and Hypotheses

1.5.1 Overall Objective

The overarching objective is to delineate the potential disease-modifying effects of BTKis in MS. Beyond short-term suppression of inflammatory activity, the study aims to build a causally interpretable chain across three levels:

Clinical level: assess whether BTKis reduce active inflammation in the CNS of patients with MS, focusing on new T1 Gd+ lesions, new or enlarging T2 lesions, and relapse-related outcomes.

Safety and sustainability: determine whether BTKis show acceptable tolerability when clinical and imaging outcomes improve, with no significant differences from control in the overall incidence of common adverse events (e.g., headache, liver enzyme elevation, upper respiratory symptoms). This level evaluates feasibility for long-term management rather than short, intensive interventions only.

Mechanistic level: test whether BTKis directly attenuate EBV-mediated B-cell activity, thereby suppressing the pathogenic B-cell phenotype and its pro-inflammatory crosstalk with myeloid cells. This will be verified in vitro using PBMCs from patients with MS. After EBV infection and induction of LCLs, the effects of Evobrutinib, Tolebrutinib, Orelabrutinib, and Fenebrutinib on key molecular networks will be compared.

The central question is whether BTKis can serve as effective disease-modifying strategies for MS, rather than functioning solely as short-term anti-inflammatory agents.

1.5.2 Specific Aims

Aim 1: Synthesize RCT evidence to evaluate the clinical efficacy and safety of BTKis in MS.

This aim will quantify effects on new T1 Gd+ lesions, new or enlarging T2 lesions, and relapse-related outcomes to answer whether BTKis suppress new inflammatory events and their structural sequelae on imaging. Safety analyses will compare the overall incidence of common adverse events with control to test tolerability during prolonged use. Dose–response will be assessed to determine whether inhibition of new T1 Gd+ lesions differs across dose levels.

Aim 2: Define the molecular mechanisms of BTKis within the patient-derived immune context and characterize their intervention profile on the EBV–B-cell–inflammation axis.

This aim will test whether BTKis downregulate EBV-driven pathogenic B-cell phenotypes to suppress the chronic inflammatory microenvironment in MS. PBMCs will be infected with EBV to generate LCLs and then treated with different BTKis. After 48 hours, cells will be harvested for qPCR and WB to evaluate effects on EBV-related molecules (e.g., EBNA1, EBNA2, LMP1, BZLF1) and host pro-inflammatory/co-stimulatory signaling molecules (e.g., CD40, NF-κB, STAT3, ADAR1, AICDA). Quantitative comparisons will

test multi-target downregulation and potential differences in breadth and potency across BTKis, informing individualized therapy.

1.5.3 Hypotheses

Hypothesis 1: BTKis significantly reduce imaging markers of active CNS inflammation in MS—new T1 Gd+ lesions and new or enlarging T2 lesions—and lower the risk of relapse-related outcomes. The effect is dose dependent, with higher doses showing greater improvements, suggesting a pharmacodynamic threshold.

Hypothesis 2: Given efficacy, BTKis meet long-term safety requirements, with the overall incidence of common adverse events (e.g., headache, liver enzyme elevation, rhinitis-like symptoms) not significantly higher than control, supporting feasibility for chronic use.

Hypothesis 3: The action of BTKis extends beyond simple BCR blockade. Through multi-node downregulation along the EBV–B-cell–inflammation axis, BTKis jointly suppress EBV-driven pathogenic B-cell phenotypes and their pro-inflammatory interactions with myeloid cells, thereby intervening in the chronic inflammatory milieu of MS.

Hypothesis 4: BTKis differ in their inhibitory profiles on the EBV–B-cell–inflammation axis, and these differences correlate with clinical trial outcomes such as MRI activity measures and relapse metrics. Such pharmacological distinctions may guide personalized treatment and inform future therapeutic choices.

CHAPTER 2.

The effects and side effects of Bruton's tyrosine kinase inhibitors for the treatment of multiple sclerosis patients: a systematic review and meta-analysis of clinical trials

2.1 Introduction

MS is a complex autoimmune disease that affects the CNS(Hafler & Weiner, 1989). Current therapies slow disease progression through immunomodulation, with notable benefits in RRMS(Piehl, 2021). However, treatment for SPMS and PPMS remains limited(Orbach et al., 2012). Many agents do not effectively prevent neurodegeneration, and numerous regimens are injectable, which challenges adherence.

As MS therapeutics evolve, BTKis have gained attention as oral immunomodulators. By inhibiting BTK, they regulate B-cell and myeloid-cell activity and show potential to suppress inflammation and mitigate neural injury(Bassani et al., 2025). BTKis not only reduce CNS inflammatory activity in MS but may also slow or prevent neurodegeneration by modulating B-cell and microglial function.

Some BTKis (e.g., Evobrutinib, Tolebrutinib, Fenebrutinib) are in clinical development and have shown efficacy (Rozkiewicz et al., 2023), particularly in reducing new T1 Gd+

lesions and T2 lesions. Nonetheless, long-term efficacy and safety remain debated, especially in progressive MS(Jakimovski et al., 2020). This study will conduct a systematic review and meta-analysis to evaluate the clinical effectiveness of BTKis, focusing on imaging outcomes (T1 Gd+ and T2 lesions) and relapse rates. It will also examine adverse events, and the influence of dose and treatment duration, to inform individualized care.

2.2 Research Question Definition

This chapter systematically evaluates placebo-controlled randomized controlled trials (RCTs) to define the clinical effects and safety of BTKis in MS. Specifically, it addresses three core questions:

Inflammatory activity: Do BTKis effectively suppress active CNS inflammation in MS? MRI T1 Gd+ lesions and T2 lesions are key markers of disease activity. We will test whether BTKis significantly reduce the number of new T1 Gd+ lesions and new or enlarging T2 lesions, thereby attenuating disease activity.

Clinical relapses: Do BTKis significantly lower the risk or frequency of clinical relapses? We will assess whether suppression observed on imaging translates into measurable clinical benefit.

Safety profile: Are BTKis tolerable for long-term use? We will evaluate whether treatment introduces a significant increase in adverse events and assess long-term safety and feasibility.

Answers to these questions will determine whether BTKis have the potential to serve as long-term management strategies in MS, particularly by reducing CNS inflammatory activity, lowering relapse rates, and maintaining an acceptable safety profile.

2.3 Methods

This study is a systematic review and meta-analysis evaluating the efficacy and safety of BTKis in MS. The design follows Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Parums, 2021)and prespecifies methodological constraints

across four domains—evidence sources, outcome definitions, data extraction and quality control, and statistical procedures—to ensure reproducibility, auditability, and interpretability.

2.3.1 Eligibility Criteria

To test, under strictly randomized, blinded, placebo-controlled conditions, whether BTKis show features consistent with disease-modifying therapy candidates—namely suppression of active inflammation, reduction in relapse, and acceptable tolerability and safety—only placebo-controlled RCTs were eligible. Non-randomized designs, open-label single-arm studies, observational cohorts, case series, pharmacokinetic/pharmacodynamic studies, and in vitro or animal mechanism studies were excluded from quantitative synthesis. This avoids basing inference on materials highly susceptible to selection bias in MS drug research (e.g., preferential allocation of highly active disease to experimental agents or physician tailoring based on prior response)(Ye & Nie, 2024). It also improves regulatory readability, as placebo-controlled RCTs are the evidence form most accepted by clinicians and regulators when assessing oral immunomodulators intended for long-term use.

Efficacy outcomes comprised three categories: (i) new gadolinium-enhancing T1 lesions (T1 Gd+), reflecting recent blood–brain barrier disruption and active CNS inflammation; (ii) new or enlarging T2 lesions (T2 lesions), reflecting the rate of new demyelination and local expansion of prior lesions; and (iii) relapse-related outcomes, including ARR or risk of relapse, representing clinically perceptible neurological worsening. T1 Gd+ indexes recent inflammatory events; T2 lesions index structural sequelae of inflammation; relapse indexes translation into clinical signs and symptoms. Safety outcomes included any adverse event, common specified events (e.g., headache, transaminase elevation, rhinitis-like upper respiratory symptoms), and serious adverse events. Together, these outcomes cover the trajectory from active immune attack to imaging-detectable tissue injury and functional deterioration.

All included studies underwent structured extraction of: participant baseline characteristics (age, sex distribution, baseline disease activity); trial design features (randomization, blinding, follow-up duration, placebo control); intervention details (BTKi agent, dose, dosing schedule); primary efficacy endpoints (new T1 Gd+, new/enlarging T2 lesions, relapse metrics); and safety endpoints (rates of any and common specified adverse

events). Two reviewers extracted data independently with adjudication by a third reviewer to reduce selection bias, consistent with high-quality systematic review practice.

Risk of bias was assessed for each trial, including adequacy of random sequence generation and allocation concealment; blinding of participants, investigators, and outcome assessors; whether MRI endpoints were centrally read under blinded conditions; handling of withdrawals and loss to follow-up; and selective reporting. Notably, MRI endpoints in MS RCTs—especially new T1 Gd+ and new/enlarging T2 lesions—are commonly determined by centralized, blinded reading, which lowers observer bias and strengthens cross-trial comparability for pooled analyses.

2.3.2 Data Synthesis and Statistical Analysis

All placebo-controlled RCTs meeting criteria were synthesized quantitatively for prespecified endpoints: imaging activity, relapse outcomes, and safety.

2.3.2.1 Data Extraction and Missing Data

From each RCT we extracted, for treatment and control groups, means, standard deviations (SDs), and sample sizes (or event counts, as applicable). Imaging endpoints included T1 Gd+ and new/enlarging T2 lesions; clinical endpoints included relapse occurrence; safety endpoints included common and serious adverse events. Some studies (e.g., Amit 2024; Jens 2021; Daniel 2021) did not report SDs for specific outcomes. To avoid excluding these trials, missing SDs were imputed using the Bracken 1992 method(Bracken, 1992), which estimates SDs from coefficients of variation (CVs) derived from fully reported studies. SD imputation was implemented with the metagear package to maximize comparability and retain sample information.

2.3.2.2 Effect Measures and Computation

To enable pooling across heterogeneous measurement scales and time windows, continuous imaging outcomes (e.g., counts of new T1 Gd+ or new/enlarging T2 lesions) were synthesized as standardized mean differences (SMDs), rather than weighted mean differences. This standardization accommodates differences such as per-patient vs per-interval lesion counts. The pooled overall treatment effect is reported as an effect size (ES). For continuous outcomes, ES corresponds to the pooled SMD; for count/rate or dichotomous outcomes (e.g., relapse, adverse events), ES corresponds to the prespecified pooled estimate for that endpoint type.

For dichotomous outcomes (e.g., relapse occurrence; specific adverse events), relative risks (RRs) with 95% confidence intervals (CIs) were calculated to compare event rates between BTKi and control groups. All effect estimates are reported with two-sided 95% CIs.

2.3.2.3 Model Selection: Fixed vs Random Effects

Both fixed-effects and random-effects models were applied, with selection guided by heterogeneity. When trials were highly consistent in outcome definitions, observation windows, and dosing schemes and showed low between-study heterogeneity, a fixed-effects model was used to obtain precise estimates under high concordance. When clinical or methodological differences were present (e.g., dose, trial duration, enrolled population) or when statistical heterogeneity was evident, a DerSimonian–Laird inverse-variance weighted random-effects model was used to account for between-study variance and produce pooled effects. This dual-model strategy aligns with common practice in drug-intervention meta-analyses, especially when early-phase trials are relatively small and not fully homogeneous.

2.3.2.4 Assessment of Heterogeneity

Between-study heterogeneity was tested with Cochran's Q and quantified with I^2. Q evaluates whether the observed dispersion exceeds that expected by sampling error; I^2 indicates the proportion of total variability attributable to true between-study differences rather than chance. I^2 at or below ~50% was treated as low heterogeneity, favoring fixed effects; clearly elevated I^2 (typically >50%) indicated substantial heterogeneity, favoring random effects.

2.3.2.5 Sensitivity Analysis

To assess dependence on any single study, leave-one-out analysis was prespecified. Each trial was excluded in turn, pooled effect sizes (SMD or RR, per endpoint type) were recalculated, and shifts in the overall estimate were examined. This procedure evaluates robustness and identifies influential studies.

2.3.2.6 Subgroup Analyses

To explore sources of heterogeneity and clinically informative differences, the following subgroup analyses were conducted:

Dose strata. Within a molecule or trial, dosing arms were split into lower vs higher dose categories, and effects were estimated separately to assess dose–response and define

effective windows within safety bounds. Multi-dose arms were treated as distinct subgroups rather than combined.

Treatment duration. Trials or trial segments were classified as relatively short vs relatively long exposure to evaluate time-on-treatment effects.

BTKi molecule. Analyses were stratified by specific BTKi to examine systematic differences across agents on the same endpoints.

2.3.2.7 Meta-regression Analysis

To further explore sources of between-study heterogeneity, we performed meta-regression. The primary objective was to assess the influence of the following study-level covariates on treatment effect:

BTKi dose: effect of different dosing levels;

Treatment duration: effect of exposure time;

BTKi type: effect differences among agents (e.g., Evobrutinib, Tolebrutinib, Fenebrutinib).

Meta-regression was used to explore systematic contributors to heterogeneity rather than to support causal inference. The dependent variable was the effect size (ES; SMD or RR, as appropriate), modeled on the covariates listed above.

2.3.2.8 Publication Bias

Publication bias was assessed via visual inspection of funnel plots and Egger's regression test. Funnel-plot asymmetry was used to screen for small-study effects; Egger's test quantified asymmetry. A significant Egger result was interpreted as suggestive of publication or small-study bias and considered in result interpretation.

2.3.2.9 Statistical Software

All meta-analyses, subgroup and sensitivity analyses, and regressions were conducted in prespecified environments. Continuous endpoints (SMD/ES), SD imputation, and effect extraction were implemented in RStudio (including metagear for SD estimation). Dichotomous endpoints (RR), fixed- and random-effects fitting, heterogeneity statistics, Egger's test, and funnel plots were conducted in Stata 15.1.

2.3.3 Literature Search Strategy

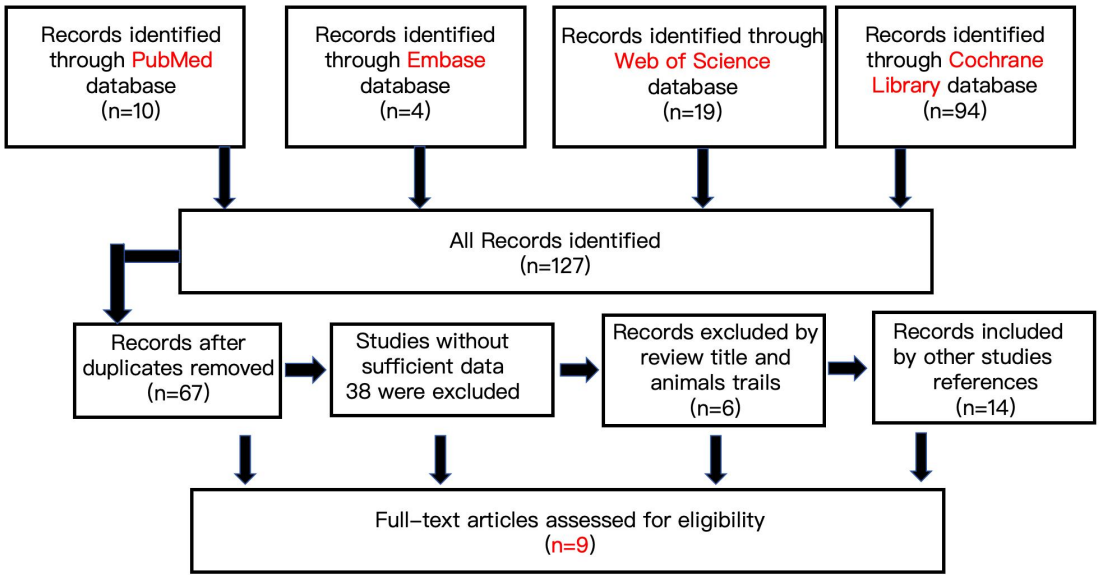

Figure 1. PRISMA flow diagram for study selection.

The search strategy sought to systematically and reproducibly identify all publicly available randomized, placebo-controlled clinical trials (RCTs) of BTKis in MS. The workflow specified sources, keyword architecture, time window, de-duplication, and eligibility criteria, and followed PRISMA requirements for traceability.

To maximize retrieval, we conducted parallel searches across multiple databases and complemented these with gray literature, conference abstracts, and clinical trial registry records to reduce publication bias and avoid reliance on formally published positive results only. Four international databases were searched—PubMed, Embase, Web of Science, and the Cochrane Library—from database inception to the data-lock (January 2025). This window was chosen to capture evidence across the clinical development of BTKis in MS, particularly interim and final reports of phase II and phase III placebo-controlled trials.

2.3.3.1 Search Details

In accordance with PRISMA, the following strategies were applied:

PubMed/MEDLINE: combined subject headings and title/abstract terms for “Multiple Sclerosis” and related variants with terms related to “Bruton’s Tyrosine Kinase”.(**Table 1**)

Web of Science: applied the same topics/keywords and limited results to randomized controlled trials.(**Table 2**)

Cochrane Library: searched “Multiple Sclerosis,” “Bruton’s Tyrosine Kinase,” and RCTs to ensure coverage of registered trials and systematic reviews.(**Table 3**)

Embase: used the terms “multiple sclerosis” and “tyrosine kinase,” combined with an RCT filter.(**Table 4**)

2.3.3.2 Supplementary Searches

To mitigate publication bias, supplementary searches covered gray literature, conference abstracts, and clinical trial registries. This approach ensured inclusion of relevant studies not yet formally published. These searches yielded 15 additional records that were incorporated into subsequent screening and analysis.

Table 1: The complete search strategy for Pubmed

Search	Query	Items found
#1	Search: ("Multiple Sclerosis"[Mesh]) OR (((((Sclerosis, Multiple[Title/Abstract]) OR (MS (Multiple Sclerosis[Title/Abstract]))) OR (Sclerosis, Disseminated[Title/Abstract])) OR (Disseminated Sclerosis[Title/Abstract])) OR (Multiple Sclerosis, Acute Fulminating[Title/Abstract]))	87,700
#2	Search: ("Agammaglobulinaemia Tyrosine Kinase"[Mesh]) OR (((((((Kinase, Agammaglobulinaemia Tyrosine[Title/Abstract]) OR (Tyrosine Kinase, Agammaglobulinaemia[Title/Abstract])) OR (B Cell Progenitor Kinase[Title/Abstract])) OR (Bruton's Tyrosine Kinase[Title/Abstract])) OR (Brutons Tyrosine Kinase[Title/Abstract])) OR (Bruton Tyrosine Kinase[Title/Abstract])) OR (Kinase,	4,501

	Bruton's Tyrosine[Title/Abstract])) OR (Tyrosine Kinase, Bruton's[Title/Abstract]))	
#3	Search:randomized controlled trial[Publication Type] OR randomized[Title/Abstract] OR placebo[Title/Abstract]	249
#4	#1 AND #2 AND #3	10

Table 2: The complete search strategy for Web of science

Search	Query	Items found
#1	TS=(Multiple sclerosis OR Sclerosis, Multiple OR MS (Multiple Sclerosis) OR Sclerosis, Disseminated OR Disseminated Sclerosis OR Multiple Sclerosis, Acute Fulminating)	160,646
#2	TS=(Agammaglobulinaemia Tyrosine Kinase OR Kinase, Agammaglobulinaemia Tyrosine OR Tyrosine Kinase, Agammaglobulinaemia OR B Cell Progenitor Kinase OR Bruton's Tyrosine Kinase OR Brutons Tyrosine Kinase OR Bruton Tyrosine Kinase OR Kinase, Bruton's Tyrosine OR Tyrosine Kinase, Bruton's)	8,228
#3	TS=(randomized controlled trial OR randomized OR placeb OR RCT)	1,187,658
#4	#1 AND #2 AND #3	19

Table 3: The complete search strategy for Cochrane library

Search	Query	Items found
#1	Multiple sclerosis	14190

#2	(Sclerosis, Multiple):ab,ti,kw OR (MS (Multiple Sclerosis)):ab,ti,kw OR (Sclerosis, Disseminated):ab,ti,kw OR (Disseminated Sclerosis):ab,ti,kw OR (Multiple Sclerosis, Acute Fulminating):ab,ti,kw	13520
#3	#1 OR #2	14199
#4	Agammaglobulinaemia Tyrosine Kinase	54
#5	(Kinase, Agammaglobulinaemia Tyrosine):ab,ti,kw OR (Tyrosine Kinase, Agammaglobulinaemia):ab,ti,kw OR (B Cell Progenitor Kinase):ab,ti,kw OR (Bruton's Tyrosine Kinase):ab,ti,kw OR (Brutons Tyrosine Kinase):ab,ti,kw OR (Bruton Tyrosine Kinase):ab,ti,kw OR (Kinase, Bruton's Tyrosine):ab,ti,kw OR (Tyrosine Kinase, Bruton's):ab,ti,kw	676
#6	#4 OR #5	676
#7	(Tyrosine Kinase, Bruton's):ab,ti,kw OR (randomized controlled trial):ab,ti,kw OR (randomized):ab,ti,kw OR (placeb):ab,ti,kw OR (RCT):ab,ti,kw	1234926
#8	#3 AND #6 AND #7	94

Table 4: The complete search strategy for Embase

Search	Query	Items found
#1	multiple AND ('sclerosis'/exp OR sclerosis)	235,695
#2	'Sclerosis, Multiple':ab,ti OR 'MS (Multiple	1,136

	Sclerosis)':ab,ti OR 'Sclerosis, Disseminated':ab,ti OR 'Disseminated Sclerosis':ab,ti OR 'Multiple Sclerosis, Acute Fulminating':ab,ti	
#3	#1 OR #2	14199
#4	agammaglobulinaemia AND tyrosine AND kinase	83
#5	'Kinase, Agammaglobulinaemia Tyrosine':ab,ti OR 'Tyrosine Kinase, Agammaglobulinaemia':ab,ti OR 'B Cell Progenitor Kinase':ab,ti OR 'Bruton*s Tyrosine Kinase':ab,ti OR 'Brutons Tyrosine Kinase':ab,ti OR 'Bruton Tyrosine Kinase':ab,ti OR 'Kinase, Bruton*s Tyrosine':ab,ti OR 'Tyrosine Kinase, Bruton*s':ab,ti	2,508
#6	#4 OR #5	2,582
#7	'randomized controlled trial ':ab,ti OR 'randomized ':ab,ti OR 'placebo':ab,ti OR 'rct':ab,ti	1,261,402
#8	#3 AND #6 AND #7	4

2.4 Results

2.4.1 Overview of Included Studies

Nine RCTs met the eligibility criteria, all evaluating the efficacy and safety of BTKis in patients with MS. Each trial used a randomized, double-blind, placebo-controlled design, supporting objectivity and internal validity. Owing to differences in treatment regimens, dose selection, and follow-up duration, study characteristics showed heterogeneity (Figure 1).

2.4.1.1 Study Characteristics

Seven studies evaluated distinct BTKis—primarily Evobrutinib, Tolebrutinib, and Fenebrutinib—across trials. Dosing ranged from 5 mg to 200 mg, according to individual trial designs. All studies prespecified follow-up windows, typically 12–24 weeks. Primary

endpoints included imaging measures (new T1 Gd+ lesions; new or enlarging T2 lesions) and clinical outcomes (annualized relapse rate, ARR).

