Dimethyl Fumarate Induced Lymphopenia in Multiple Sclerosis: A Review of the Literature

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Abstract

Dimethyl fumarate (DMF) is a first line medication for multiple sclerosis. It has a favourable safety profile, however, there is concern regarding the occurrence of moderate-severe and sustained lymphopenia and the associated risk of progressive multifocal leukoencephalopathy. We carried out an extensive literature review to understand the molecular mechanisms underlying this adverse reaction. Dynamic changes in certain components of the immune system are likely to be important for the therapeutic effects of DMF, including depletion of memory T cells and decrease in activated T cells together with expansion of naïve T cells. Similar modifications were reported for the B cell components. $CD8^+$ T cells are particularly susceptible to DMF-induced cell death, with marked reductions observed in lymphopenic subjects. The reasons underlying such increased sensitivity are not known, nor it is known how expansion of other lymphocyte subsets occurs. Understanding the molecular mechanisms underlying DMF action is challenging: *in vivo* DMF is rapidly metabolized to monomethyl fumarate (MMF), a less potent immunomodulator *in vitro*. Pharmacokinetics indicate that MMF is the main active species *in vivo*. However, the relative importance of DMF and MMF in toxicity remains unclear, with evidence presented in favour of either of the compounds as toxic species. Pharmacogenetic studies to identify genetic predictors of DMF-induced lymphopenia are limited, with inconclusive results. A role of the gut microbiome in the pharmacological effects of DMF is emerging. It is clear that further investigations are necessary to understand the mechanisms of DMF-induced lymphopenia and devise preventive strategies. Periodic monitoring of absolute lymphocyte counts, currently performed in clinical practise, allows for the early detection of lymphopenia as a risk-minimization strategy.

Keywords: Dimethyl fumarate, Monomethyl fumarate, Lymphopenia, Multiple Sclerosis, Psoriasis, Pharmacokinetics, Pharmacogenetics, Microbiome.

1. Introduction

Dimethyl Fumarate (DMF) is the most commonly prescribed oral first-line medication for multiple sclerosis (MS). The drug has a favourable safety profile with the most frequent adverse reactions being flushing and gastrointestinal complaints. The adverse reaction which has caused most concern however is lymphopenia. In the post-marketing setting, rare cases of progressive multifocal leukoencephalopathy (PML) have occurred, mainly in the presence of prolonged moderate-severe lymphopenia, *i.e.* absolute lymphocyte counts (ALCs) below 0.8 x 10^9/L for more than 6 months. Although the incidence of PML due to DMF is relatively low (Jordan et al., 2020), predicting which subjects are at greater risk for developing sustained moderate-severe lymphopenia may have important implications for clinical decisions, including the choice of disease-modifying therapy and discontinuation of DMF in order to prevent complications.

At present, the mechanisms underlying the therapeutic actions and toxicity of DMF have not been fully elucidated. DMF exerts complex immunomodulatory actions including a reduction in the different components of the immune system. Decreased lymphocyte counts are consistently observed with DMF treatment, although values tend to stabilize above the lower limit of normal in most subjects. It is not clear if lower lymphocyte counts are directly correlated with drug efficacy in MS because of conflicting data. Observational studies have also shown that depletion of memory T cells, which are the major autoreactive T cells in MS, and a decrease in activated T cells, which sustain inflammation, together with the expansion of naïve T cells can contribute to the therapeutic effects of DMF. DMF treatment also induces changes in the levels of other immune cell components which may contribute to its pharmacological action. In this article, we review what is currently known about DMF-induced lymphopenia as a basis for recognising subjects at higher risk and devising strategies to minimize the occurrence of this adverse reaction. We also highlight areas of research that need further investigation in order to understand the mechanisms underlying the effects of DMF *in vivo*, including efficacy and toxicity.