Among the nine RCTs, four were full peer-reviewed articles with detailed methods, analyses, and results; four were conference abstracts providing preliminary data; and one was a registered trial record that, while not reporting all outcomes, supplied key design and results information. Quality appraisal indicated that six included articles achieved the highest rating (Jadad score = 5) (Table 5).

Table 5. Quality of included studies upon on the Jadad scale for randomized control trials studies.

Article	First author	Year	Randomization*	Blinding £	Destination€	Jadad score	Quality
1	Amit	2024	2	2	1	5	High
2	Jens	2021	2	2	1	5	High
3	Daniel	2021	2	2	1	5	High
4	Anthony	2023	2	2	1	5	High
5	Montalban	2019	2	2	1	5	High
6	Montalban	2024	2	2	1	5	High
7	Oh	2024	2	2	1	5	High
8	Guehring	2017	2	2	1	5	High
9	Reich	2021	2	2	1	5	High

In Jadad, scale quality is High if total score was 4-5; Fair if total score was 2-3; Poor if total score was 0-1.

* Deduct 1 point if the study described as randomized and give 1 additional point if the method to generate the sequence of randomization was described and it was appropriate.

£ Deduct 1 point if the study described as double blind and give 1 additional point If the method of double blinding was described and it was appropriate.

€ Deduct 1 point if there was a description of withdrawals and dropouts.

2.4.2 Efficacy Results for BTKis

2.4.2.1 Heterogeneity Test and Analysis for T1 Gd+ Lesions

Based on four publications (pooled as seven dose-defined groups), the meta-analysis yielded I^2 = 53.5%, with an overall heterogeneity test P = 0.044 (Figure 2), indicating moderate heterogeneity. Accordingly, a random-effects model was applied. The DerSimonian–Laird (D+L) pooled SMD was 0.340 (95% CI, 0.108–0.573; P = 0.004), demonstrating a significant overall treatment effect favoring BTKis over placebo. The z statistic was 3.84 (P < 0.001; Figure 3), further confirming the significance of the pooled effect. These results indicate that BTKi therapy significantly reduced the number of new T1 Gd+ lesions.

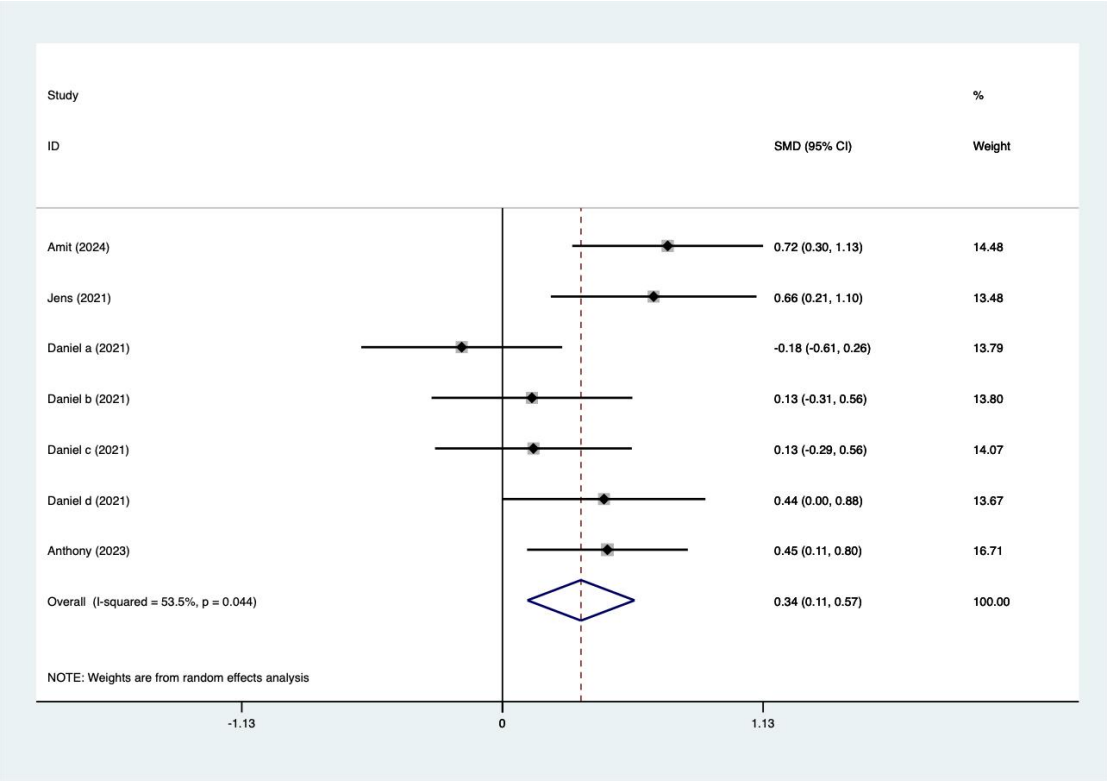

Figure 2. Forest plot of the meta-analysis for T1 Gd+ lesions.

Study	SMD	[95% Conf.	Interval]	% Weight
Amit (2024)	0.716	0.302	1.130	14.48
Jens (2021)	0.655	0.209	1.101	13.48
Daniel a (2021)	-0.177	-0.612	0.259	13.79
Daniel b (2021)	0.128	-0.307	0.563	13.80
Daniel c (2021)	0.134	-0.292	0.561	14.07
Daniel d (2021)	0.440	0.000	0.880	13.67
Anthony (2023)	0.455	0.107	0.803	16.71
D+L pooled SMD	0.340	0.108	0.573	100.00

Heterogeneity chi-squared = 12.92 (d.f. = 6) p = 0.044
I-squared (variation in SMD attributable to heterogeneity) = 53.5%
Estimate of between-study variance Tau-squared = 0.0524

Test of SMD=0 : z= 2.87 p = 0.004

Figure 3. Effect sizes and heterogeneity test results for the meta-analysis of T1 Gd+ lesions.

2.4.2.2 Sensitivity Analysis and Exploration of Heterogeneity Sources for T1 Gd+ Lesions

A sensitivity analysis was conducted on the four publications (grouped into seven arms by BTKi dose) (Figure 4). After excluding each study in turn, the overall combined effect was 0.3404 (95% CI, 0.1083–0.5725), indicating that the pooled effect remained significant and stable, thereby supporting the reliability and consistency of the overall findings.

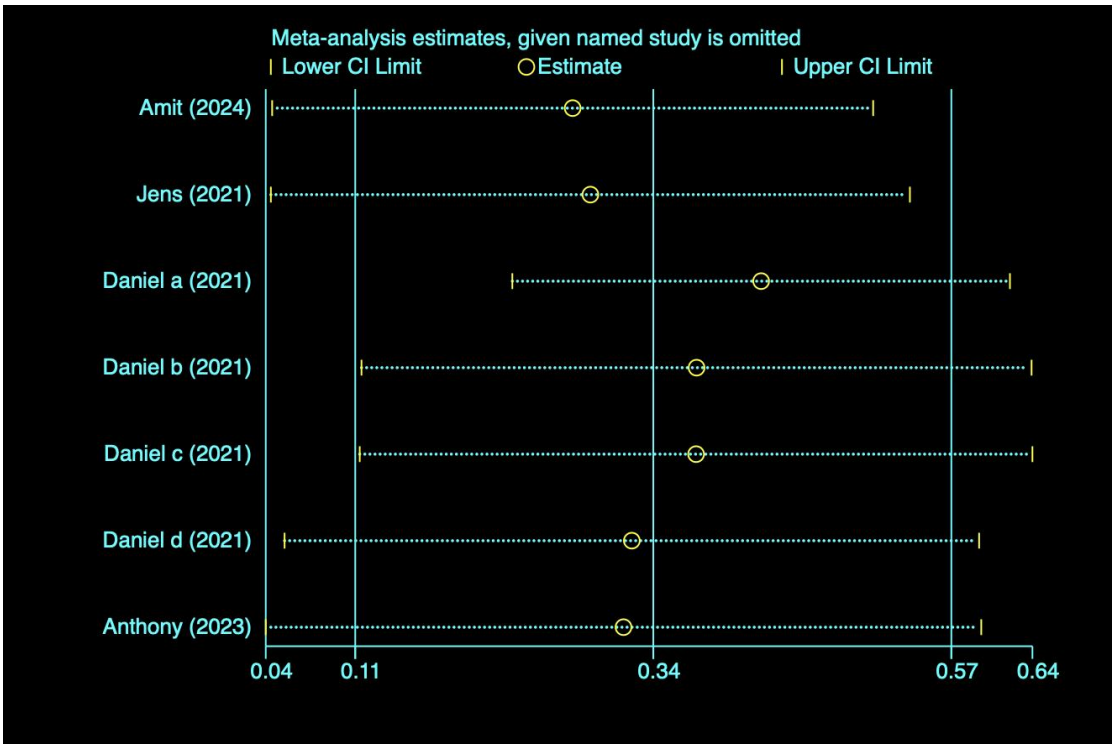

Figure 4. Sensitivity analysis: meta-analytic effect for T1 Gd+ lesions after excluding individual studies.

2.4.2.3 Subgroup Analysis

To identify which factor accounted for heterogeneity—dose, duration, or BTKi type—we conducted meta-regression significance testing. The meta-regression for BTKi dose (Figure 5) yielded a regression coefficient of −0.5259948 ($p = 0.024$; 95% CI, −0.9478765 to −0.1041131), indicating a statistically significant dose effect. The adjusted R^2 was 100%, suggesting that dose explained virtually all variability in effect sizes; this very high value implies that dose was the principal driver of between-study variation. The model estimated $\tau^2 = 0$, indicating minimal residual between-study variance after accounting for dose. Consistently, I^2 was 0%, reflecting very high agreement across studies. In sum, dose had a significant impact on the magnitude of the treatment effect, and the model explained nearly all observed heterogeneity.

```
. metareg _ES dosage, wsse(_seES) bsest(reml)

Meta-regression                                     Number of obs  =       7
REML estimate of between-study variance             tau2           =       0
% residual variation due to heterogeneity           I-squared_res  =   0.00%
Proportion of between-study variance explained      Adj R-squared  = 100.00%
With Knapp-Hartung modification
```

_ES	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
dosage	-.5259948	.1641192	-3.20	0.024	-.9478765	-.1041131
_cons	1.082101	.2430632	4.45	0.007	.4572869	1.706915

Figure 5. Meta-regression results: effect of BTKi dose on effect size.

Duration meta-regression. The meta-regression for BTKi treatment duration (Figure 6) showed a positive trend between duration and effect size, but the association was not statistically significant (p = 0.345). This indicates that treatment duration did not have a statistically significant impact on the effect size.

```
Meta-regression                                     Number of obs  =       7
REML estimate of between-study variance             tau2           =  .05235
% residual variation due to heterogeneity           I-squared_res  =  53.77%
Proportion of between-study variance explained      Adj R-squared  =   1.30%
With Knapp-Hartung modification
```

_ES	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
duration	.3638746	.348856	1.04	0.345	-.5328884	1.260638
_cons	-.0725221	.4134165	-0.18	0.868	-1.135243	.9901989

Figure 6. Meta-regression results: effect of BTKi treatment duration on effect size.

Meta-regression for BTKi type. The analysis (Figure 7) showed that although the regression coefficient for the typesofbtk covariate was negative and approached significance (p = 0.084), the 95% CI included zero. Thus, we cannot conclude that BTKi type has a statistically significant negative effect on the effect size.

```
. metareg _ES typesofbtk, wsse(_seES) bsest(reml)

Meta-regression                                     Number of obs  =       7
REML estimate of between-study variance             tau2           =  .01683
% residual variation due to heterogeneity           I-squared_res  =  26.01%
Proportion of between-study variance explained      Adj R-squared  =  68.26%
With Knapp-Hartung modification
```

_ES	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
typesofbtk	-.2785404	.1291639	-2.16	0.084	-.6105667	.053486
_cons	1.061912	.3463074	3.07	0.028	.1717	1.952123

Figure 7. Meta-regression results: effect of BTKi type on effect size.

In summary, dose had a significant impact on the effect size. We therefore conducted a subgroup meta-analysis to further examine heterogeneity (Figure 8), dividing studies by BTK dosage into a low-dose group (<60 mg QD) and a high-dose group (≥60 mg QD), and included the dose group as a covariate in meta-regression. High-dose group (Group 1): heterogeneity test p = 0.716, I^2 = 0%, indicating high within-group consistency and non-significant heterogeneity; the SMD p = 0.000, showing a significant effect in the high-dose group. Low-dose group (Group 2): heterogeneity test p = 0.525, I^2 = 0%; heterogeneity was likewise non-significant with consistent effects across studies, but the SMD p = 0.813, indicating no statistically significant effect in the low-dose group. Overall: heterogeneity test p = 0.044, I^2 = 53.5%, suggesting between-group heterogeneity and a clear difference across dose strata. These results indicate that the high-dose regimen yields a significant therapeutic effect, whereas the low-dose regimen does not reach statistical significance (Figure 9).

```
Test(s) of heterogeneity:
               Heterogeneity  degrees of
                 statistic     freedom      P    I-squared**   Tau-squared

1                     1.36          3      0.716      0.0%        0.0000
2                     1.29          2      0.525      0.0%        0.0000
Overall              12.92          6      0.044     53.5%        0.0524
** I-squared: the variation in SMD attributable to heterogeneity)

Note: between group heterogeneity not calculated;
only valid with inverse variance method

Significance test(s) of SMD=0

1                     z=  5.37     p = 0.000
2                     z=  0.24     p = 0.813
Overall               z=  2.87     p = 0.004
------------------------------------------------------------------------------
```

Figure 8. Heterogeneity testing by dose group for BTKi treatment effect.

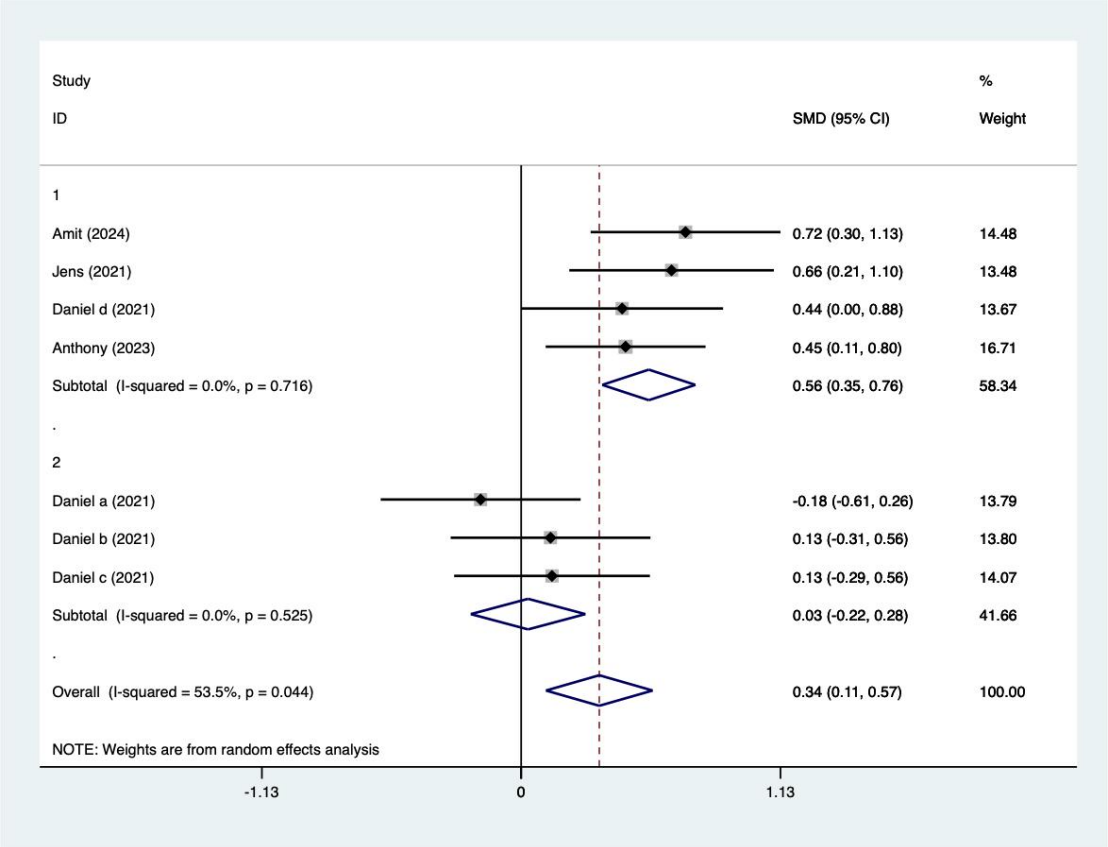

Figure 9. Meta-regression: effect of dose on effect size.

2.4.2.4 Publication Bias Assessment

We assessed publication bias in the overall and subgroup analyses using funnel plots. Visual symmetry generally indicates no publication bias (Figure 10). In the overall analysis (Figure 11), Egger's test yielded P = 0.611 (>0.05), indicating a symmetrical funnel plot and no evidence of publication bias. In the high-dose group (Group 1), P = 0.547 (>0.05), also showing no publication bias; in the low-dose group (Group 2), P = 0.629 (>0.05), likewise indicating no bias. For the dose-stratified set as a whole, P = 0.499 (>0.05), suggesting that none of the strata showed publication bias. Therefore, neither the overall analysis nor the subgroup analyses revealed meaningful bias.

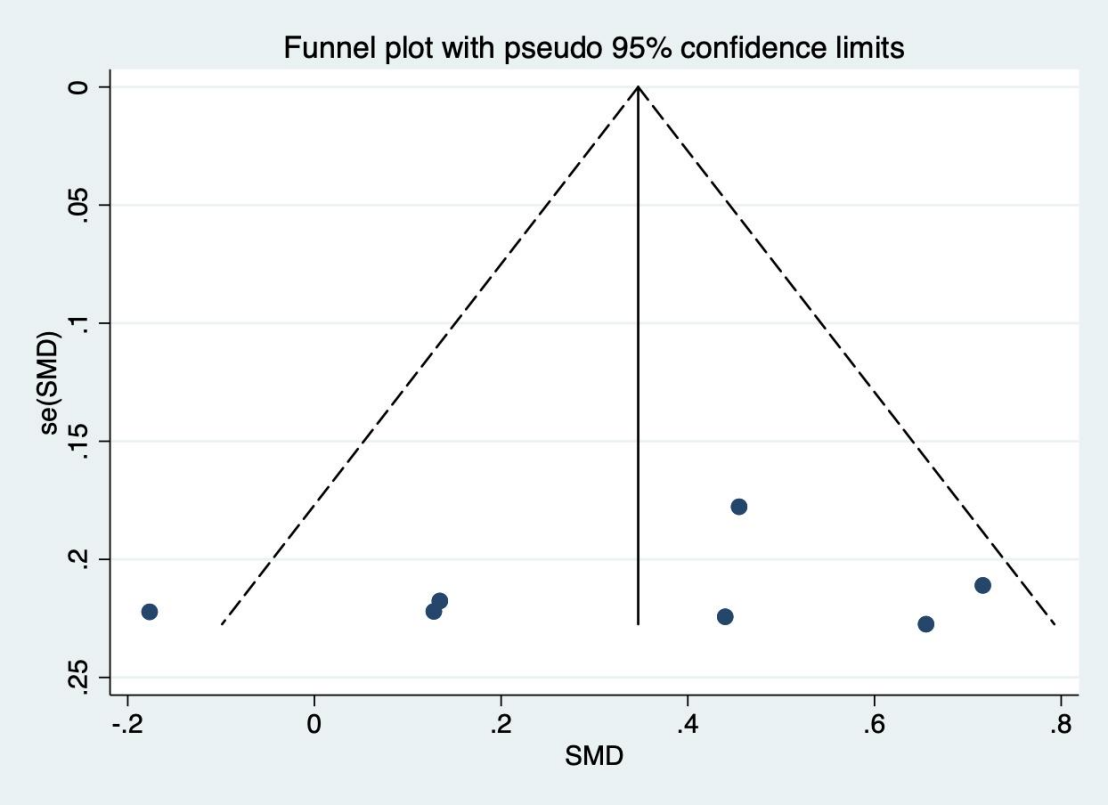

Figure 10. Overall publication bias assessment for T1 Gd+ lesions under BTKi treatment.

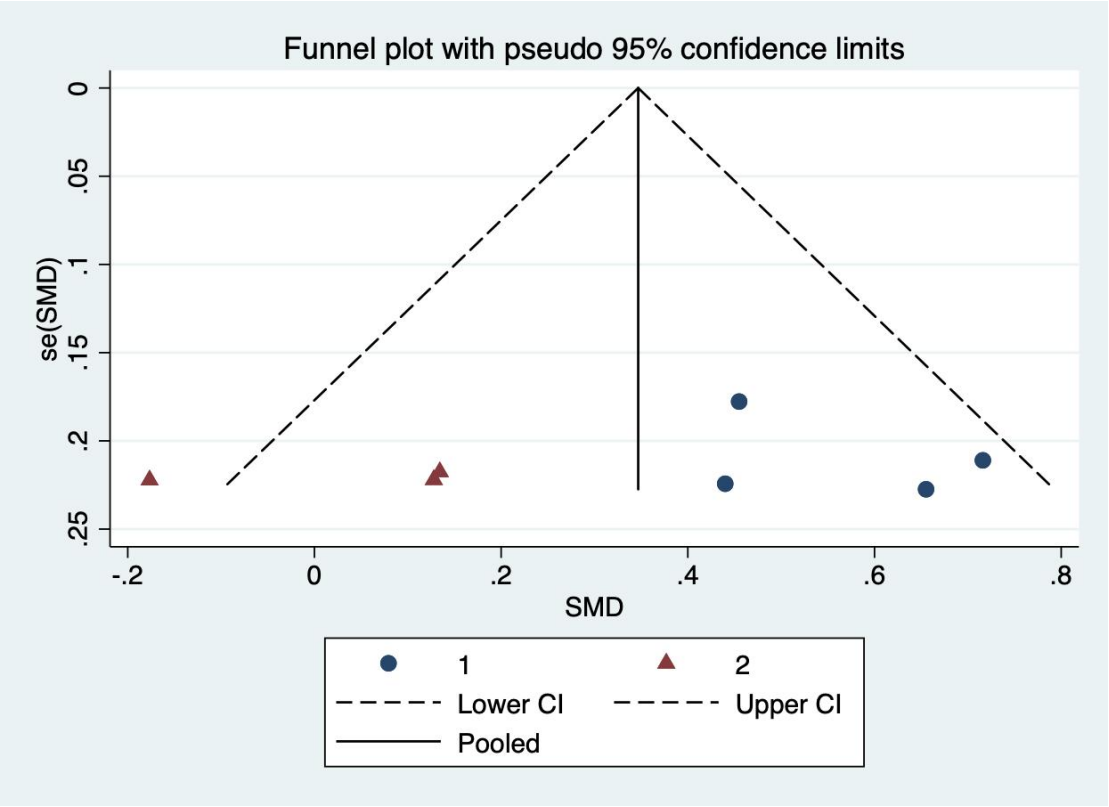

Figure 11. Publication bias assessment for the high-dose and low-dose groups.

Egger's linear regression test estimating the slope and intercept (bias) terms. The p value for the intercept (bias term) was 0.611 (>0.05), indicating no significant small-study effects or publication bias in the overall analysis (Figure 12). This table summarizes publication bias test results after dose stratification (Group 1: high-dose BTKi; Group 2: low-dose BTKi) as well as overall. Begg's rank correlation test and Egger's regression both yielded p values > 0.05 across groups (Group 1: Egger P = 0.547; Group 2: Egger P = 0.629; overall: Egger P = 0.499), providing no statistical evidence of publication bias (Figure 13).

Egger's test

Std_Eff	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
slope	1.152117	1.489804	0.77	0.474	-2.677546	4.98178
bias	-3.802476	7.009991	-0.54	0.611	-21.82223	14.21728

Figure 12. Overall publication bias statistics for T1 Gd+ lesions under BTKi treatment

dosage	n	Begg's score	Begg's s.d.	Begg's z	Begg's p	cont. corr. z	cont. corr. p	Egger's bias	Egger's p
1	4	2	2.944	0.68	0.497	0.34	0.734	2.59	0.547
2	3	-3	1.915	-1.57	0.117	1.04	0.296	-37.04	0.629
overall	7	-1	3.512	-0.28	0.776	0.00	1.000	2.43	0.499

Figure 13. Begg's and Egger's tests after dose stratification

2.4.3 Heterogeneity Test and Analysis for the Number of New or Enlarging T2 Lesions

for both GDE and T2 lesions (pooled into seven dose-defined groups by BTKi dose). Fixed-effects analysis showed a significant reduction in T2 lesions [n = 7; SMD (95% CI) = 0.349 (0.192, 0.506); $p < 0.001$] (Figure 14). $I^2 = 0\%$, and the heterogeneity test P = 0.556, indicating no significant heterogeneity between studies. Figure 15 presents the standardized mean differences (SMD) and their 95% confidence intervals across the seven groups. The fixed-effects model results showed that oral BTKi treatment significantly reduced the number of new or enlarging T2 lesions, with a pooled effect size of SMD = 0.349 (95% CI: 0.192–0.506; $p < 0.001$). The heterogeneity test across studies showed $\chi^2 = 4.90$, P = 0.556, and $I^2 = 0\%$, suggesting no significant heterogeneity among the studies. The overall effect was statistically significant, confirming that BTKi treatment consistently reduces the number of T2 lesions.

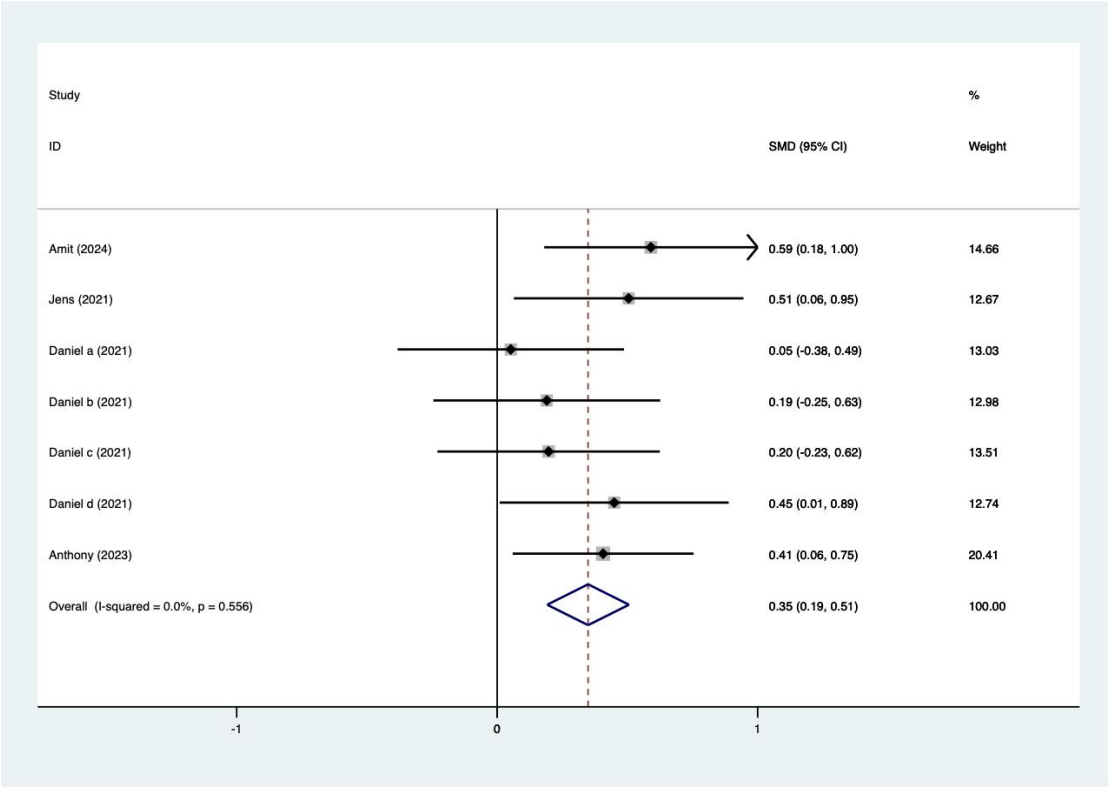

Figure 14. Forest plot of the meta-analysis for the number of new or enlarging T2 lesions.

Study	SMD	[95% Conf.	Interval]	% Weight
Amit (2024)	**0.591**	**0.181**	**1.000**	**14.66**
Jens (2021)	**0.505**	**0.064**	**0.946**	**12.67**
Daniel a (2021)	**0.052**	**-0.383**	**0.487**	**13.03**
Daniel b (2021)	**0.191**	**-0.245**	**0.626**	**12.98**
Daniel c (2021)	**0.198**	**-0.229**	**0.625**	**13.51**
Daniel d (2021)	**0.449**	**0.010**	**0.889**	**12.74**
Anthony (2023)	**0.407**	**0.060**	**0.754**	**20.41**
I-V pooled SMD	**0.349**	**0.192**	**0.506**	**100.00**

Heterogeneity chi-squared = **4.90** (d.f. = **6**) p = **0.556**
I-squared (variation in SMD attributable to heterogeneity) = **0.0%**

Test of SMD=0 : z= **4.36** p = **0.000**

Figure 15. Meta-analysis effect sizes and heterogeneity test results for the number of new or enlarging T2 lesions.

2.4.3.1 Sensitivity Analysis and Exploration of Heterogeneity Sources for the Number of New or Enlarging T2 Lesions

A sensitivity analysis was conducted on the four studies (grouped into seven dose-defined groups by BTKi dose) (Figure 16). After excluding each study in turn, the overall combined effect remained significant and stable, with a pooled effect size of 0.3492 and a 95% confidence interval of [0.1922, 0.5061]. This indicates that the overall effect remained robust, even after excluding individual studies, confirming the reliability and consistency of the findings.

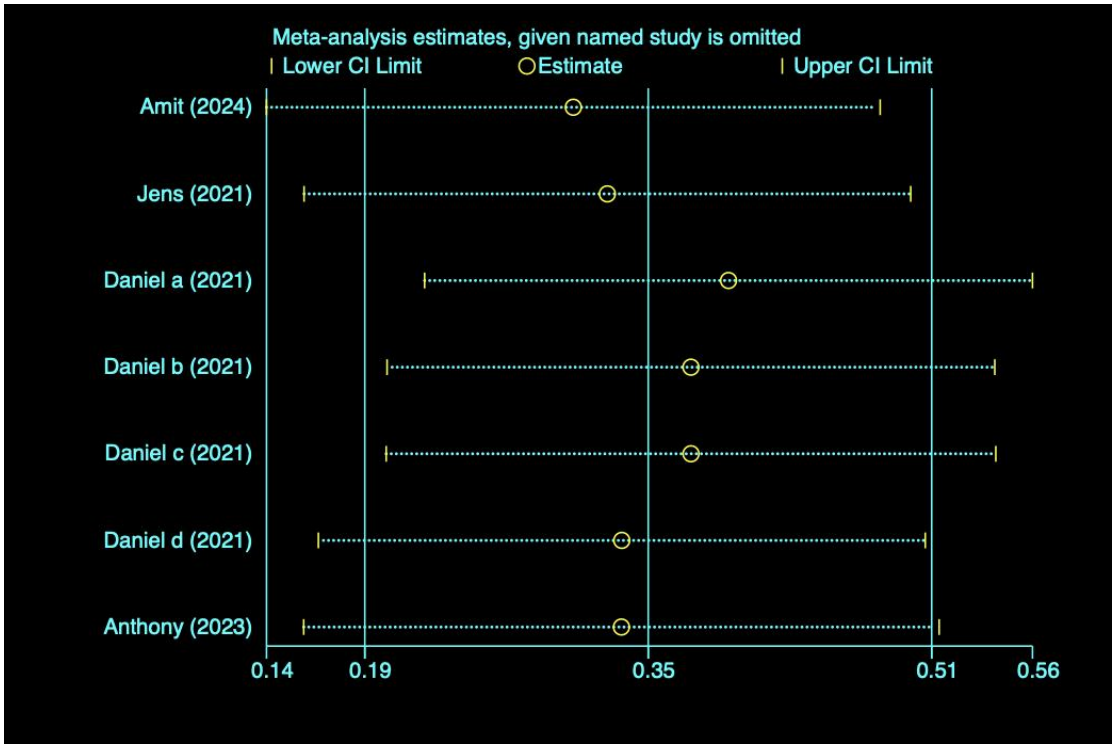

Figure 16. Sensitivity analysis.

2.4.3.2 Subgroup Analysis

To identify whether specific risk factors contributed to heterogeneity, we conducted meta-regression significance testing. The meta-regression for BTKi dose (Figure 17) showed that treatment dose did not significantly affect the effect size ($p = 0.097$, >0.05), indicating that dosage was not the main factor contributing to heterogeneity between studies.

Similarly, the meta-regression for treatment duration (Figure 18) showed no significant effect on the effect size (p = 0.492, much greater than 0.05), suggesting that treatment duration was not a key factor driving heterogeneity. The meta-regression for types of BTKi (Figure 19) also showed no significant impact on effect size (p = 0.183, >0.05), indicating that the type of BTKi did not contribute to heterogeneity between studies. Overall, the meta-regression and subgroup analysis results indicate that BTKi dose, duration, and type do not cause heterogeneity in this study, further confirming the reliability and consistency of the overall findings.

```
. metareg _ES dosage, wsse(_seES) bsest(reml)

Meta-regression                                 Number of obs  =       7
REML estimate of between-study variance         tau2           =       0
% residual variation due to heterogeneity       I-squared_res  =   0.00%
Proportion of between-study variance explained  Adj R-squared  =      .%
With Knapp-Hartung modification
```

_ES	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
dosage	-.3336303	.1638108	-2.04	0.097	-.7547193	.0874587
_cons	.8146641	.2421775	3.36	0.020	.1921269	1.437201

Figure 17. Meta-regression results: Effect of BTKi dose on effect size.

```
. db metareg

. metareg _ES duration, wsse(_seES) bsest(reml)

Meta-regression                                 Number of obs  =       7
REML estimate of between-study variance         tau2           =       0
% residual variation due to heterogeneity       I-squared_res  =   0.00%
Proportion of between-study variance explained  Adj R-squared  =      .%
With Knapp-Hartung modification
```

_ES	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
duration	.1786262	.2407949	0.74	0.492	-.4403568	.7976092
_cons	.1479251	.2828683	0.52	0.623	-.5792111	.8750613

Figure 18. Meta-regression results: Effect of treatment duration on effect size.

```
. metareg _ES typesofbtk, wsse(_seES) bsest(reml)

Meta-regression                                     Number of obs  =       7
REML estimate of between-study variance             tau2           =       0
% residual variation due to heterogeneity           I-squared_res  =   0.00%
Proportion of between-study variance explained      Adj R-squared  =      .%
With Knapp-Hartung modification
```

_ES	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
typesofbtk	-.1686774	.1093025	-1.54	0.183	-.4496484	.1122937
_cons	.7843753	.2931592	2.68	0.044	.0307856	1.537965

Figure 19. Meta-regression results: Effect of BTKi type on effect size.

2.4.3.3 Publication Bias for the Number of New or Enlarging T2 Lesions

We assessed publication bias in the overall analysis using funnel plots. Visual symmetry generally indicates the absence of publication bias (Figure 20). In the overall analysis (Figure 21), Egger's regression test yielded P = 0.580 (> 0.05), indicating a symmetrical funnel plot and no evidence of publication bias.

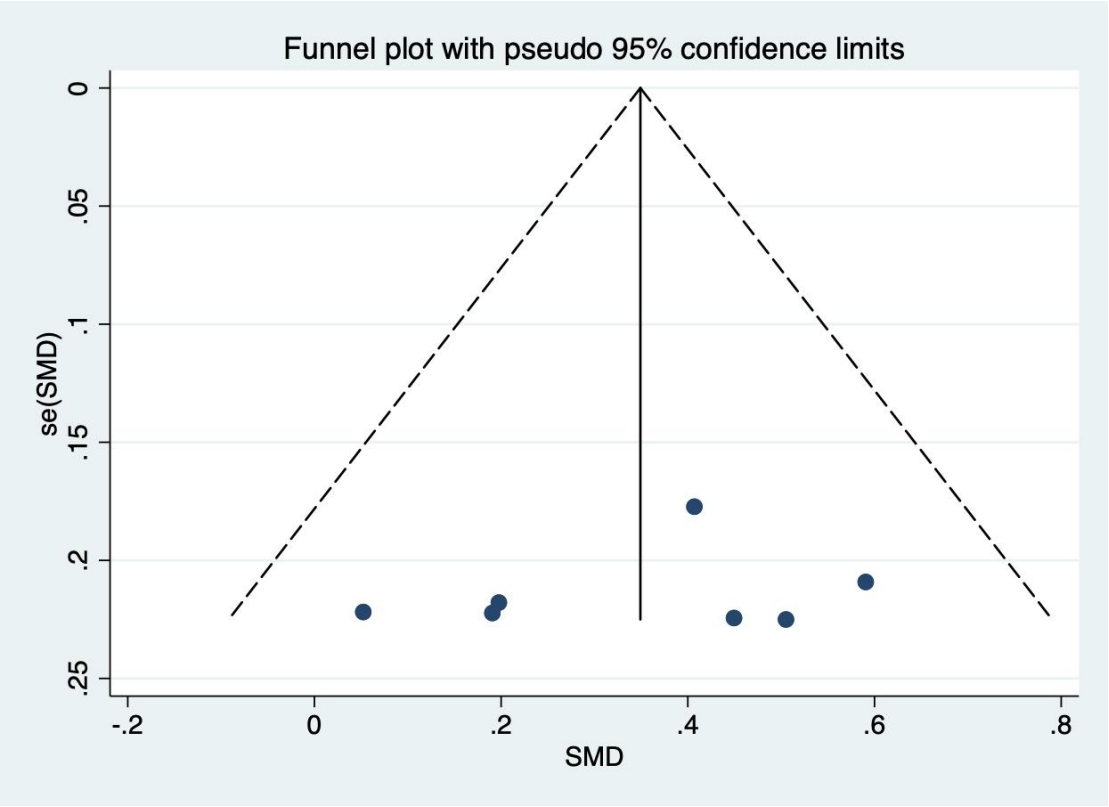

Figure 20. Overall publication bias assessment for the number of new or enlarging T2 lesions under BTKi treatment

Egger's test

Std_Eff	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
slope	.8849142	.9097855	0.97	0.375	-1.453764	3.223592
bias	-2.537408	4.293676	-0.59	0.580	-13.57465	8.499838

Figure 21. Overall publication bias statistics for the number of new or enlarging T2 lesions under BTKi treatment

2.4.4 Effect of BTKi Treatment on Relapse Rate

Only two studies reported relapse rates following BTKi treatment (pooled into three dose-defined groups). The random-effects analysis yielded a pooled effect size (ES) of 0.13 (95% CI: 0.10, 0.17)(Figure 22), indicating that BTKi treatment significantly reduced the relapse rate. The overall I^2 was 12.1%, with a P value of 0.321, suggesting low

heterogeneity between studies and consistent results. Therefore, BTKi treatment's impact on relapse rate was consistently confirmed in these studies, with the combined effect size indicating a significant effect in the treatment group.

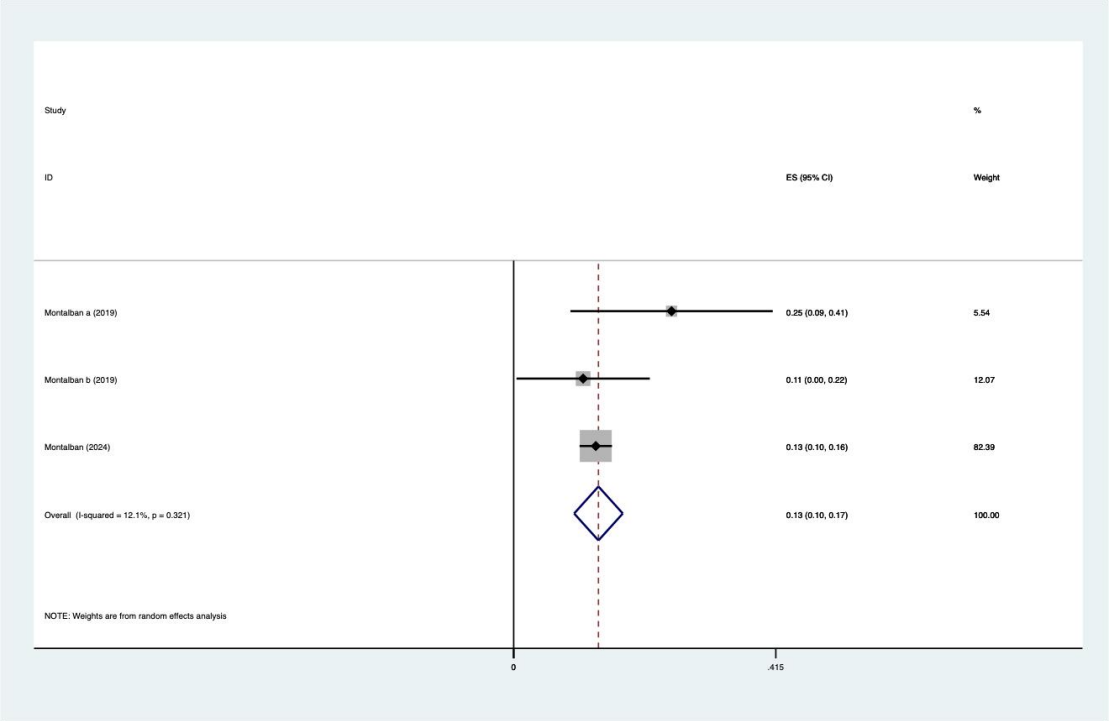

Figure 22. Forest plot of the meta-analysis for relapse rate under BTKi treatment.

2.4.4.1 Sensitivity Analysis for Relapse Rate

Under the random-effects model, after excluding each study in turn, the pooled ES from the meta-analysis did not significantly change, and the confidence intervals for all studies remained relatively consistent. Even after excluding any single study, the combined effect size remained around 0.13 (95% CI: 0.10, 0.17), with P values all less than 0.001, indicating that the effect of BTKi treatment on relapse rate was consistent and significant across these studies(Figure 23).

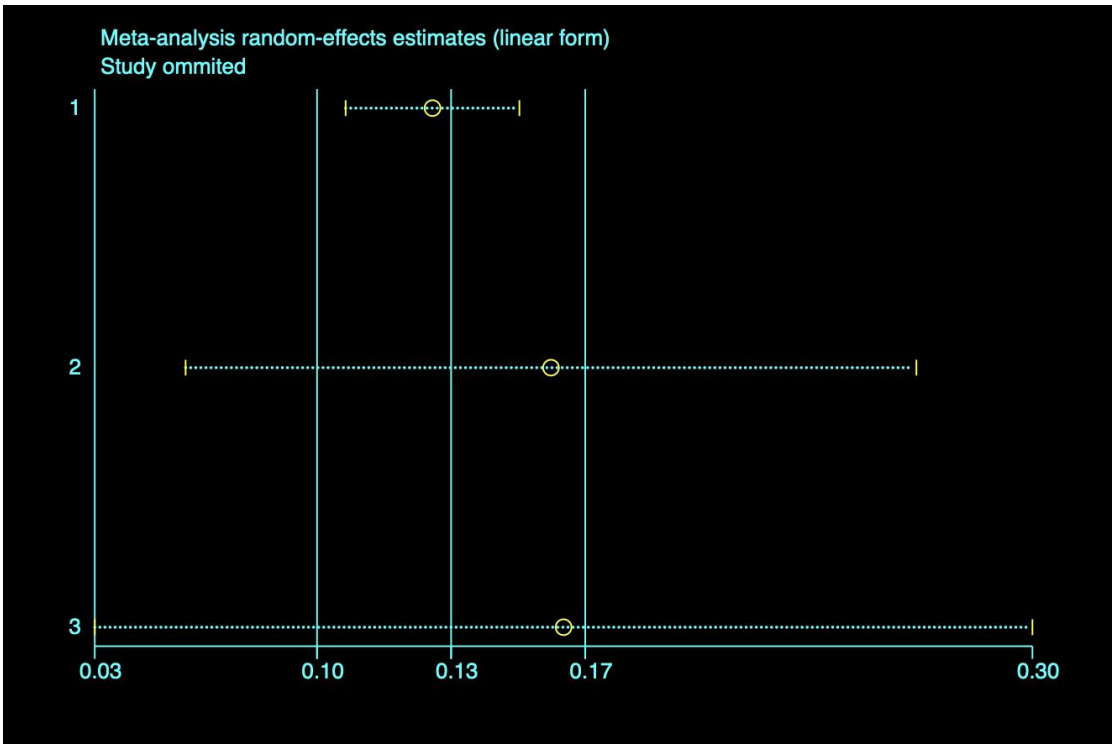

Figure 23. Meta-analysis random-effects estimates for relapse rate: sensitivity analysis.

2.4.4.2 Publication Bias for Relapse Rate

We assessed publication bias in the overall analysis using funnel plots. Visual symmetry generally indicates the absence of publication bias. In the overall analysis, Egger's test yielded P = 0.580 (> 0.05), indicating a symmetrical funnel plot and no evidence of publication bias(Figure 24).

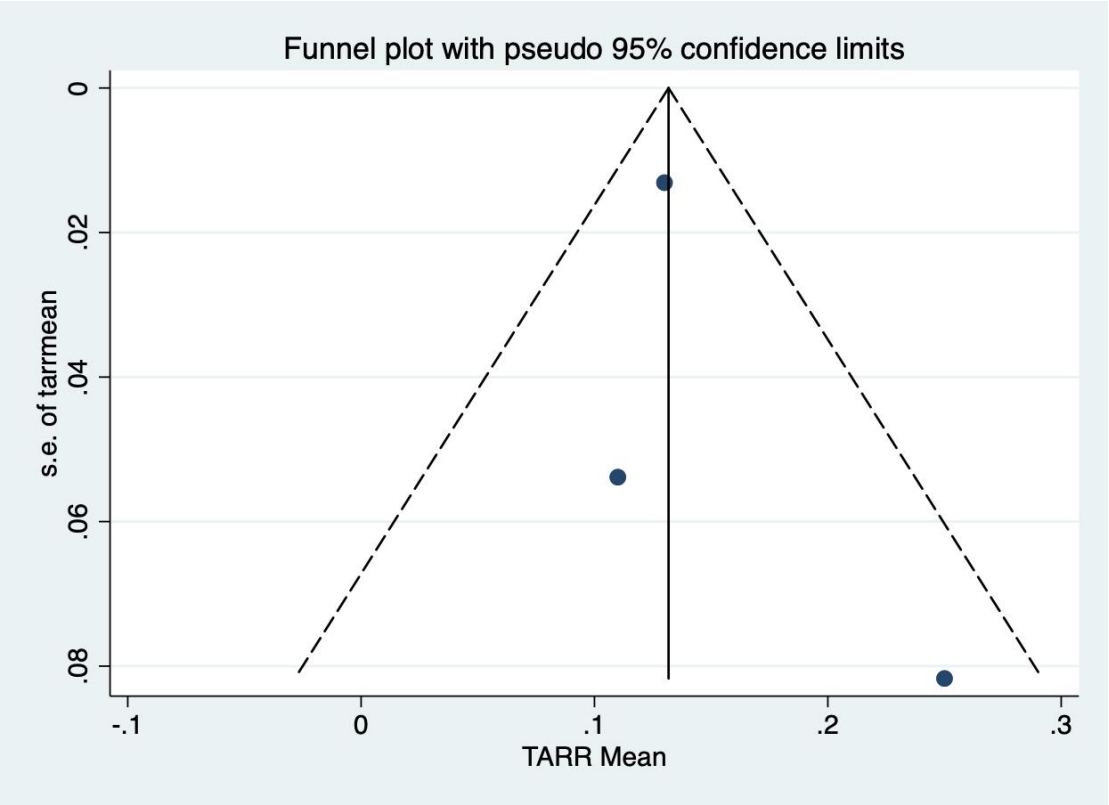

Figure 24. Overall publication bias assessment for relapse rate under BTKi treatment.

2.4.4.3 Side Effects of BTKi Treatment

Five studies included in the analysis reported all adverse events. Fixed-effects analysis (Figure 25) showed no significant difference in the occurrence of adverse events between the treatment and control groups [n = 5, OR (95% CI) = 0.99 (95% CI: 0.92, 1.06), p = 0.759]. I^2 = 0%, P = 0.547, indicating no significant heterogeneity between studies.

For headache reported in three studies, fixed-effects analysis (Figure 28) showed no significant association between headache and BTKi treatment [n = 3, RR = 0.648 (95% CI: 0.180, 2.337), p = 0.942].

For AST/ALT reported in four studies, fixed-effects analysis (Figure 27) showed no significant association between AST/ALT and BTKi treatment [n = 4, RR = 0.91 (95% CI: 0.79, 1.04), p = 0.161].

For rhinitis reported in two studies, fixed-effects analysis (Figure 26) showed no significant association between rhinitis and BTKi treatment [n = 2, RR = 1.39 (95% CI: 0.71, 2.69), p = 0.337].

The sensitivity analysis results showed that under the fixed-effects model, the exclusion of any individual study had a minimal impact on the overall effect size. The change in the overall effect size was limited after excluding any single study, indicating that the study results were robust. Specifically, the effect sizes remained consistent across different exclusion scenarios, demonstrating minimal variability between studies. Therefore, the sensitivity analysis under the fixed-effects model further validated the stable effect of BTKi treatment in patients with multiple sclerosis (Figure 29).

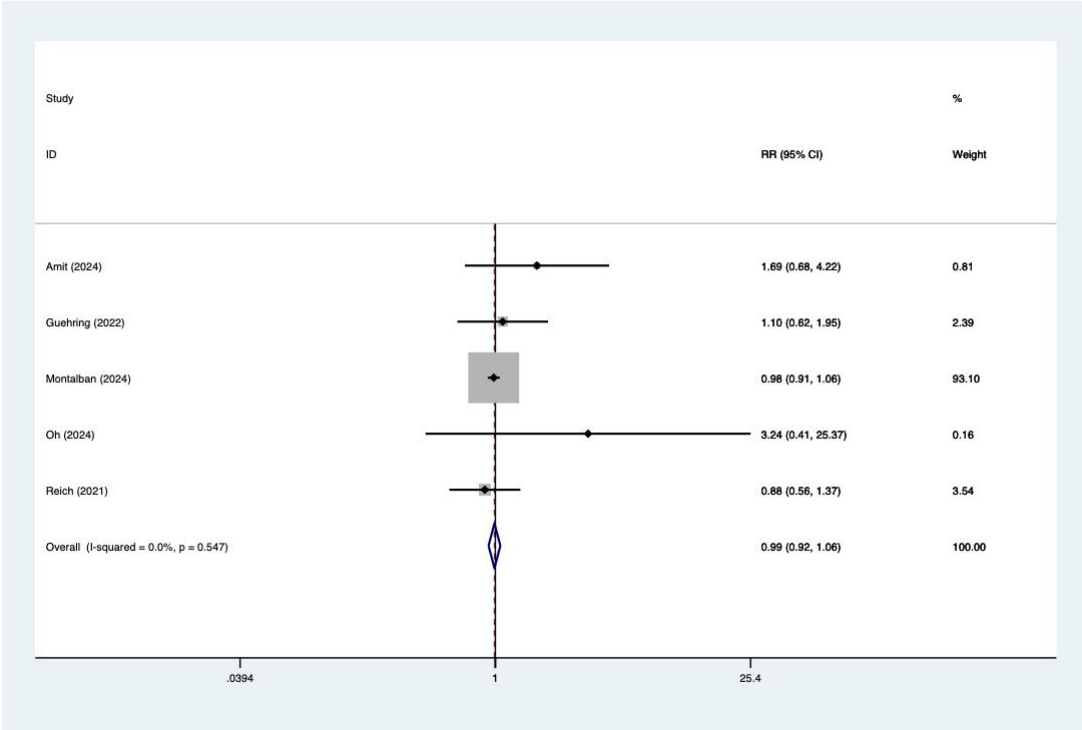

Figure 25. Forest plot of the meta-analysis for adverse events.

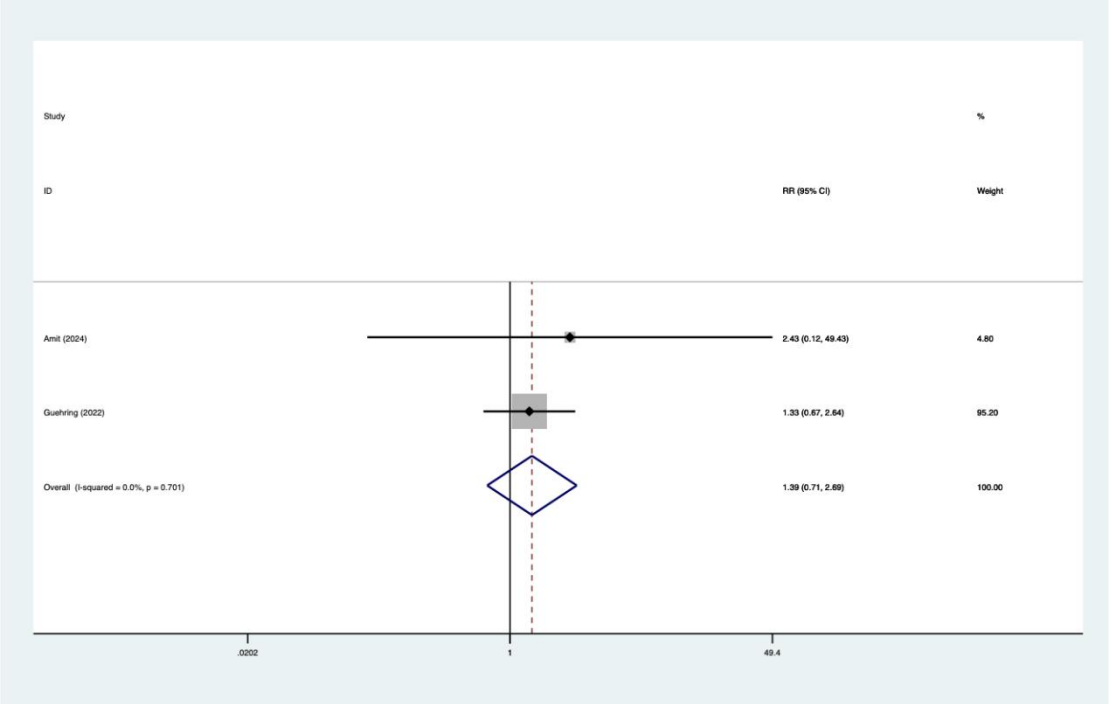

Figure 26. Rhinitis heterogeneity analysis.

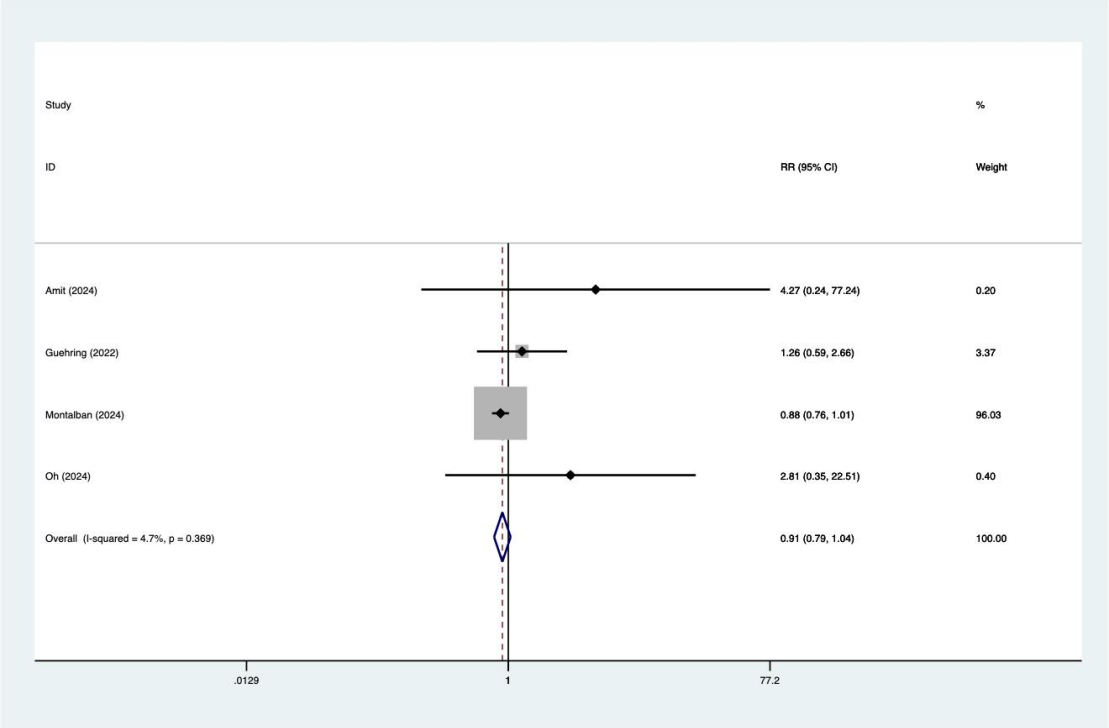

Figure 27. AST/ALT heterogeneity analysis.

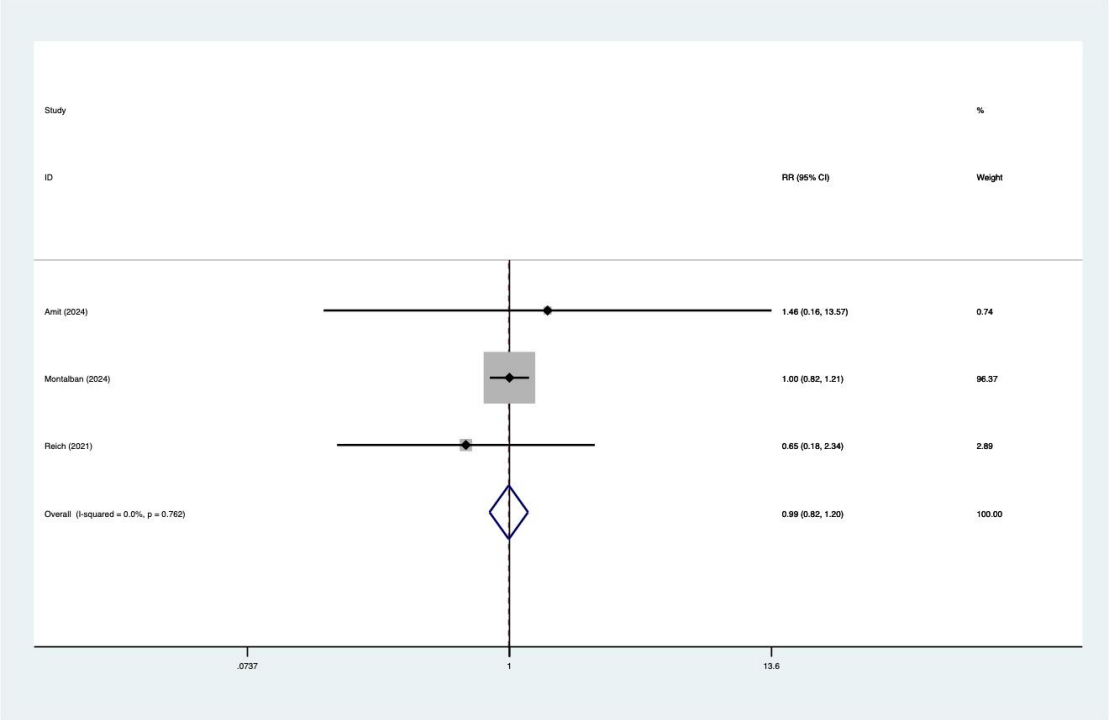

Figure 28. Headache heterogeneity analysis.

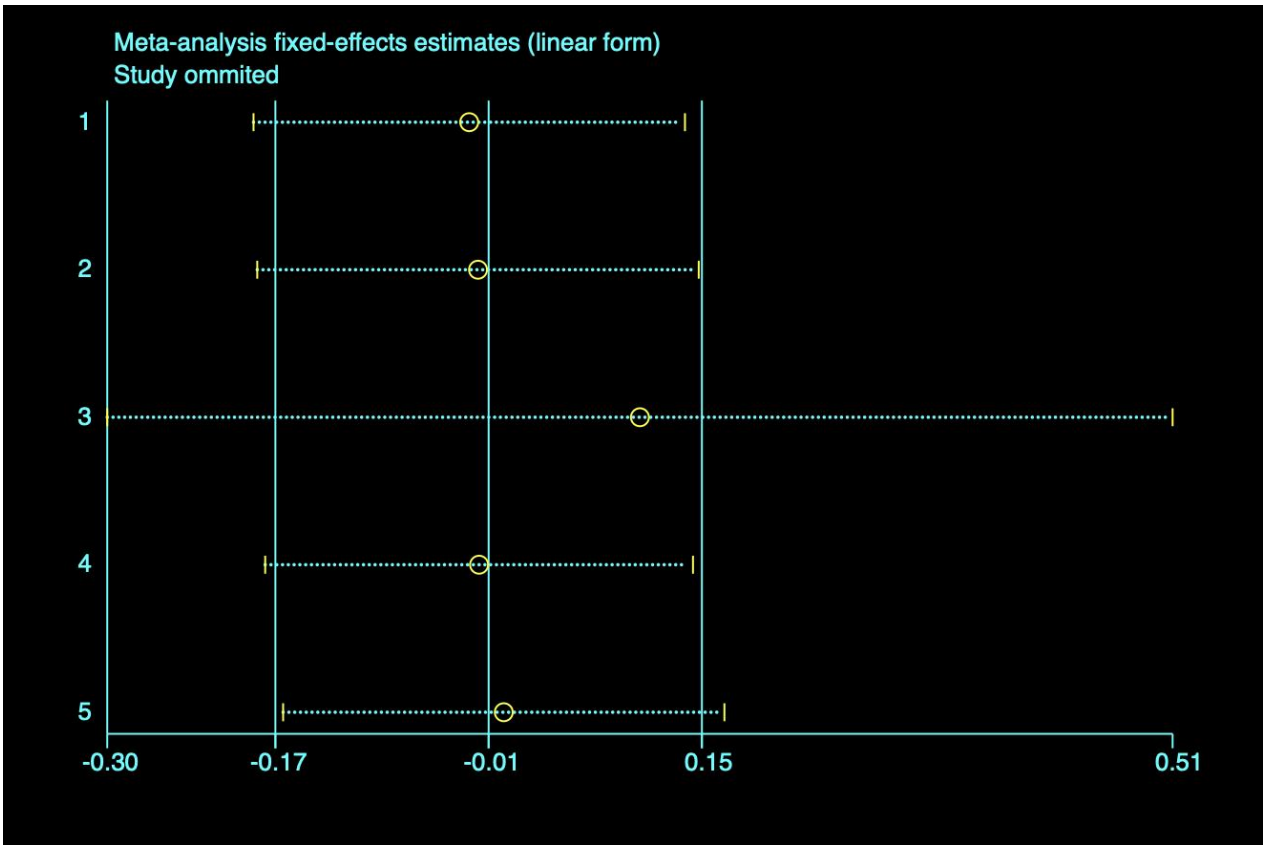

Figure 29. Sensitivity analysis.

2.4 Discussion

2.4.1 Summary of Findings

This study assessed the efficacy and safety of BTKi treatment in MS patients through a systematic review and meta-analysis. The overall results indicate that BTKi treatment significantly reduced the number of new T1 Gd+ lesions and new/enlarging T2 lesions, demonstrating its effectiveness in suppressing CNS inflammation. Additionally, BTKi treatment significantly lowered relapse rates, further confirming its potential in improving clinical function.

2.4.2 The Key Role of Treatment Dosage

The dosage of BTKi significantly impacted the therapeutic effect. The high-dose group (≥60 mg QD) showed a significantly better treatment effect than the low-dose group (<60 mg QD), highlighting the critical role of dosage in enhancing the therapeutic effect. This finding provides a reference for personalized treatment, suggesting that drug dosage should be adjusted based on the patient's condition to achieve optimal efficacy. However, the low-dose group did not show statistical significance, suggesting that low doses may have limited effects, particularly in reducing CNS inflammation and preventing neurodegeneration.

2.4.3 Side Effects and Safety

The current analysis found no significant differences in the occurrence of adverse events between the BTKi treatment group and the control group. Common adverse events such as headache, elevated liver enzymes, and upper respiratory symptoms were similar in both groups, indicating that BTKi treatment is relatively safe. However, the study only reported limited side effect data, and it is unclear whether BTKi treatment could lead to other adverse effects in larger-scale, long-term studies. Therefore, future research should focus on the long-term safety of BTKi treatment.

2.4.4 Heterogeneity and Sensitivity Analysis

While this study demonstrated the significant efficacy of BTKi treatment, some degree of heterogeneity was observed, particularly in the low-dose group. Meta-regression analysis showed that dosage was a major factor influencing the effect size, which may account for the differences in findings across studies. Sensitivity analysis further confirmed that the effect sizes between studies remained relatively consistent, with minimal variation, indicating that despite some heterogeneity, BTKi treatment demonstrated stable efficacy overall.

2.4.5 Publication Bias and Study Quality

Regarding publication bias, sensitivity and funnel plot analyses did not indicate any significant bias, which strengthens the credibility of the study's conclusions. All included studies were RCTs, and most of them had high quality. Therefore, the results of this study are reliable and can provide strong support for clinical practice. However, some studies reported preliminary data, and some were conference abstracts, which may have limited the transparency and completeness of the data. Future research should ensure complete transparency of data to further improve the reproducibility of the studies.

2.4.6 Limitations of the Study

The limitations of this study include the relatively small number of included studies, particularly the limited long-term data on relapse rates and side effects. Furthermore, some studies were conference abstracts, with limited detail and transparency in the data. Although we used missing data imputation and sensitivity analysis to minimize these issues, future research will require more high-quality, long-term follow-up RCTs to further validate the efficacy and safety of BTKi treatment in different MS subtypes.

2.4.7 Future Research Directions

This study provides preliminary evidence for the effectiveness and safety of BTKi treatment in MS, but further research is needed to assess long-term treatment effects, particularly in patients with chronic progressive MS (SPMS and PPMS). Future studies could also explore the long-term efficacy of different types of BTKi (e.g., Evobrutinib, Tolebrutinib, Fenebrutinib) and optimize strategies for personalized treatment. Larger clinical trials will help verify the sustained efficacy of BTKi treatment for MS patients and further evaluate its safety.

2.4.8 Clinical Application Prospects

In conclusion, BTKi, as a novel immunomodulatory drug, shows great therapeutic potential, especially in reducing CNS inflammation and relapse rates. Due to the convenience of oral administration, BTKi treatment offers patients a more comfortable treatment option compared to traditional injectable therapies. However, since efficacy is closely related to dosage, future studies need to further evaluate the most suitable dosage and treatment strategy for different patient populations to achieve personalized treatment.

CHAPTER 3.

The Bruton Tyrosine Kinase (BTK) inhibitors on Epstein-Barr Virus (EBV)-host interactions in Multiple Sclerosis

3.1. Introduction

Multiple sclerosis (MS) is a complex chronic demyelinating disease characterized by significant immunological and neurodegenerative features(Trapp & Nave, 2008). The clinical manifestation of MS typically involves progressive damage to the nervous system, with patients experiencing varying degrees of functional impairment as the disease progresses(McDonald & Ron, 1999). These impairments affect motor, sensory, and cognitive functions. The pathological mechanisms underlying MS are not yet fully understood, but research suggests that it involves the interaction of multiple genetic and environmental factors(Fugger et al., 2009). The interplay between genetic susceptibility and external environmental factors triggers abnormal immune responses, leading to neuronal damage and demyelination(Olsson et al., 2017). As immunological research continues to advance, the pathogenesis of MS is becoming clearer, especially regarding the relationship between immune responses and neurodegenerative changes. Despite numerous breakthroughs in MS research, an effective cure for MS remains elusive, with current treatments primarily focused on disease alleviation and the control of functional impairments(Gholamzad et al., 2019).

The immunopathological characteristics of MS are evident in the abnormal activation of peripheral adaptive immune responses(Hemmer et al., 2015). In the early stages of the disease, localized immune responses lead to damage in the central nervous system (CNS) and initiate neurodegenerative processes(Leszek et al., 2016). The interaction between peripheral immune cells and CNS cells results in the immune system attacking neural tissue, causing demyelination(Mayo et al., 2012). This immune dysregulation is one of the core features of MS pathogenesis. Although current disease-modifying therapies (DMTs) have made progress in reducing inflammation and clinical relapses, they still face significant limitations, particularly in inhibiting progressive neurodegeneration(Stamatellos & Papazisis, 2023). Effective solutions for treating persistent neurodegenerative changes are yet to be found. Therefore, modulating immune responses and controlling immune cell attacks on neurons remains one of the focal points of MS research.

Recent studies have increasingly highlighted the relationship between Epstein-Barr virus (EBV) and MS(Soldan & Lieberman, 2023). EBV is a widely disseminated virus, with approximately 95% of the global population being infected at some point in their lives(Wong et al., 2022). EBV spreads through saliva, initially infecting B cells in the upper respiratory tract, and under specific viral activation factors, promotes the proliferation and transformation of B cells(Küppers, 2003). EBV infection not only affects B cell proliferation but also alters the immune response by interacting with the host cell signaling pathways via LMP1 protein, thereby exacerbating immune dysregulation and neuronal damage(Hatton et al., 2014). LMP1 protein mimics the CD40 signaling pathway, further activating the immune response, leading to excessive immune activation and CNS damage(Graham et al., 2010). This abnormal virus-host interaction mechanism is a key factor in the pathogenesis of MS.

Studies have traced the relationship between EBV and MS back over twenty years. Epidemiological data suggest that individuals who are seronegative for EBV have an almost zero risk of developing MS, while those who have been infected with EBV have a significantly increased risk of MS, particularly after experiencing an episode of infectious mononucleosis(Pakpoor et al., 2013). Numerous studies have found that MS patients exhibit enhanced immune responses to EBV prior to disease onset, suggesting a potential defect in immune surveillance(Houen et al., 2020). This imbalance in immune response may make individuals more susceptible to EBV infection, triggering the immune pathological changes associated with MS. Research by Bjornevik (Bjornevik et al., 2022) further confirmed that EBV infection is not only a necessary condition for MS onset but may also trigger neuronal

damage before the onset of clinical symptoms, significantly increasing the risk of MS. This finding provides new perspectives for the early diagnosis and intervention of MS.

Although there is a close association between EBV infection and MS, not all individuals infected with EBV will develop MS. This phenomenon may be due to a combination of host genetic susceptibility, viral strain characteristics, and environmental factors. Studies have shown that host gene polymorphisms and viral genetic variations together determine whether EBV infection leads to MS. Research by Mechelli (Mechelli et al., 2024) revealed differences between genotypes, indicating that certain genetic backgrounds may make individuals more susceptible to developing MS after EBV infection. Variations in the virus's genetic makeup may also influence its pathogenic mechanism, leading to different immune responses and MS risk across individuals.

Given the central role of EBV in MS pathogenesis, treatment strategies targeting this mechanism have become a major focus of current research. BTKi (Bruton's tyrosine kinase inhibitors), as an emerging disease-modifying therapy, has shown promising potential in alleviating CNS immune inflammation and neurodegeneration(García-Merino, 2021). However, no study has specifically focused on the role of BTKi in modulating EBV-related immune mechanisms. Therefore, a deeper understanding of how BTKi modulates immune abnormalities induced by EBV will not only aid in further understanding the pathogenesis of MS but may also provide new insights and biomarkers for individualized treatment strategies. By better understanding the interaction between EBV infection and immune responses, we can offer new targeted therapeutic strategies for MS treatment and accelerate the progress towards curing the disease.

3.1.1 Pathogenic Mechanism of EBV in Multiple Sclerosis

Currently, several theories have been proposed regarding how EBV initiates and sustains the pathogenic process of multiple sclerosis MS. Studies suggest that EBV infection induces the host's autoimmune response and releases immune clones through its specific viral proteins interacting with unique antigens in the CNS. Through this mechanism, EBV infection may trigger immune system dysregulation, thereby promoting the onset of MS (Jilek et al., 2008; Jog et al., 2020; Lanz et al., 2022; Tengvall et al., 2019). In this process, EBV-infected memory B cells can migrate to the peripheral blood, further activate T cells, and promote their migration to the CNS (Walther & D'addario, 2001). Additionally, these EBV-infected B cells produce an EBV-encoded interleukin-10-like protein that antagonizes the body's immune regulatory factor, IL-10, thereby exacerbating the immune response.

This mechanism intensifies the overreaction of the immune system, leading to neuronal damage through sustained immune attacks (Kang & Kieff, 2015).

In this process, the lack of CD8+ T cells plays a significant role in the clearance of EBV-infected B cells. Due to insufficient CD8+ T cell activity, EBV-infected B cells cannot be effectively cleared, leading to their accumulation and proliferation in the meninges and subcortical follicles of the CNS (Serafini et al., 2007; Serafini & Del Rio, 2004). These EBV-infected B cells exacerbate the local inflammatory response in the CNS through cytotoxic damage, interactions with autoreactive T cells, and the production of autoantibodies, further promoting neurodegeneration. The continuous development of this local inflammation accelerates the pathological progression of MS, resulting in severe neurological dysfunction as the disease progresses (Bar-Or et al., 2022). Moreover, increasing evidence suggests that EBV may synergistically interact with other pathogens such as human herpesvirus 6 (HHV6) and human endogenous retrovirus, intensifying neurotoxic responses and antiviral immune responses. These interactions among pathogens could make the immune response more destructive, further promoting the onset and progression of MS(Fierz et al., 2017).

In both clinical and radiological presentations of MS, the reactivation of EBV is often associated with relapses, a process that has been confirmed by several studies. It is known that during relapse phases of MS patients, levels of EBV early antigen (EA) and EBV-DNA significantly increase (Buljevac et al., 2005; Rodman & Wandinger-Ness, 2000; Torkildsen et al., 2008). These findings further support the critical role of EBV in the pathological mechanism of MS and suggest that EBV reactivation may be a key trigger for MS relapses.

In recent years, the effectiveness of B-cell-depleting disease-modifying therapies (DMTs) in clinical practice has supported the crucial role of EBV in MS(Stamatellos & Papazisis, 2023). One of the mechanisms of these therapies is the effective prevention of disease relapse by targeting and destroying the EBV latent reservoir. For example, anti-CD20 monoclonal antibodies (mAbs) can clear EBV-infected B cells in peripheral blood, preventing their reactivation and thus limiting the entry of these pathogenic B cells and cross-reactive T cells into the CNS(Alrashoudi, 2024). Recent studies have shown that anti-CD20 therapy effectively reduces the incidence of relapses and, to some extent, slows the progression of MS(Pham et al., 2021) However, the application of anti-CD20 monoclonal antibodies in the EBV-driven pathological mechanism has certain limitations. The main issue is that these monoclonal antibodies cannot cross the blood-brain barrier (BBB), preventing them from effectively targeting EBV-infected cells within the CNS(Rani et al.,

2024). Furthermore, anti-CD20 antibodies cannot clear plasma cells and plasmablasts that do not express CD20, limiting their efficacy in the progressive phase of MS(Margoni et al., 2022). The progression of MS largely depends on immune cell dysregulation within the CNS, where these cells persist in the CNS and participate in the expansion of neurodegeneration during chronic inflammation. Therefore, the efficacy of anti-CD20 therapy in treating progressive MS is limited.

3.1.2 BTK Inhibitors: Pharmacology and Evidence for Multiple Sclerosis (MS)

Bruton's tyrosine kinase inhibitors (BTKis) can be classified into two categories: irreversible BTKis and reversible BTKis(Gupta et al., 2025).

Irreversible BTKis: These inhibitors covalently bind to the ATP-binding site of the kinase, providing long-lasting inhibition. Representative drugs include Ibrutinib, Evobrutinib, Tolebrutinib, and Orelabrutinib. These drugs block the enzyme's function by covalently binding to the target enzyme, resulting in long-term suppression.

Reversible BTKis: Drugs like Fenebrutinib and BIIB091 fall into this category(Airas et al., 2024). These inhibitors bind to the kinase pocket via hydrogen bonds or hydrophobic interactions, leading to enzyme inactivation. These drugs typically exhibit weaker binding, making their inhibitory effects reversible, which is suitable for short-term suppression needs.

The first-generation BTKis, such as Ibrutinib, have achieved significant results in treating lymphoid malignancies. However, clinical application has shown notable safety issues. Specifically, Ibrutinib and other first-generation BTKis exhibit off-target activity against other kinases, such as TEC, ITK, BMX, TXK, EGFR, and JAK, leading to a range of adverse effects, including bleeding, arrhythmia, and immunosuppression. These side effects significantly limit their clinical use and therapeutic safety (Ringheim et al., 2021) As a result, researchers began developing more selective BTKis, which show stronger targeting properties and selectively inhibit BTK with minimal effects on other kinases, thereby effectively reducing side effects and improving therapeutic outcomes (Liang et al., 2018).

Initially, BTKis were primarily used to treat lymphoid malignancies, especially chronic lymphocytic leukemia (CLL) and other B-cell-associated cancers. However, as research progressed, it became apparent that BTKis also have potential therapeutic effects on various autoimmune diseases, including MS. MS is a chronic CNS disease in which the immune system abnormally attacks the body's own nerve tissue. BTKis, due to their oral administration and good CNS penetration, have emerged as a promising therapeutic strategy

for both relapsing and progressive forms of MS. Furthermore, BTKis can address the dysfunction of adaptive immune cells (such as B cells and T cells) and innate immune cells (such as microglia), making them a potential new option for treating MS (Yong & Yong, 2022).

In preclinical animal models of MS, particularly the experimental autoimmune encephalomyelitis (EAE) mouse model, BTKis have been shown to effectively reduce the ability of B cells to present antigens to T cells, thereby inhibiting T-cell overactivation. Studies also revealed that BTKis alleviate CNS damage by suppressing the production of pro-inflammatory cytokines. These effects significantly reduce the degree of neurodegeneration associated with MS (Torke et al., 2020). Specifically, in the EAE mouse model, treatment with Evobrutinib effectively reduces meningeal inflammation and decreases B cells in ectopic follicles, effects that traditional anti-CD20 drugs cannot achieve. This further demonstrates the potential and advantages of BTKis in MS treatment (Bhargava et al., 2021).

Currently, several BTKi drugs are undergoing clinical trials for MS and are expected to enter the disease-modifying therapy (DMT) field in the coming years, providing new treatment options. These drugs include:

Tolebrutinib: As an irreversible BTKi, Tolebrutinib has a low affinity for JAK3 and EGFR, making it highly selective. Phase I studies have shown that Tolebrutinib can effectively increase the permeability of the blood-brain barrier (BBB), enhancing the drug's CNS penetration and addressing the challenge of CNS drug delivery. In a phase 2b randomized, double-blind, placebo-controlled, crossover trial involving 130 relapsing MS patients, treatment with 60 mg of Tolebrutinib significantly reduced the number of new gadolinium-enhanced lesions and T2 high-signal lesions, with good safety results(Reich et al., 2021). Tolebrutinib is currently undergoing several phase 3 clinical studies to evaluate its efficacy in both relapsing and progressive MS (NCT04411641, NCT04458051, NCT04410991, and NCT04410978).

Evobrutinib: As another irreversible BTKi, Evobrutinib was evaluated in a phase II clinical trial, which found that 75 mg of Evobrutinib daily reduced gadolinium-enhanced lesions in T1-weighted MRI but did not significantly decrease relapse rates or disability progression (Montalban et al., 2019). Evobrutinib is currently undergoing two phase III studies (NCT04338022 and NCT04338022), with final results expected in 2026.

Ibrutinib: A reversible, non-covalent BTKi, Ibrutinib inhibits enzymes by binding to their inactive forms. This reversible inhibition offers more flexibility in clinical applications.

Currently, Fenebrutinib is undergoing phase 3 clinical trials for MS patients, with study identifiers including NCT04586023, NCT04586010, and NCT04544449.

Orelabrutinib: As an irreversible BTKi, Orelabrutinib is undergoing a phase 2 placebo-controlled study involving 160 relapsing MS patients, expected to be completed in 2024 (NCT04711148). Additionally, a phase 1 clinical trial is evaluating the tolerance and pharmacokinetics of the reversible BTKi inhibitor BIIB091, initially developed for treating MS (Bame et al., 2021; NCT03943056).

As an emerging disease-modifying treatment strategy, BTKis have shown significant therapeutic potential. With further research and clinical data accumulation, BTKis may bring new breakthroughs in the treatment of MS and provide more effective therapeutic options for patients. The development and clinical application of these new drugs not only drive innovation in MS treatment but also offer new approaches and choices for treating other autoimmune diseases.

3.2 Project Objectives and Research Design

The objective of this project is to evaluate the role of BTKi as a host-directed therapy against EBV in the context of MS. To this aim we examined how BTKi modulates the interaction between EBV and the host immune system, with a particular focus on its potential therapeutic effects in MS.

3.3 Relevance and Innovation

To date, no study has specifically explored the relationship between BTKi and EBV in MS. This study aims to delve into how BTKi influences the interaction between EBV and the host immune system during MS treatment. Through this research, we hope to uncover new mechanisms of action for Tolebrutinib (a BTKi) in MS, providing new perspectives on the pathogenesis of the disease. This discovery could represent a significant breakthrough in MS treatment, potentially paving the way for future cure strategies. Furthermore, the identification of drug response biomarkers based on EBV genetic background may provide innovative and highly relevant biomarkers for MS. This would offer strong support for personalized treatment and precision medicine in the future.

3.4 Methods

3.4.1 In Vitro Testing

To assess the impact of BTKi on virus-host interactions, we will use the LCL as an in vitro model. This model can replicate EBV-induced B cell pathology. The LCLs will be obtained by infecting B cells with the B95.8 EBV strain. This model will help investigate the effects of Tolebrutinib on EBV-infected B cells and assess whether viral genetic variations influence these effects.

3.4.2 Participant Selection and Blood Sample Collection

All participants will sign a written informed consent form before participating in the study, ensuring their understanding of the study's objectives, procedures, potential risks, and possible benefits, and confirming voluntary participation. The study protocol will be approved by the local ethics committee to ensure that all experimental procedures comply with ethical standards and protect the participants' rights and privacy. We plan to collect peripheral blood samples from 15 patients diagnosed with MS according to the 2017 McDonald criteria, as well as from 15 HD. Each participant will provide 30 milliliters of blood. When selecting MS patients, we will specifically ensure that they have not received DMTs for at least three months before the sample collection, or have not been undergoing treatment on the day of sampling. This criterion aims to avoid treatment interference, ensuring that the collected samples reflect the natural disease course of the patients. For healthy donors, participants must have no immune system diseases or other health conditions that could affect immune responses to ensure the quality and reliability of the blood samples. Basic information, clinical diagnoses, medical histories, and other relevant data of all participants will be carefully recorded and kept confidential.

3.4.3 PBMC Isolation and EBV Infection

To extract B lymphocytes, peripheral blood mononuclear cells (PBMC) will first be separated using the Ficoll-Hypaque density gradient centrifugation method. The blood will be mixed with sterile PBS at a 1:1 ratio, layered gently onto a tube containing Ficoll, and centrifuged at 1,800 rpm for 25 minutes. After centrifugation, the lymphocyte ring will be collected from the top layer and washed 2-3 times with PBS to remove any impurities. The lymphocytes will then be resuspended in an appropriate amount of culture medium for further use. The PBMCs will be infected with the EBV B95.8 strain to generate B95.8-LCLs

(lymphoblastoid cell lines), which will serve as the experimental model for studying virus-host immune interactions.

To prepare for B lymphocyte infection, a mixture of virus, cyclosporine A, and RPMI culture medium will be used. Cyclosporine A will selectively kill T cells to ensure B cell infection with EBV. The resuspended B lymphocytes will be mixed with the virus and cyclosporine A and incubated for 1.5 to 2 hours, with gentle stirring every 10 minutes to promote the contact and infection of the virus with the B cells. After incubation, additional culture medium and cyclosporine A will be added, and the cells will be distributed into 24-well culture plates for further incubation. PBS solution will be added around the wells to maintain the humidity and stable pH of the culture environment. In the infected cell cultures, the virus will be added, and the cells will be incubated in the culture medium, followed by further media supplementation to increase the virus concentration and facilitate the infection process. The cells' reactions will be assessed through further culture and observation. After the experiment, all tools and incubators used will be cleaned to ensure no contamination.

3.4.4 Grouping

We will isolate cells from MS patients and HD and infect them with the B95.8 EBV strain to generate B95.8-LCLs. The groups will be as follows:

MS Group (B95.8-LCLs derived from MS patients): PBMCs from MS patients will be used to generate B95.8-LCLs. These cells will be used to assess the impact of Tolebrutinib on EBV-infected B cells in the context of MS.

HD Group (B95.8-LCLs derived from healthy donors): PBMCs from healthy donors will be used to generate B95.8-LCLs. These cells will serve as a control group to compare the responses of EBV-infected B cells to Tolebrutinib in healthy individuals.

3.4.4 BTK Inhibitor Dose-Response Analysis

To evaluate the efficacy of BTKi, we conducted a dose-response analysis to determine the half-maximal inhibitory concentration (IC50) for several BTKi compounds. The appropriate cell lines were selected and cultured under standard conditions. The cells were exposed to different concentrations of BTKi. In order to comprehensively assess the dose-response relationship of BTKi, the following concentration range was selected: 0.01 μM, 0.1 μM, 1 μM, 5 μM, 10 μM, 15 μM, 20 μM, 50 μM, 75 μM, and 100 μM. The culture medium was RPMI containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The cells were exposed to different concentrations of the drug

for 24 or 48 hours, with the exposure time determined according to the experimental design. As a control group, cells were treated with 0.1% DMSO solution.

After incubation, cell viability was assessed using the Trypan Blue exclusion assay. The Trypan Blue staining method stains dead cells, allowing for the measurement of cell survival percentage. By calculating the ratio of viable cells to total cells in each group, the cell survival rate for each group was obtained. The survival rate data at different concentrations were plotted as a dose-response curve, with the X-axis representing BTKi concentration and the Y-axis representing the percentage of viable cells. The IC50 value is the drug concentration that reduces cell viability by 50%.

Non-linear regression analysis was performed using the four-parameter logistic regression model to fit the dose-response data and calculate the IC50 values. The formula is as follows:

$$Y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + (X/IC50)^{\text{Hill coefficient}}}$$

Where:

Y represents the cell survival percentage,X is the drug concentration,Min and Max are the minimum and maximum values of the inhibition curve,The Hill coefficient represents the slope of the curve.All experimental data were processed using the Quest Graph™ IC50 Calculator (https://www.aatbio.com/tools/ic50-calculator) to calculate the IC50 values. This tool uses the four-parameter logistic regression model to fit the data, providing accurate IC50 values.

3.4.5 Cell Treatment with BTKi and Extraction

Before beginning the experiment, cell counting is performed using a hemocytometer to ensure the cell concentration reaches 5×10^6 /ml, ensuring an adequate number of cells for subsequent experiments. If the cell concentration is below the required standard, cells must be re-prepared and adjusted to the desired concentration. Once the cell concentration meets the requirement, cells are distributed into two culture bottles for drug treatment and control treatment.

Cells are assigned to four experimental groups, each with an equal number of cells to ensure consistency between the experimental and control groups. The specific grouping is as follows:

MS-E group (Multiple Sclerosis patient-derived, BTKi treatment group): Cells from MS patients are treated with BTKi.

MS-0.1% group (Multiple Sclerosis patient-derived, DMSO control group): Cells from MS patients are treated with 0.1% DMSO solution as a control group.

HD-E group (Healthy Donor-derived, BTKi treatment group): Cells from healthy donors are treated with BTKi.

HD-0.1% group (Healthy Donor-derived, DMSO control group): Cells from healthy donors are treated with 0.1% DMSO solution as a control group.

The required volume of BTKi solution is calculated for each group to ensure the correct drug concentration. BTKi is added to the treatment groups, and an equal volume of 0.1% DMSO solution is added to the control groups. All drug treatments should strictly follow the designed concentrations to ensure accurate dosing. After treatment, all culture bottles are incubated at 37°C in a 5% CO_2 incubator for 48 hours. During the incubation period, cells are regularly observed for morphological changes and growth conditions, and the effect of the drug on cell proliferation and survival is recorded. After 48 hours of incubation, cell survival rate is assessed using the trypan blue exclusion method. By staining dead cells, the survival rate is calculated by the ratio of surviving cells to total cells, evaluating the effect of the drug in each group. To ensure the accuracy of the data and the reproducibility of the experiment, cells are counted again after the experiment, and cell concentration for each group is checked under the microscope to meet the following standards: WB 1.5×10^6 /ml and RNA 1.0×10^6 /ml. The experiment continues only if these standards are met. If the cell numbers are appropriate, cell pellets are collected for subsequent experiments such as protein or RNA extraction. If not immediately used, cell pellets are stored at -20°C to prevent degradation until further experiments.

3.4.6 Western Blot Experimental Procedure

3.4.6.1 Sample Preparation and Lysis

The day before the experiment, prepare the necessary reagents, including samples, loading buffer, running buffer, transfer buffer, and TTBS (TBS with 0.1% Tween 20).

Cells are separated from the culture medium using Ficoll-Hypaque density gradient centrifugation. To ensure efficient lysis, cells are lysed using RIPA lysis buffer, and protease and phosphatase inhibitors are added to prevent protein degradation. The lysis buffer is prepared as follows: 50 μL RIPA lysis buffer, 442.5 μL water, 2.5 μL protease inhibitor, and 5 μL phosphatase inhibitor.

After lysis, each sample is mixed with 40 μL of mix and incubated on ice for 20 minutes. After incubation, the samples are centrifuged at 300 rpm for 15 minutes, and the supernatant is transferred to a new tube. The supernatant is then subjected to high-speed centrifugation (8000 g, 4°C, 10 minutes). The final supernatant is collected and stored at -20°C for future experiments.

3.4.6.2 Gel Electrophoresis and Sample Loading

After lysis, protein separation is performed. Prepare the loading buffer and β-mercaptoethanol at a 1:9 ratio. For each sample, add 3.75 μL of 4X loading buffer and 6.1 μL of β-mercaptoethanol. The sample and buffer are mixed well and heated in a 70°C water bath for 10 minutes to denature the proteins.

During sample loading, add 15 μL of the treated sample to each well. Electrophoresis is carried out initially at 100V, and once the proteins have migrated to an appropriate position, the voltage is adjusted to 120V to complete the electrophoresis.

3.4.6.3 Transfer to Membrane

After electrophoresis, protein transfer is performed. Prepare the transfer apparatus, assemble the membrane and gel, and prepare the transfer buffer. The membrane and gel are cut to appropriate sizes, avoiding direct contact with the gel. Transfer is performed at 350 mA for 1.5 hours to ensure proteins are transferred from the gel to the membrane.

After transfer, the membrane is stained with Ponceau solution for protein confirmation, and excess dye is washed off with distilled water. The membrane is then washed with TTBS for 10 minutes to remove any residual Ponceau solution. The membrane is further blocked with 5% non-fat milk at room temperature for 1 hour to reduce non-specific binding.

3.4.6.3 Primary Antibody Incubation

After blocking, the membrane is transferred to a solution containing the appropriate concentration of primary antibodies （Table 1）. The membrane is fully immersed in the primary antibody solution and incubated overnight at 4°C to ensure adequate binding of the primary antibody to the target protein.

Table.1 Antibody Information

Antibody	Catalog Number	Supplier	Dilution
CD40 antibody (Rb mAb to CD40)	ab224639	Abcam	1:1000
EBNA2 antibody (Ms mAb to EBNA2 [PE2])	AB90543	Abcam	1:1000
ADAR1 antibody (Rb mAb to ADAR1)	ab126745	Abcam	1:1000
PI3 kinase p110β antibody (Rb mAb to PI3 Kinase p110 Beta)	EPR5512	Abcam	1:1000
EBV latent membrane protein 1 antibody (Ms mAb to EBV Latent Membrane Protein 1)	ab78113	Abcam	1:1000
IKBα antibody (Rb mAb to IKB alpha [E130])	AB32518	Abcam	1:1000
NF-κB P65 antibody (Rb pAb to NF-KB P65)	AB16502	Abcam	1:1000
BTK antibody (Rb mAb to BTK [EPR20445])	AB208937	Abcam	1:1000
STAT3 antibody (Rb mAb to STAT3 [EPR787Y])	AB68153	Abcam	1:1000
β-Actin antibody (Beta actin [C4] - Mouse)	J2320	Santa Cruz	1:1000

3.4.6.4 Secondary Antibody Incubation

After primary antibody incubation, the membrane is washed three times with TTBS for 5 minutes each to remove any unbound primary antibody. The membrane is then transferred to a solution containing the secondary antibody at a 1:5000 dilution. The membrane is incubated for 1 hour to allow the secondary antibody to form a stable complex with the primary antibody.

3.4.6.5 Signal Detection

After incubation, the protein signals on the membrane are detected using an appropriate chemiluminescent reagent. The chemiluminescent reaction captures and records the signal

intensity of the target proteins, allowing us to evaluate the inhibitory effect of BTKi on cell proteins.

3.4.7 RT-PCR Experimental Method

3.4.7.1 Cell Treatment and RNA Extraction

The cultured cells were taken from the 37°C incubator, and 12 mL of cell suspension was aspirated using a pipette and transferred to a 15 mL Falcon tube. The tube was then placed at 4°C and centrifuged at 1200 rpm for 8 minutes. After centrifugation, the supernatant was discarded, and the cell pellet was transferred to a 1.5 mL Falcon tube. RPMI-1640 medium (without antibiotics) was added, and the contents were mixed thoroughly. Subsequently, a chloroform-containing solution was added for RNA extraction, gently mixed, and incubated for 10 minutes. The sample was then transferred to a centrifuge and centrifuged at 8000 rpm for 15 minutes at 4°C to separate the RNA pellet. The transparent liquid from the upper phase was transferred to a new 1.5 mL tube. Next, 50 μL of isopropanol was added for RNA precipitation, and the tube was stored at -20°C for 2 hours to allow the RNA to precipitate. After this step, the sample was retrieved, and centrifuged again, with the RNA pellet being washed using 75% ethanol.

3.4.7.2 RNA Resuspension and Concentration Measurement

After washing the RNA pellet, it was resuspended in 30 μL of DEPC-treated water and incubated on ice for 5-10 minutes to ensure complete dissolution. The RNA concentration was measured using a spectrophotometer (e.g., Nanodrop), ensuring that each sample had an RNA concentration of 40 ng/μL.

3.4.7.3 Reverse Transcription Reaction

An appropriate reverse transcription kit was used for RNA to cDNA synthesis. The required RNA amount was calculated based on the RNA concentration of the samples, with 40 ng/μL typically used for each reaction. The reverse transcription reaction was carried out with the following components:

10X RT Buffer: 2.0 μL

25X dNTP Mix: 0.8 μL

10X RT Random Primer: 2.0 μL

MultiScribe™ Reverse Transcriptase: 1.0 μL

RNase Inhibitor: 1.0 μL

Nuclease-free Water: Adjusted to a final volume of 10 μL

3.4.7.4 Quantitative PCR Reaction System and Experimental Steps

In this study, quantitative polymerase chain reaction (qPCR) experiments were carried out with the following reaction system, with a final reaction volume of 20 μL. The composition is as follows:

10 μL Premix Ex Taq: PCR premix reagent containing buffer, dNTPs, and Taq DNA polymerase, ensuring smooth reaction.

1 μL Taqman Probe: Specific probe for target gene amplification, using fluorescent labeling to monitor the PCR reaction in real-time.

8 μL Sterile Water: Used to adjust the total reaction volume and avoid unnecessary impurities or interference.

1 μL cDNA Template: PCR template derived from cDNA synthesized from RNA samples to ensure amplification of the target gene.

Quantitative PCR was performed to measure the mRNA levels of the following genes:

Viral transcripts: EBNA1, EBNA2, EBV, LMP1, BZLF1

Human transcripts: CD40, AICDA, GAPDH

3.4.7.5 PCR Amplification Conditions

PCR reactions were performed under the following temperature and time conditions for 40 cycles. The specific steps were as follows:

The reaction system was heated to 95°C and maintained for 30 seconds to fully activate the Taq polymerase and ensure complete denaturation of the DNA template, preparing it for the subsequent amplification steps.At the beginning of each cycle, the reaction temperature was raised to 95°C and maintained for 5 seconds to ensure the complete separation of DNA strands, generating single-stranded DNA templates.The temperature was then lowered to 60°C, and the reaction continued for 30 seconds for both annealing and extension. During this phase, primers bound to the target DNA templates, and DNA polymerase extended the new DNA strands. This step is crucial for amplification specificity and efficiency.

3.4.8 Statistical Analysis

Data analysis was performed using GraphPad Prism software, with paired t-tests used to evaluate differences between groups. All data followed a normal distribution, and paired t-tests were applied for analysis. Results were expressed as p-values, with statistical significance set at $p < 0.05$.

3.5 Results

3.5.1 Dose-Response Analysis: 24-hour and 48-hour Treatment Results

The dose-response curves for different BTKi drugs after 24 hours (Figure 1a) and 48 hours (Figure 1b) of treatment are shown. Based on the regression results of the IC50 values, we can observe the extent of the effects of each drug on cell responses and how their inhibitory efficacy changes with concentration. The IC50 values for each BTK inhibitor after 24-hour treatment (Figure 1a) are as follows:

Evobrutinib: 42.4913 μM

Tolebrutinib: 25.1281 μM

Fenebrutinib: 20.8674 μM

Orelabrutinib: 20.6257 μM

For the 48-hour treatment, the IC50 values are:

Evobrutinib: 17.6804 μM

Tolebrutinib: 9.7745 μM

Fenebrutinib: 20.2539 μM

Orelabrutinib: 15.0138 μM

Evobrutinib exhibited the highest IC50 values in both 24-hour and 48-hour response curves (42.4913 μM and 17.6804 μM, respectively). Tolebrutinib showed relatively lower IC50 values (25.1281 μM and 9.7745 μM, respectively). Fenebrutinib and Orelabrutinib displayed similar response trends after 24-hour treatment, with their IC50 values falling between those of Evobrutinib and Tolebrutinib.

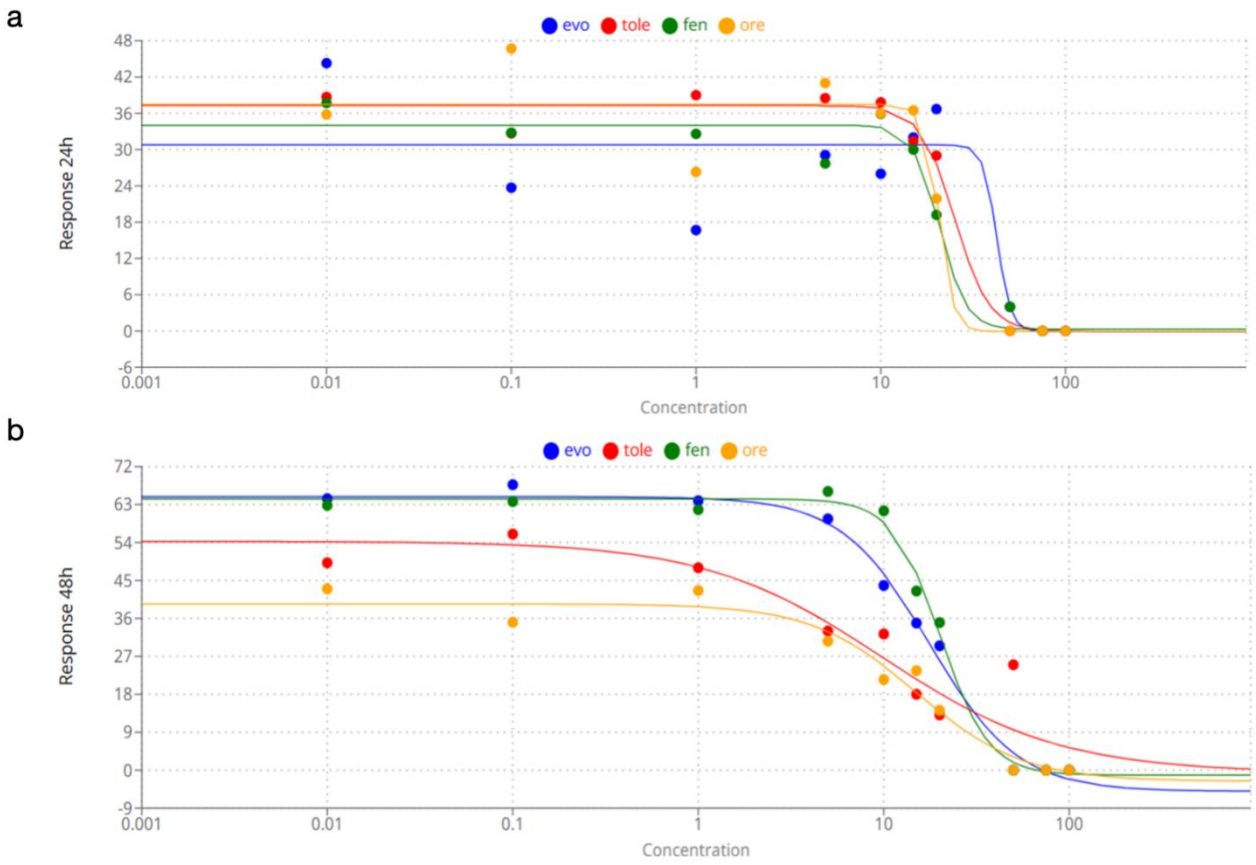

Figure 1. a) shows the dose-response curve for BTKi drugs after 24 hours of treatment. b) displays the dose-response curve after 48 hours of treatment.

3.5.2 Regulatory Effects of Evobrutinib on BTK, LMP1, and EBNA2 Expression in MS and HD Group Cells

To evaluate the effects of Evobrutinib on BTK, LMP1, EBNA2, and β-actin in cells from the MS group and HD group, we used Western blotting to assess the expression of these four proteins. In the MS group, compared to the control group (DMSO 0.1%), the expression of BTK in the Evobrutinib-treated group significantly decreased (Figure 2b, $p<0.001$). Similarly, LMP1 expression was significantly reduced in the Evobrutinib-treated group (Figure 2c, $p<0.01$). For EBNA2, the Evobrutinib-treated group showed a decrease in expression, and this difference was statistically significant (Figure 2d, $p<0.05$). In the HD group, the Evobrutinib-treated group also showed a significant decrease in BTK expression compared to the DMSO 0.1% control group (Figure 2f, $p<0.0001$). Additionally, LMP1 expression was significantly reduced in the Evobrutinib-treated group (Figure 2g, $p<0.001$).

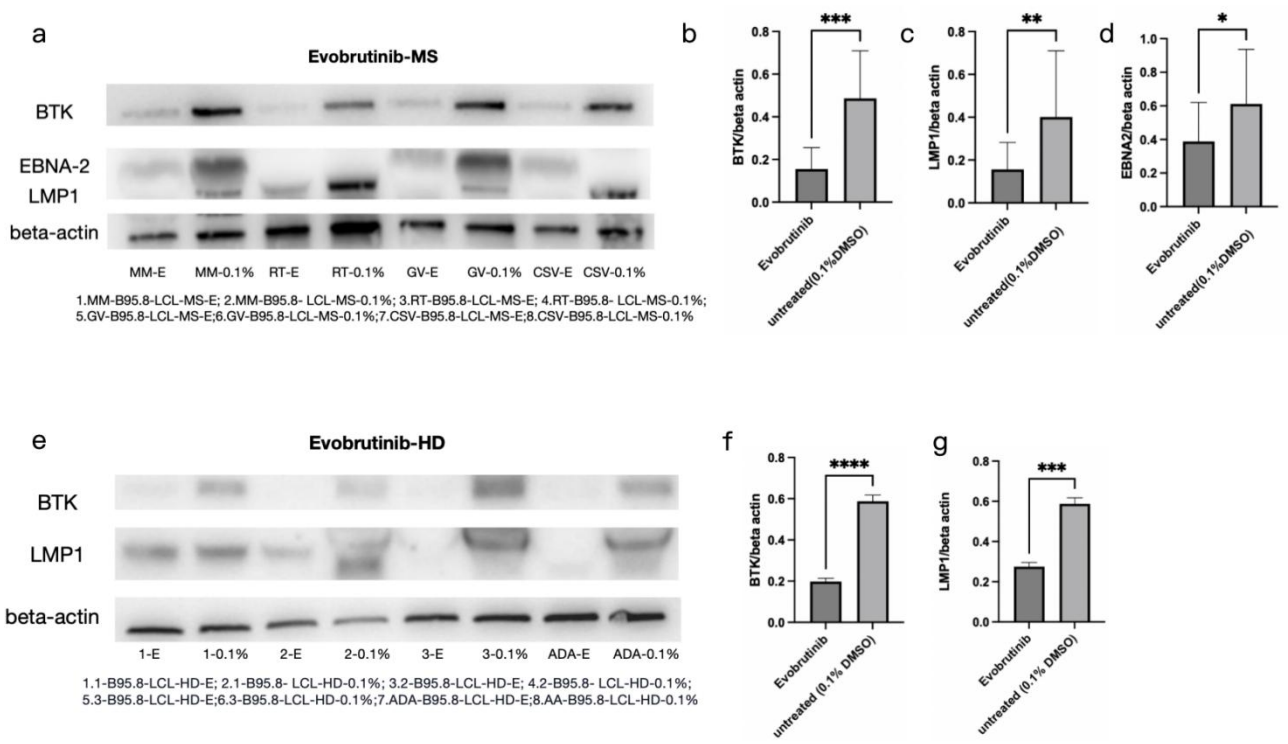

Figure 2. Effects of Evobrutinib on BTK, LMP1, and EBNA2 Expression in MS and HD Group Cells.

(a) Expression of BTK, LMP1, and EBNA2 in MS group cells after Evobrutinib treatment. (b) Western blot results show a significant decrease in BTK expression in the Evobrutinib-treated group. (c) LMP1 expression significantly decreased. (d) EBNA2 expression also decreased. (e) Expression of BTK and LMP1 in HD group cells after Evobrutinib treatment. (f) BTK significantly decreased in the Evobrutinib-treated group, and LMP1 expression significantly decreased. (g) *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.5.3 Regulatory Effects of Tolebrutinib on BTK and LMP1 Expression in MS and HD Group Cells

To evaluate the effects of Tolebrutinib on BTK and LMP1 expression in cells from the MS group and HD group, we assessed the expression levels of these two proteins using Western blotting. In the MS group, the BTK expression in the Tolebrutinib-treated group was significantly lower than that in the control group (Figure 3b, $p<0.05$), indicating that Tolebrutinib significantly downregulated BTK expression in the MS group. Furthermore, LMP1 expression was also significantly lower in the Tolebrutinib-treated group compared

to the control group (Figure 3c, $p<0.05$). In the HD group, the BTK expression in the Tolebrutinib-treated group was also significantly lower than in the control group (Figure 3e, $p<0.0001$). Additionally, LMP1 expression was significantly decreased in the Tolebrutinib-treated group compared to the control group (Figure 3f, $p<0.05$).

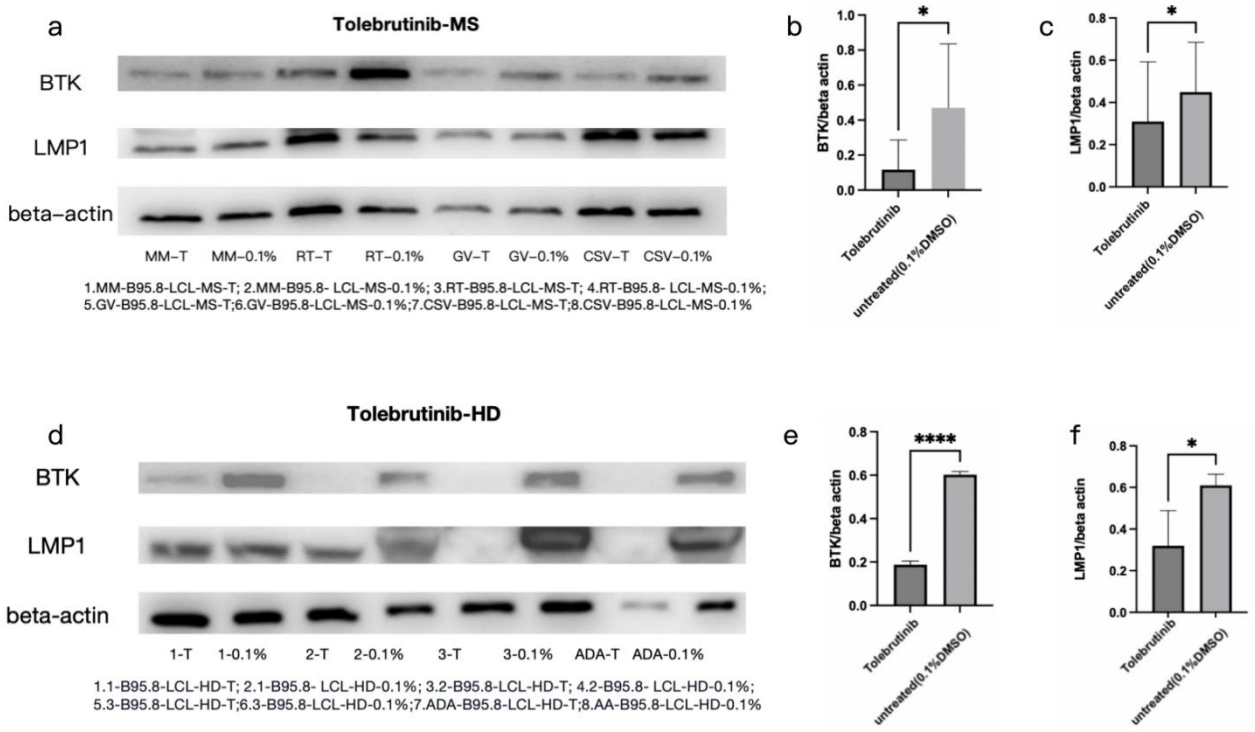

Figure 3. Effects of Tolebrutinib on BTK, LMP1, and EBNA2 Expression in MS and HD Group Cells

a) Expression of BTK and LMP1 in MS group cells after Tolebrutinib treatment. Western blot results show significant decrease in BTK expression (b) and LMP1 expression (c) in the Tolebrutinib-treated group. (e) Expression of BTK and LMP1 in HD group cells after Tolebrutinib treatment. Western blot results show significant decrease in BTK expression (f) and LMP1 expression (g). *$p<0.05$, ****$p<0.0001$.

3.5.4 Effects of Orelabrutinib on BTK and LMP1 Expression in MS and HD Group Cells

To evaluate the effects of Orelabrutinib on BTK and LMP1 expression in cells from the MS group and HD group, we performed Western blotting to detect the expression levels of BTK, LMP1, and β-actin. In the MS group, compared to the control group (DMSO 0.1%), the Orelabrutinib-treated group showed a significant decrease in BTK expression (Figure 4b, $p<0.01$). Additionally, the expression of LMP1 was significantly reduced in the Orelabrutinib-treated group (Figure 4c, $p<0.05$). In the HD group, the BTK expression in the Orelabrutinib-treated group was significantly lower than in the control group (Figure 4e, $p<0.05$). Similarly, LMP1 expression was significantly decreased in the Orelabrutinib-treated group (Figure 4f, $p<0.01$).

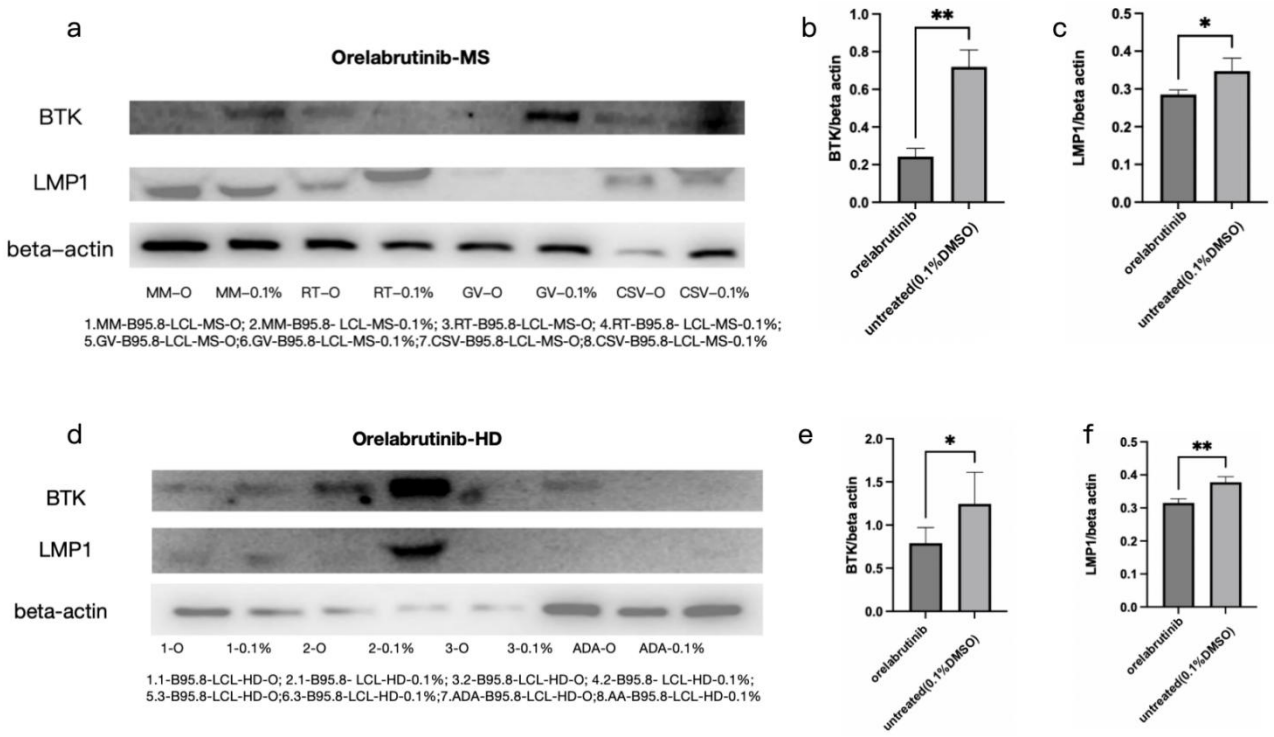

Figure 4: Effects of Orelabrutinib on BTK, LMP1, and EBNA2 Expression in MS and HD Group Cells

(a) Expression of BTK and LMP1 in MS group cells after Orelabrutinib treatment. Western blot results show significant decrease in BTK expression (b) and LMP1 expression (c). (d) Expression of BTK and LMP1 in HD group cells after Orelabrutinib treatment. Western blot results show significant decrease in BTK expression (e) and LMP1 expression (f). *$p<0.05$, **$p<0.01$.

3.5.5 Effects of Fenebrutinib on BTK and LMP1 Expression in MS and HD Group Cells

To evaluate the effects of Fenebrutinib on BTK and LMP1 expression in cells from the MS group and HD group, Western blot analysis was performed. In the MS group, compared to the control group, the BTK expression in the Fenebrutinib-treated group was significantly reduced (Figure 5b, $p<0.05$). Additionally, the expression of LMP1 was significantly decreased in the Fenebrutinib-treated group (Figure 5c, $p<0.05$). In the HD group, the BTK expression in the Fenebrutinib-treated group was significantly lower (Figure 5e, $p<0.0001$), and the LMP1 expression was also significantly reduced (Figure 5f, $p<0.05$).

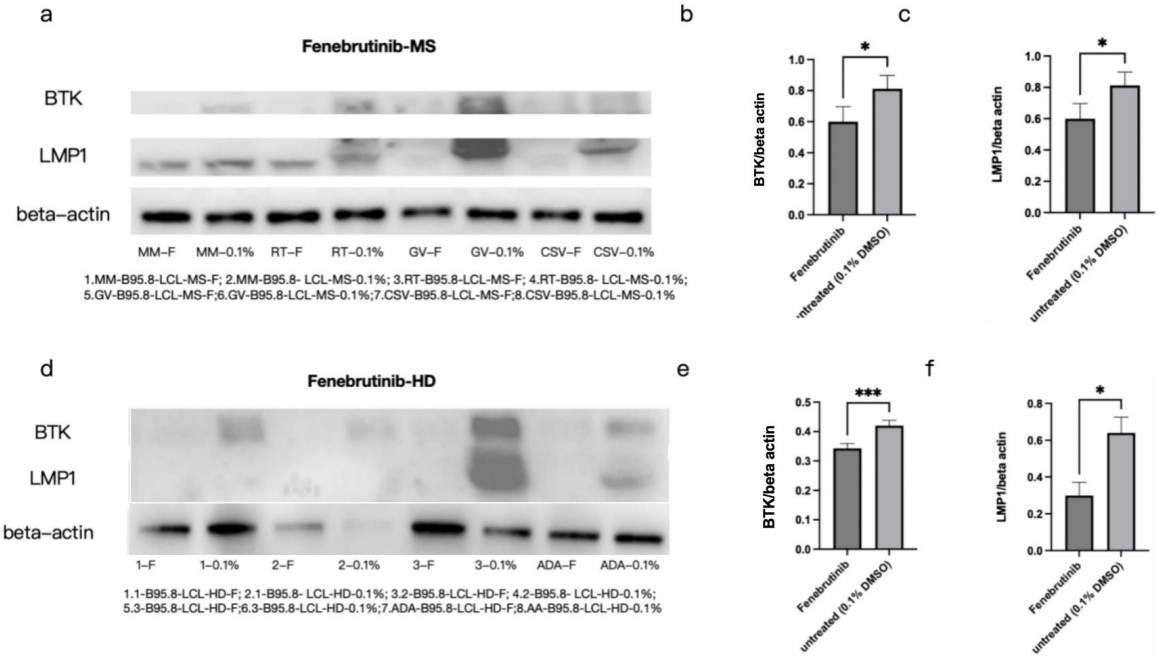

Figure 5: Effects of Fenebrutinib on BTK, LMP1, and EBNA2 Expression in MS and HD Group Cells

(a) Expression of BTK and LMP1 in MS group cells after Fenebrutinib treatment. Western blot results show significant decrease in BTK expression (b) and LMP1 expression (c). (d) Expression of BTK and LMP1 in HD group cells after Fenebrutinib treatment. Western blot results show significant decrease in BTK expression (e) and LMP1 expression (f). *$p<0.05$, ***$p<0.001$.

3.6 Effects of Different BTKi Drugs on Target Gene Expression in MS Group Cells

3.6.1 Evobrutinib

Figure 6 shows the effect of Evobrutinib on the mRNA expression of EBNA1, EBNA2, LMP1, EBV, CD40, BTK,STAT3,AICDA, and BZLF1 in MS and HD group cells. By comparing the Evobrutinib treatment group with the control group (DMSO 0.1%), we observe significant changes in the expression of certain genes. Figure 6a Further statistical analysis indicates that the expression of EBNA2, LMP1, EBV, BTK,STAT3 and CD40 in the Evobrutinib treatment group was significantly lower than that in the control group, with statistical significance (EBNA2, LMP1: $p < 0.05$; EBV,BTK: $p < 0.01$; CD40,STAT3: $p < 0.05$).Figure 6b Further statistical analysis indicates that the expression of EBNA2, LMP1, EBV, BTK and CD40 in the Evobrutinib treatment group was significantly lower than that in the control group, with statistical significance (EBNA2, LMP1: $p < 0.05$; EBV,BTK: $p < 0.001$; CD40: $p < 0.05$).

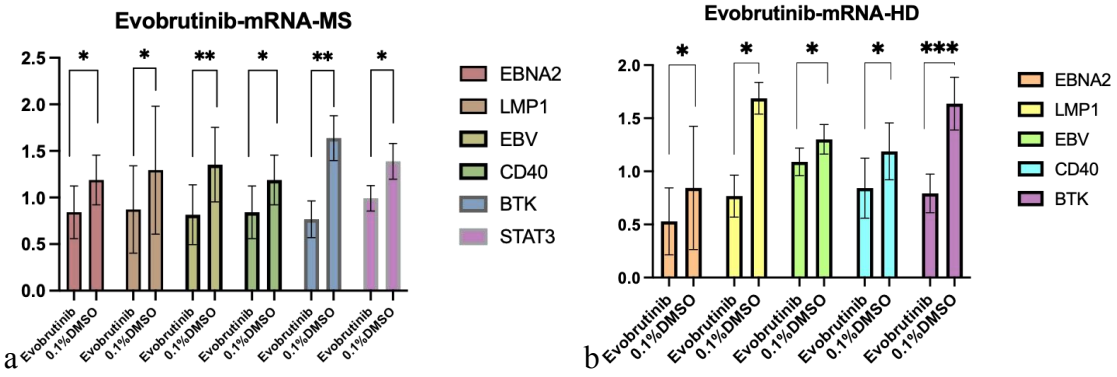

Figure 6. The Effect of Evobrutinib Treatment on mRNA Expression in MS Group Cells

a. Compare the differences in mRNA expression of various genes between the Evobrutinib treatment group and the control group in MS patients. Statistical analysis showed that after Evobrutinib treatment, the expression of EBNA2 ($p < 0.05$), LMP1 ($p < 0.05$), EBV ($p < 0.01$), BTK ($p < 0.01$), STAT3 ($p < 0.05$), and CD40 ($p < 0.05$) was significantly downregulated. b. Compare the differences in mRNA expression of various genes between the Evobrutinib treatment group and the control group in HD. Statistical

analysis showed that after Evobrutinib treatment, the expression of EBNA2 ($p < 0.05$), LMP1 ($p < 0.05$), EBV ($p < 0.05$), CD40 ($p < 0.05$), and BTK ($p < 0.001$) was significantly downregulated.

3.6.2 Tolebrutinib

Figure 7 illustrates the effect of Tolebrutinib on the mRNA expression of EBNA1, EBNA2, LMP1, EBV, CD40, BTK, STAT3, AICDA, and BZLF1 in MS and HD group cells. By comparing the Tolebrutinib treatment group with the control group (DMSO 0.1%), significant changes in the expression of certain genes were observed, further confirming the efficacy of Tolebrutinib treatment. The significance analysis in Figure 7a shows that, compared to the DMSO control group, the mRNA expression of LMP1 ($p < 0.05$), EBV ($p < 0.05$), and BTK ($p < 0.01$) was significantly downregulated in the Tolebrutinib treatment group of the MS group. The significance analysis in Figure 7b shows that, compared to the DMSO control group, the mRNA expression of LMP1 ($p < 0.05$), EBV ($p < 0.05$), and BTK ($p < 0.05$) was significantly downregulated in the Tolebrutinib treatment group of the HD group.

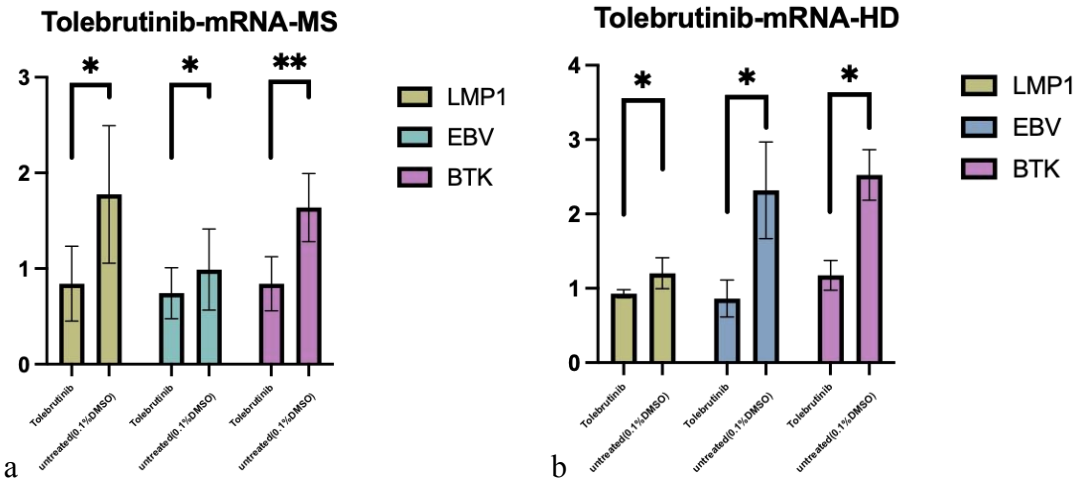

Figure 7. The Effect of Tolebrutinib Treatment on mRNA Expression in MS Group Cells

a. Compare the differences in mRNA expression of various genes between the Tolebrutinib treatment group and the control group in MS patients. Statistical analysis showed that after Tolebrutinib treatment, the expression of LMP1 ($p < 0.05$), EBV ($p < 0.05$), BTK ($p < 0.01$) was significantly downregulated. b. Compare the differences in

mRNA expression of various genes between the Tolebrutinib treatment group and the control group in HD. Statistical analysis showed that after Tolebrutinib treatment, the expression of LMP1 ($p < 0.05$), EBV ($p < 0.05$), BTK ($p < 0.05$) was significantly downregulated.

3.6.3 Orelabrutinib

Figure 8 shows the trend of mRNA expression for various genes (EBNA1, EBNA2, LMP1, EBV, BTK,STAT3,CD40, AICDA, BZLF1) in MS and HD group cells after Orelabrutinib treatment. Figure 8a presents the statistical results of gene mRNA expression in MS group cells after Orelabrutinib treatment. Statistical analysis shows that the mRNA expression of EBNA2 ($p < 0.05$), LMP1 ($p < 0.05$), EBV ($p < 0.05$) and BTK ($p < 0.05$) was significantly downregulated in the Orelabrutinib treatment group.Figure 8b presents the statistical results of gene mRNA expression in HD group cells after Orelabrutinib treatment. Statistical analysis shows that the mRNA expression of EBNA2 ($p < 0.05$), LMP1 ($p < 0.05$), EBV ($p < 0.05$) and BTK ($p < 0.05$) was significantly downregulated in the Orelabrutinib treatment group.

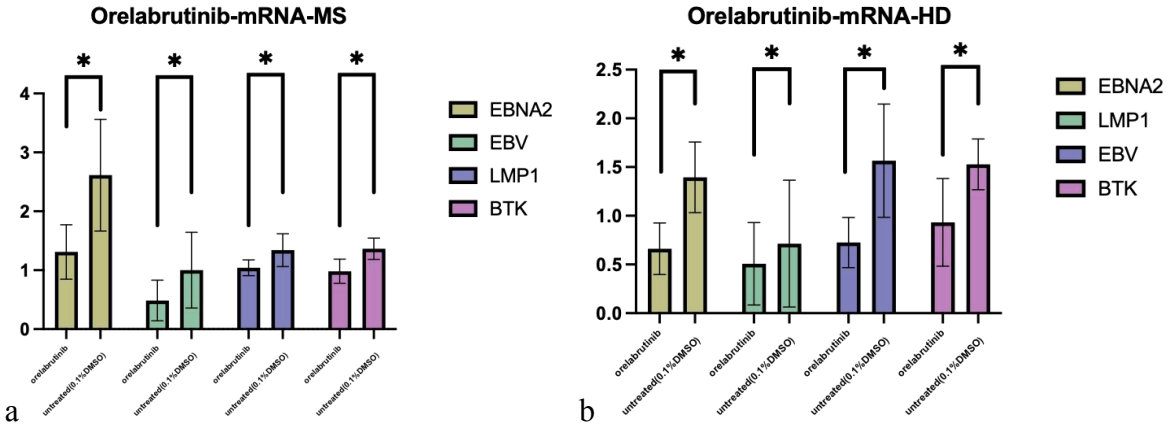

Figure 8. The Effect of Orelabrutinib Treatment on mRNA Expression in MS Group Cells

a. Compare the differences in mRNA expression of various genes between the Orelabrutinib treatment group and the control group in MS patients. Statistical analysis showed that after Orelabrutinib treatment, the expression of EBNA2 ($p < 0.05$),LMP1 ($p < 0.05$), EBV ($p < 0.05$), BTK ($p < 0.05$) was significantly downregulated. b. Compare the differences in mRNA expression of various genes between the Orelabrutinib treatment group and the control group in HD. Statistical analysis showed that after Orelabrutinib treatment, the expression of EBNA2 ($p < 0.05$),LMP1 ($p < 0.05$), EBV ($p < 0.05$), BTK ($p < 0.05$) was significantly downregulated.

3.6.4 Fenebrutinib

Figure 9 shows the mRNA expression of key genes (EBNA1, EBNA2, LMP1, EBV,BTK,STAT3, CD40, AICDA, and BZLF1) in MS group cells after Fenebrutinib treatment. Figure 9a shows the statistical comparison between the Fenebrutinib treatment group and the control group in the MS patients. The statistical chart reveals that the expression of the LMP1 ($p < 0.05$) and BTK ($p < 0.05$) was significantly downregulated in the Fenebrutinib treatment group. Figure 9b shows the statistical comparison between the Fenebrutinib treatment group and the control group in HD group. The statistical chart reveals that the expression of the BTK ($p < 0.05$) was significantly downregulated in the Fenebrutinib treatment group, while no significant differences were observed for other genes.

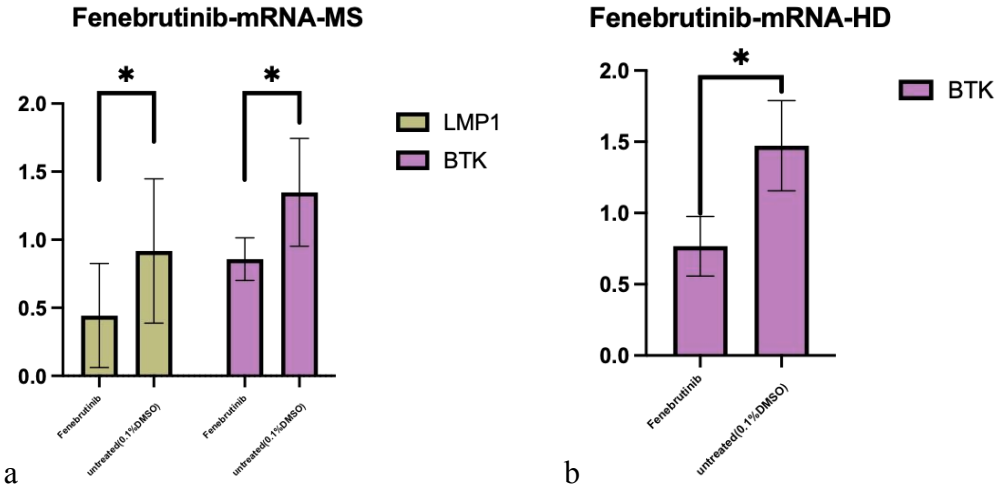

Figure 9: The Effect of Fenebrutinib Treatment on mRNA Expression in MS Group Cells

a. Compare the differences in mRNA expression of various genes between the Fenebrutinib treatment group and the control group in MS patients. Statistical analysis showed that after Fenebrutinib treatment, the expression of LMP1 ($p < 0.05$) and BTK ($p < 0.05$) was significantly downregulated. b. Compare the differences in mRNA expression of various genes between the Fenebrutinib treatment group and the control group in HD. Statistical analysis showed that after Fenebrutinib treatment, the expression of BTK ($p < 0.05$) was significantly downregulated.

3.7 Discussion

3.7.1 Summary of Findings

This study explores the regulatory effects of four BTKi — Evobrutinib, Tolebrutinib, Fenebrutinib, and Orelabrutinib — on Epstein-Barr virus (EBV)-related proteins and immune responses in Multiple Sclerosis (MS) patients. The results show that BTK inhibitors significantly suppress EBV-related proteins (such as LMP1 and EBNA2) and modulate the immune responses in MS patients, indicating the potential of BTK inhibitors in MS treatment. These findings provide new data supporting the clinical application of BTK inhibitors in MS, particularly in immune modulation and viral suppression. Understanding the interaction mechanisms between EBV, BTK inhibitors, and MS is crucial for advancing MS treatments. EBV infection has been identified as a major trigger for MS, and BTK inhibitors might offer a new therapeutic approach for MS. The novelty of this study lies in its focus on how BTK inhibitors regulate the interaction between EBV and the host immune system, filling a gap in the current literature.

3.7.2 The Role of EBV in MS Pathogenesis

The relationship between EBV and MS has been extensively studied, and our findings are consistent with existing literature, showing that EBV infection activates immune responses, particularly in B cells, leading to immune system attacks on nerve tissues, thereby triggering MS. EBV's LMP1 protein mimics the CD40 signaling pathway, further activating immune responses and exacerbating immune dysregulation and neurodegeneration. The role of immune responses, especially in the activation of B cells, is

crucial in MS. EBV infection promotes B cell proliferation and immune activation, initiating autoimmune responses. These B cells produce autoantibodies and interact with autoreactive T cells, contributing to neurodegenerative lesions. Moreover, host genetic susceptibility plays a decisive role in the relationship between EBV infection and MS, as individuals with certain genetic backgrounds may be more prone to developing MS after EBV infection.

3.7.3 Dose-Response Analysis and Its Significance in MS Treatment

The dose-response analysis revealed the half-maximal IC50 for four BTK inhibitors. Evobrutinib showed relatively high IC50 values at both 24 hours (42.49 μM) and 48 hours (17.68 μM), indicating weaker efficacy that requires higher drug concentrations for half-maximal inhibition. Although Evobrutinib's response curve gradually approached maximal inhibition after 48 hours, a longer time was required to exhibit noticeable suppressive effects.

Tolebrutinib, on the other hand, displayed relatively lower IC50 values (25.13 μM at 24 hours and 9.77 μM at 48 hours), suggesting stronger inhibitory efficacy, especially after 48 hours, where significant effects were observed even at lower concentrations.

Fenebrutinib and Orelabrutinib exhibited IC50 values of 20.87 μM and 20.63 μM at 24 hours, and 20.63 μM and 15.01 μM at 48 hours, respectively. Both drugs showed a similar response trend, with IC50 values between those of Evobrutinib and Tolebrutinib. While their suppressive effects were stronger than Evobrutinib, they were still weaker than Tolebrutinib.

The dose-response curves for these four BTK inhibitors show their inhibitory effects at different time points. Evobrutinib's effects were weaker, requiring higher concentrations for noticeable inhibition, while Tolebrutinib exhibited strong inhibition, especially after 48 hours. Fenebrutinib and Orelabrutinib fell between the two, demonstrating moderate inhibitory effects. Therefore, 48 hours was selected as the optimal time point for experiments, as all four BTK inhibitors showed stronger effects at this time, with Tolebrutinib demonstrating the most significant effect with the lowest IC50 value, indicating more substantial inhibition at the same concentration.

3.7.4 Western Blot Results Analysis

In this study, WB was used to evaluate the expression of BTK, LMP1, EBNA2, and other related proteins in cells from the MS and HD groups, and to assess the regulatory effects of different BTKi In both the MS and HD groups, Evobrutinib significantly downregulated the expression of BTK and LMP1, indicating a notable inhibition of the BTK pathway, which might exert its effects by interfering with EBV-related immune responses. In the MS group, the expression of BTK decreased significantly ($p<0.001$), and there was also a marked reduction in the expression of LMP1 and EBNA2, suggesting that Evobrutinib inhibits the synthesis of viral proteins in MS patient cells and may help alleviate the inflammatory process of MS.

Similarly, Tolebrutinib showed a similar effect in both the MS and HD groups, significantly downregulating the expression of BTK and LMP1 ($p<0.05$). In the MS group, the downregulation of BTK expression was particularly significant ($p<0.05$), indicating that Tolebrutinib may play a strong role in immune modulation, especially against virus-related immune responses. However, the effects of Tolebrutinib were also significant in the HD group, suggesting its potential for cross-population immunosuppressive action.

Orelabrutinib also demonstrated a strong downregulation effect in both the MS and HD groups, particularly inhibiting the expression of BTK and LMP1. In the MS group, the expression of BTK and LMP1 significantly decreased ($p<0.01$ and $p<0.05$, respectively). This finding, similar to those of Evobrutinib and Tolebrutinib, highlights the commonality and effectiveness of Orelabrutinib in regulating the BTK pathway and EBV-related protein expression.

In both the MS and HD groups, Fenebrutinib also significantly downregulated the expression of BTK and LMP1. Notably, in the HD group, the expression of BTK significantly decreased ($p<0.0001$), suggesting that Fenebrutinib also exerts an inhibitory effect on immune responses in healthy donor cells. The performance of Fenebrutinib in the MS group was similar to the other BTK inhibitors, further supporting its potential in modulating immune responses in MS patients.

These results demonstrate that all four BTK inhibitors (Evobrutinib, Tolebrutinib, Orelabrutinib, and Fenebrutinib) significantly downregulated the expression of BTK, LMP1,

and EBNA2 in cells from both the MS and HD groups. In the HD group, all four inhibitors (Evobrutinib, Tolebrutinib, Orelabrutinib, and Fenebrutinib) significantly downregulated the expression of BTK and LMP1, showing that these inhibitors can effectively modulate immune responses, even in the absence of disease. Although the effects were less pronounced in healthy individuals compared to MS patients, the results still demonstrate the broad immunosuppressive potential of these BTK inhibitors. By regulating these proteins, BTK inhibitors may suppress EBV-related immune responses, reducing neuroinflammation and immune responses in MS patients. This provides support for the potential of BTK inhibitors as therapeutic agents for MS. However, despite the significant inhibitory effects observed in both groups, further clinical data are needed to confirm the efficacy of these drugs, particularly regarding their long-term impact on the immune system.

3.7.5 qPCR Results Analysis

By comparing the effects of four BTK inhibitors (Evobrutinib, Tolebrutinib, Orelabrutinib, and Fenebrutinib) on the mRNA expression of target genes (EBNA1, EBNA2, LMP1, EBV, CD40, AICDA, and BZLF1) in MS group cells, we revealed the potential of BTK inhibitors in regulating EBV-related immune responses.

In the Evobrutinib-treated group, the mRNA expression of EBNA2, LMP1, EBV, and CD40 significantly decreased ($p<0.05$ to $p<0.01$), indicating that Evobrutinib can suppress EBV-related immune and inflammatory responses. The reduction in EBNA2 and LMP1 expression particularly suggests that Evobrutinib inhibits EBV replication and protein expression, thus decreasing the contribution of EBV to the pathogenesis of MS.

Tolebrutinib similarly significantly downregulated the expression of LMP1 and EBV in the MS group ($p<0.05$ and $p<0.01$). Similar to Evobrutinib, Tolebrutinib appears to play a role in immune regulation in MS by inhibiting EBV-related gene expression. Compared to Evobrutinib, Tolebrutinib showed a trend of downregulating more target genes, but only LMP1 and EBV showed statistically significant changes.

Orelabrutinib also showed significant suppression of EBNA2, LMP1, and EBV expression ($p<0.05$). These results suggest that Orelabrutinib may inhibit the activity of EBV in MS patient cells by downregulating key EBV-related genes, thus affecting the immune response in MS.

In the Fenebrutinib-treated group, the expression of LMP1 significantly decreased ($p<0.05$), but other genes, such as EBNA2, EBV, and CD40, did not show significant differences. While Fenebrutinib had a notable suppressive effect on LMP1, it did not exhibit the same broad inhibitory effects on other target genes as the other BTK inhibitors, which may suggest that Fenebrutinib's effect on immune modulation is more limited or may require higher doses or longer treatment periods to become more pronounced.

Although each drug showed a clear trend in the downregulation of target gene expression in MS group cells, statistical analysis indicated that these changes did not reach significance. Despite observing trends, the smaller sample size may have led to insufficient sensitivity in statistical testing, meaning that the effects of BTK inhibitors on some genes might be small, with changes not large enough to reach statistical significance. Therefore, increasing the sample size, optimizing experimental design, and adjusting treatment conditions in future studies may reveal more significant effects. These trending results provide valuable clues for further research, which may require additional experiments to confirm their clinical relevance.

The results of this study show that all four BTK inhibitors significantly downregulated the expression of multiple EBV-related genes in MS group cells, particularly EBNA2, LMP1, and EBV. These findings support the potential of BTK inhibitors in the treatment of multiple sclerosis (MS), particularly in suppressing EBV-related immune responses. However, there are differences in the effects of the drugs: Evobrutinib, Tolebrutinib, and Orelabrutinib significantly inhibited the expression of multiple genes, while Fenebrutinib had a more limited effect on some genes, suggesting that it may require further optimization of dosage or treatment protocols.

3.7.6 WB and qPCR Results Analysis

In this study, although both WB and qPCR results generally showed significant reductions in the target genes BTK and LMP1 across different BTK inhibitor treatment groups, the results were not entirely consistent. WB analysis indicated that all BTK inhibitors (Evobrutinib, Tolebrutinib, Orelabrutinib, Fenebrutinib) led to significant reductions in the protein levels of BTK and LMP1, with statistically significant differences. This suggests that these drugs suppressed the synthesis or stability of the target proteins during translation. However, in qPCR analysis, although most drugs showed significant

decreases in mRNA, some genes (such as EBNA2, AICDA, etc.) did not exhibit synchronized changes in mRNA levels with protein expression.

This inconsistency may be due to several factors. First, there is a complex regulatory relationship between transcription and translation, and a decrease in mRNA does not always directly lead to a corresponding change in protein levels. While qPCR technology is highly sensitive to changes in mRNA, protein stability, post-translational modifications, and degradation mechanisms may cause protein changes to lag behind mRNA changes. For example, the degradation rate of LMP1 protein may be relatively fast, making its significant reduction in WB less sensitive compared to the mRNA decline observed in qPCR. Additionally, differences in the sensitivity of the experimental methods could also contribute to the inconsistency. qPCR can sensitively detect low-level transcriptional changes, while WB's detection of protein relies on antibody specificity and sample processing, which may not fully reflect changes in low-level proteins. Therefore, even though mRNA levels significantly decreased, the changes in protein expression in WB might be slower, particularly when protein degradation is relatively slow.

The mechanisms of action of different BTK inhibitors may also contribute to this inconsistency. Under certain drug treatments, although mRNA levels significantly decrease, their impact on protein translation and stability may vary. For example, some drugs may mainly reduce mRNA expression by inhibiting transcriptional activity, with a weaker effect on protein translation inhibition. Conversely, some drugs may directly influence protein synthesis or degradation pathways, leading to more significant changes at the protein level. BTK inhibitors significantly downregulated the mRNA and protein expression of target genes in both MS and HD group cells. However, mRNA and protein changes were not fully synchronized, which may be due to post-transcriptional regulation, experimental sensitivity differences, and variations in the mechanisms of action of the drugs. Future research should combine additional analytical methods, such as protein stability studies and post-translational modification analysis, to further explore the regulatory mechanisms of BTK inhibitors in the transcription and translation processes.

3.7.7 Evobrutinib's Broad Inhibitory Effect in Immune Regulation

In the WB experiments, we observed that Evobrutinib significantly inhibited EBNA2 expression in B-cell models derived from MS patients compared to other drugs. Furthermore, qPCR analysis also showed a significant downregulation of CD40. This result piqued our interest because both EBNA2 and CD40 are key molecules closely related to immune responses and play crucial roles in the pathogenesis of MS. EBNA2 is a critical protein of EBV that is involved in the virus's latency and reactivation. It promotes abnormal activation of B cells through interaction with host cells. CD40, as a co-stimulatory molecule for B cells, directly participates in the amplification of immune responses. Therefore, the inhibition of these two important molecules by Evobrutinib prompted further consideration of its potential role in immune regulation. We further examined the effects of Evobrutinib on pro-inflammatory signaling pathways such as NF-κB and STAT3, to verify whether Evobrutinib has a multifaceted action, rather than merely targeting a single pathway.

Evobrutinib showed significant inhibitory effects on several key proteins. The results (Figure 10) indicated that Evobrutinib significantly downregulated proteins associated with EBV infection (such as LMP1, EBNA2) as well as molecules involved in the host immune response (such as CD40, NF-κB, STAT3). This suggests that Evobrutinib not only affects the activation process of EBV-related B cells but also regulates the immune response by inhibiting immune amplification pathways (such as NF-κB and STAT3). LMP1 and EBNA2 are crucial proteins in the EBV infection process, involved in the virus's latency and immune evasion. The significant downregulation of these proteins by Evobrutinib indicates that it can break the immune evasion mechanism by suppressing EBV activation, thus impacting B-cell function. NF-κB and STAT3 are transcription factors closely associated with immune and inflammatory responses. Evobrutinib's downregulation of these two molecules suggests that it effectively inhibits the overactivation of the immune system and reduces the occurrence of persistent inflammation. This is an important mechanism for controlling MS progression and reducing neurodegeneration. The downregulation of ADAR1 further indicates that Evobrutinib can weaken the adaptive mechanisms in chronic inflammation. ADAR1 plays an important role in maintaining high activity of immune

responses and cell survival, and the inhibitory effect of Evobrutinib offers potential for treating chronic active lesions.、

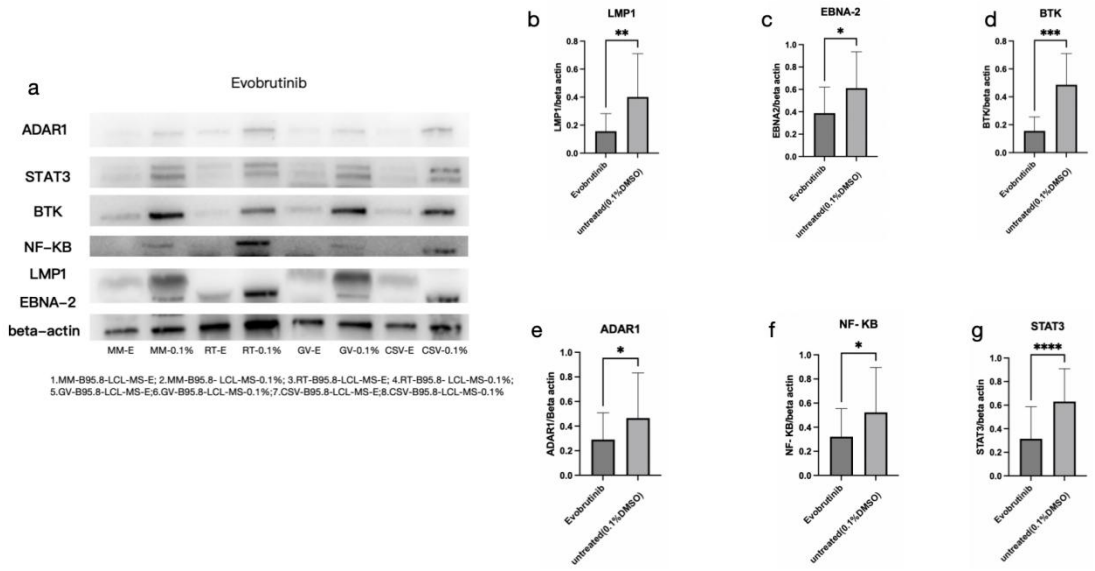

Figure 10. Effect of Evobrutinib on Key Protein Expression in MS Group

(a) shows the Western Blot analysis results of Evobrutinib in MS patient-derived B-cell models, involving proteins such as (b,c,d,e,f,g)ADAR1, STAT3, BTK, NF-κB, LMP1, EBNA-2, and β-actin. through g show the quantitative analysis of protein expression levels between the Evobrutinib-treated and untreated groups. The results demonstrate that Evobrutinib significantly downregulated the expression of LMP1, EBNA-2, BTK, NF-κB, STAT3, and ADAR1, particularly showing significant differences in the inhibition of EBNA-2 and BTK ($p < 0.05$). These results suggest that Evobrutinib exerts its effects in the immune regulation process of MS through influencing multiple key protein pathways.

3.7.8 Comparison with Existing Treatment Regimens

This study compares BTK inhibitors with existing DMTs. Current anti-CD20 monoclonal antibody therapies control immune responses by depleting B cells, but they have limitations in crossing the blood-brain barrier and regulating immune responses within the central nervous system. In contrast, BTK inhibitors can more effectively modulate the

immune environment within the central nervous system by targeting B cells and other immune cells.

The advantages of BTK inhibitors lie not only in their strong blood-brain barrier penetration capabilities but also in their targeted action, specifically inhibiting key signaling pathways in the immune system. However, these drugs still have some limitations, such as concerns about their long-term safety and varying efficacy in different MS subtypes. Further evaluation of their long-term efficacy and safety is needed in future studies.

3.7.9 Outlook

Although this study provides valuable experimental data, there are some limitations. First, the sample size is small, and the evaluation was conducted only through in vitro cell models, which may limit the generalizability of the results. Second, while the study used MS patient-derived cell lines, the immune mechanisms of MS are complex, and in vitro experiments cannot fully replicate the intricate immune responses in vivo. Therefore, future research should focus on the long-term efficacy and safety of BTK inhibitors in MS treatment, especially in patients with a larger sample size and at different disease stages. Combining clinical data will help better assess their therapeutic effects and side effects, particularly in terms of safety with long-term use. Research should explore the combination of BTK inhibitors with other immune-modulating drugs to provide more comprehensive treatment options. Additionally, combining genomics and immunology research could help us understand how different MS subtypes respond to BTK inhibitors, thus enabling personalized treatment approaches.

CHAPTER 4.

GENERAL DISCUSSION

4.1 Overall Framework Review and Key Conclusions

MS is a chronic immune-mediated disease characterized by CNS inflammation, demyelination, and axonal damage. Its clinical phenotype includes recurrent focal neurological deficits and progressively accumulating irreversible disability. Traditionally, the activity phase of MS has been understood as a process in which the peripheral immune system enters the CNS and triggers widespread inflammation. However, recent histological, imaging, and immunological data indicate that this model is inadequate for explaining the active lesions in relapsing disease. Active lesions are not solely driven by diffuse, transient inflammatory infiltrates, but rather by relatively stable, focal, cell-interaction-driven inflammatory niches. These niches are often enriched with abnormally activated B cells, infiltrating myeloid cells/microglial-like cells, and highly pro-inflammatory cytokine and co-stimulatory signal networks, closely associated with the formation of acute active lesions (new gadolinium-enhancing T1 lesions), the expansion of pre-existing white matter lesions (new or enlarged T2 lesions), and the occurrence of recent relapse events.

In this structured inflammatory niche, B cells are no longer merely sources of antibodies, but play a central role as local inflammation orchestrators. Abnormally activated B cells in the CNS (including lymphoid structures associated with meninges and chronic active

regions at the lesion borders) can simultaneously perform three functions: presenting antigens to autoreactive T cells and providing co-stimulatory signals (e.g., through the CD40 axis), continuously secreting pro-inflammatory cytokines and chemotactic signals to recruit and sustain the pro-inflammatory polarization of myeloid/microglial cells, and supporting the long-term presence of local inflammation through stable cell–cell interactions. This framework suggests that MS activity does not solely arise from systemic immune activation, but from an immune lesion structure that "lands" in the CNS, where its existence and maintenance drive pathological processes.

EBV has been highly suspected as an upstream driver of this pathogenic structure. Almost all MS patients exhibit serological evidence of past EBV infection, often significantly preceding the clinical diagnosis of MS. EBV can establish a latent infection in B cells and impart a range of non-physiological properties to these B cells through viral-encoded molecules (e.g., LMP1, EBNA2), including sustained co-stimulatory activity, abnormal survival advantages, pro-inflammatory output, and some immune evasion capabilities. These EBV-positive "pathogenic B cells" can be detected in peripheral blood and are also identified as enriched populations in patients' meningeal lymphoid-like structures and chronic active white matter lesion borders. The conclusion drawn from this is that MS activity is not merely a non-specific autoimmune response, but significantly depends on an EBV-maintained, pathogenic B cell-driven inflammatory network, at least in the relapsing phase of the disease.

Starting from this hypothesis, this dissertation progressively constructs the logic from pathogenic mechanisms to therapeutic targets and mechanism validation. Chapter 2, through a systematic review and strict inclusion of RCTs, established the evidence base: BTKi can alter short-term and medium-term activity markers of MS in a clinical setting. The included RCT evidence suggests that BTKi can significantly reduce the number of new gadolinium-enhancing T1 lesions within relatively short observation windows. Over longer assessment periods, they are associated with reduced rates of new or enlarged T2 lesion accumulation and exhibit suppressive signals in relapse risk. This indicates that BTKi can influence three interrelated outcomes with different time scales: the formation of acute central inflammation lesions (gadolinium-enhancing T1 lesions), the expansion of lesion structural burden (new or enlarged T2 lesions), and clinically identifiable relapse events (relapse rate). The cascade nature of these endpoints implies that BTKi do not only exert a mild immune-modulating

effect at the peripheral level, but might be intervening in a more core pathological process. While they demonstrate the ability to inhibit active lesion formation and influence relapse-related endpoints, the molecular question remains unanswered: do BTKi produce these clinical signals through nonspecific suppression of inflammatory output, or by directly intervening in the EBV-maintained pathogenic B cell network, rendering this network incapable of sustaining itself and amplifying the inflammatory response?

Chapter 3 provides deeper mechanistic evidence. We used an in vitro experimental model derived from MS patients: peripheral blood cells from relapsing MS patients and healthy donors were collected and infected with the EBV B95.8 strain to form individualized LCLs. These LCLs represent the pathological B cell state sustained by EBV activation in MS patients, serving as a key model for studying MS active lesions. In pharmacologically relevant concentrations of BTKi, exposed for 48 hours, we observed simultaneous inhibition of two key signaling networks. First, BTKi downregulated the EBV-dependent pathogenic B cell program: LMP1 and EBNA2 proteins were significantly reduced, their corresponding transcript levels dropped, and BZLF1, a gene associated with EBV reactivation, also showed a suppressive trend. This pattern suggests that the molecular basis of EBV maintaining these B cells in a hyperactive, long-lived, and highly stimulatory state is directly weakened. Second, BTKi inhibited the host inflammation amplification and co-stimulatory networks: CD40 expression decreased, activation signals of NF-κB and STAT3 were weakened, AICDA transcription levels were reduced, and molecules related to chronic inflammatory survival adaptation (such as ADAR1) were suppressed. This set of changes indicates that BTKi not only reduced these B cells' co-stimulatory and pro-inflammatory amplification capacities but also interfered with the driving forces required to maintain their hyperactivated state. BTKi does not merely suppress downstream inflammatory readouts of the host immune system, but weakens the coupling between the EBV-dependent pathogenic B cell program and its amplification of immune responses.

Active lesions, lesion expansion, and relapse in MS cannot be fully explained by traditional immune activation models but should be understood through the sustained interaction between the EBV-maintained pathogenic B cell network and the CNS inflammatory niche. The ability of BTKi in clinical trials to rapidly reduce new gadolinium-enhancing T1 lesions and reduce new or enlarged T2 lesions is due to its molecular mechanism of disrupting the link between this pathogenic B cell network and the host

immune system, thus inhibiting the amplification of inflammation. BTKi should be seen as a direct intervention in the pathogenic driver axis, rather than a downstream suppression of inflammation phenomena. This insight provides a new explanatory framework for the positioning of BTKi in MS treatment: they are not merely anti-inflammatory drugs, but candidate disease-modifying therapies whose targets are not only the inflammatory activity itself but also the structural network that sustains that activity.

4.2 Clinical Translation and Significance of Drug Stratification

This study presents the potential mechanisms of action of BTKi in multiple sclerosis MS and explores how to stratify drug use based on different pathological conditions. The research suggests that BTKi are not a uniform class of drugs, but rather a group of interventions with varying molecular actions, signal coverage depth, and targeted pathological layers. Specifically, different BTKi drugs exhibit varying degrees of inhibition of EBV-dependent pathogenic B cell programs and host immune amplification networks, which directly impact clinical treatment.

In the EBV-positive B cell model of MS patients, Evobrutinib is able to simultaneously inhibit multiple immune signaling pathways within 48 hours, affecting EBV maintenance and reactivation programs (e.g., LMP1, EBNA2, and BZLF1), while also suppressing co-stimulatory signals in host B cells (e.g., CD40) and inflammatory transcription networks (e.g., NF-κB, STAT3). Additionally, Evobrutinib interferes with the adaptive mechanisms that sustain B cell hyperactivity and survival in chronic inflammation (e.g., ADAR1).

The action profile of Tolebrutinib is similar to that of Evobrutinib. It not only suppresses the pathogenic programs on the viral side but also inhibits host immune amplification signals and co-stimulatory pathways. However, its suppression of certain chronic adaptive molecules, such as ADAR1, is slightly weaker compared to Evobrutinib. In contrast, Orelabrutinib and Fenebrutinib primarily affect specific nodes of the host immune amplification network, such as reducing NF-κB and STAT3 activation, but their suppression of EBV-related molecules and chronic inflammation adaptation programs is not as stable.

Therefore, the clinical application of BTKi should be more refined, rather than being used as a class of drugs interchangeably. The study recommends that future clinical trial designs should not only focus on the question of “whether BTKi are effective,” but should match the drug to the patient’s specific pathological type and the drug’s mechanism of action. For patients with acute high activity, broad-spectrum or dual-axis inhibitors would

be more appropriate, while for patients with low activity but chronic lesion expansion, drugs that target specific nodes may not be effective enough to change the disease.

Moreover, the relationship between BTKi dose and efficacy needs to be re-examined at the pharmacodynamic level. Research shows that at higher doses, BTKi can simultaneously inhibit both EBV-related pathogenic programs and host immune amplification networks, while lower doses typically only suppress some inflammatory signals and cannot fully disrupt the core functions of pathogenic B cells. Therefore, the minimum effective exposure of a drug is not simply a pharmacokinetic concept, but rather a pharmacodynamic threshold related to the pathological network layer. Only when this threshold is reached can BTKi effectively intervene in the disease's root cause. Clinical use of BTKi should consider the molecular-level differences in drug actions and select drugs based on the patient's pathological ecological niche. Additionally, future clinical trials should delve deeper into the non-linear relationship between different doses and efficacy. This strategy will help optimize the use of BTKi, enhance the treatment of MS, and provide actionable pathways for BTKi as disease-modifying therapies.

4.3 Research Limitations

Although this study proposes a coherent pathogenic model at the clinical, pharmacological, and molecular mechanism levels, and attempts to position BTKi as an intervention strategy targeting the EBV–B cell–host inflammation axis, there are several limitations that need to be clearly acknowledged. These limitations mainly relate to the extrapolation scope of the experimental system, the time scale, clinical endpoint types, and the integrity of the evidence chain.

Firstly, all intervention experiments in Chapter 4 used a unified 48-hour exposure window, with protein levels (Western blot) and transcriptional levels (qPCR) measured at this time point. This time scale does not address the issue of long-term exposure. It remains unclear whether these changes will remain stable over weeks or even months, whether adaptive compensation will occur, or whether it will induce B cells to differentiate into another non-pathogenic phenotype, or simply lead to the gradual apoptosis and clearance of these cells. Similarly, it is uncertain whether long-term BTK inhibition will induce alternative signaling pathways, allowing the remaining cells to re-establish a pro-inflammatory axis or reshape their communication with myeloid cells through non-BTK

dependent pathways. The value of disease-modifying therapies depends on whether they can persistently alter the lesion energizing structure, not just transiently suppress its activity.

The clinical endpoints integrated in Chapter 3 mainly focus on short-term activity indicators, including the number of new gadolinium-enhanced T1 lesions, the accumulation rate of new or enlarging T2 lesions, and trends in relapse rates. These endpoints are standard indicators in relapsing MS trial designs, with practical advantages like high sensitivity, short follow-up periods, and strong statistical power. However, they are not the most direct long-term functional outcomes. For patients, the most critical clinical questions are often not "whether new enhanced lesions appear in the short term," but "whether disability continues to accumulate in the medium and long term," "whether walking ability declines," "whether cognitive speed continues to slow," and "whether chronic activity lesions still expand in imaging." BTKi has been shown to quickly reduce the rate of activity lesion formation, but there is still insufficient evidence to prove it can slow irreversible disability accumulation over sufficiently long time scales and with sufficiently high statistical power.

Currently, there are no direct comparisons of different BTKi within the same patient population, under the same dosage constraints, and with the same follow-up windows, to assess their impact on the same lesion behavior. Therefore, although this study suggests that mechanisms should be differentiated between BTKi rather than treating them as interchangeable drugs, this differentiation remains prospective rather than confirmatory at the clinical level. At this stage, it cannot be ruled out that molecular differences between drugs, when exposed over sufficiently long treatment durations and high exposure levels, might eventually converge in overall clinical outcomes; or conversely, early molecular suppression differences might be amplified into long-term efficacy differences. Both possibilities have yet to undergo systematic validation.

These limitations do not undermine the main conclusions of this paper: BTKi not only reduce short-term activity indicators of multiple sclerosis in clinical trials but also inhibit host inflammation maintaining disease activity in EBV^+ B cell systems derived from patients, under pharmacologically relevant exposure conditions. However, these limitations

clearly indicate that this conclusion is not the endpoint, but rather lays the foundation for the next phase of validation work.

4.4 Future Research Directions

Future research should focus on validating the effects of BTKi in environments closer to the central nervous system. The experiments in Chapter 4 have already shown that BTKi can inhibit the aberrant activation of B cells in MS patients, but these studies were conducted in a single-cell model, lacking components such as microglia, myeloid cells, and the blood-brain barrier. Future research should adopt more complex experimental models, such as multi-cell co-culture systems or brain-like follicular models, to better simulate the actual immune environment of the central nervous system. If BTKi can still effectively inhibit B cell function and attenuate inflammation in these models, it would support the potential of BTKi as a treatment for chronic inflammatory lesions.

Long-term follow-up of MS patients receiving BTKi treatment should be conducted to verify whether “bidirectional suppression” truly occurs in vivo. Specifically, peripheral blood samples should be collected from patients at multiple time points before and after treatment (e.g., 2 weeks, 6 weeks, 12 weeks) to quantitatively detect molecular biomarkers related to EBV and inflammation amplification. These molecular changes should then be matched with the patients’ imaging results (e.g., the number and size of new lesions). If the changes in molecular biomarkers align with improvements in imaging indicators, it would confirm that BTKi intervene in the pathological process of MS through their effects on viral and host immune signaling.

It is also recommended to introduce mechanism-based patient stratification into clinical trial designs. Current trials often group all patients who meet MS diagnostic criteria under one treatment framework, overlooking differences in their underlying pathological mechanisms. This study proposes that different BTKi drugs should be selected based on the activity levels of EBV and immune response in patients. For example, patients with high EBV activity may be more suitable for “network-level suppression” or “dual-axis inhibition” BTKi, while those with low activity but chronic lesion expansion might benefit more from “partial node inhibition” BTKi. This stratification strategy can help us find the most appropriate treatment plan for each patient.

Future studies need to clarify the relationship between BTKi dosage and its efficacy. Current research has only defined the “working concentrations” of BTKi in vitro, but actual clinical use requires consideration of whether the drug concentrations in the human body are

high enough to achieve the desired molecular inhibitory effects. Future research should combine pharmacokinetic and pharmacodynamic data to determine if different doses affect the molecular mechanisms of BTKi in different ways. If higher doses of BTKi can effectively intervene in the EBV-related pathogenic B cell network, these drugs would not merely be anti-inflammatory agents but would serve as intervention tools targeting the MS pathological network.

Overall, the direction of future research will determine the role of BTKi in MS treatment. If these studies can prove that BTKi not only quickly reduce active lesions but also long-term modify the pathogenic B cell network, BTKi will no longer be seen as just anti-inflammatory drugs, but as treatment tools capable of altering the disease course. This will open up new avenues for MS treatment, breaking away from the traditional approaches of merely suppressing inflammation or depleting immune cells, and offering a new method to disrupt the immune microenvironment that sustains inflammation.

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