1.1. The history of dimethyl fumarate use

DMF (Tecfidera®) is an oral fumaric acid ester (FAE) registered for the treatment of individuals with relapsing-remitting multiple sclerosis (RRMS). It was approved by the US Food and Drug Administration (FDA) in 2013 and by the European Medicines Agency (EMA) in 2014 based on positive outcomes from two phase 3 clinical trials. The first study, DEFINE (NCT00420212), showed that DMF treatment resulted in a 49-50% reduction in the relative risk of relapse within a 2-year time period compared to placebo (Gold et al., 2012). DMF was administered at 240 mg either twice or thrice daily and was equally effective at both doses. Approximately 60% of the enrolled subjects were naïve to drug therapy; the remaining subjects were previously treated with other MS medications including interferon beta (IFNβ), glatiramer acetate (GA) and natalizumab. Secondary outcomes at 2 years included the assessment of the radiological evolution of disease, annualized relapse rate and time to progression of disability. All endpoints were positively affected by DMF treatment with no significant difference observed between the two dosing regimens (Gold et al., 2012). The CONFIRM study (NCT00451451), which included a cohort of GA-treated subjects as a comparator, also showed that DMF treatment significantly reduced the annualized relapse rate in people with RRMS compared with placebo. There was an improvement in the secondary outcomes of number of new radiological lesions and time to disability progression (Fox et al., 2012). This trial included a higher percentage (>70%) of

drug naïve subjects. The study was not designed to test the superiority or non-inferiority of DMF versus GA, however, the observed effect of DMF was similar to or greater than with GA (Fox et al., 2012). An ongoing long-term extension study of DEFINE/CONFIRM termed ENDORSE (NCT00835770) has largely confirmed the long-term efficacy of DMF treatment in RRMS (Gold et al., 2017).

FAEs have also been used as a treatment for psoriasis since 1959 when their therapeutic potential was discovered by the German chemist Schweckendiek (Mrowietz et al., 1998). The FAE mixture was further developed by the German physician Schafer who prescribed the treatment in association with a strict diet (Nieboer et al., 1989). Controlled clinical studies have since confirmed the efficacy of FAE mixtures and characterized DMF as the most effective compound (Nieboer et al., 1989, Nugteren-Huying et al., 1990; Altmeyer et al., 1994). Based on these results, the FAE mixture Fumaderm® was approved for the treatment of moderate-severe psoriasis in Germany in 1994. It is currently available in Germany where the dosing regimen begins at 30 mg daily of DMF with gradual weekly or monthly escalation up to 720 mg daily to prevent adverse effects. In the UK, clinical use has been limited to subjects that failed or were intolerant to the standard systemic therapy for psoriasis (Harries et al., 2005; Wain et al., 2010). In 2017, the EMA approved the first gastro-resistant oral formulation of DMF (Skilarence®), which has DMF as a single active component, for the treatment of adults with moderate-severe psoriasis (Brück et al., 2018).

2. Safety of dimethyl fumarate.

In clinical trials, DMF has been shown to have a favourable safety profile with the most frequent adverse reactions being flushing and gastrointestinal complaints, such as diarrhoea, nausea and upper abdominal pain (Gold et al., 2012; Fox et al., 2012). Transient elevations in transaminase levels, but not hepatic failure, were observed in 6% of the enrolled subjects in the DEFINE study. From studies in the psoriasis setting, it emerged that the gastrointestinal adverse reactions were mostly related to DMF, whereas abnormal liver function occurred at greater frequency in subjects treated with monoethylfumarate (MEF) (Nieboer et al., 1989). The long-term ENDORSE study provided additional evidence for the safety of DMF in people with MS. As for earlier studies, flushing and gastrointestinal complaints were the most common adverse reactions observed in the first year of treatment among those who switched from placebo or GA to DMF. Over the longer term, MS relapse is the most prevalent adverse event (as opposed to adverse reaction) in subjects continuing DMF treatment (Gold et al., 2017).

2.1 DMF-induced lymphopenia

Lymphopenia is the adverse reaction which has caused most concern. We have therefore carried out a literature review aiming i) to inform work to identify subgroups of MS subjects at higher risk of lymphopenia, ii) to understand the molecular mechanisms underlying this condition, and iii) to retrieve evidence that could be used to discriminate drug efficacy from drug toxicity. Since FAEs have been long used in the psoriasis clinical setting, epidemiologic data from this condition were also analysed aiming: i) to address if the risk of lymphopenia was similar among the two diseases and, if not, why, and ii) to identify common risk factors and pathogenic mechanisms underlying this condition in both diseases.

2.1.1. Search strategy

The literature search was carried out through the NCBI PubMed database, as detailed in Fig. 1. A total of 117 original articles, including 17 case reports were selected. Case reports primarily described infectious complications during DMF treatment, possibly linked to lymphopenia, and the main findings are reported in the section below. Of the remaining 100 original articles, 66 concerned the use of DMF in MS and 34 in the psoriasis setting. Among the 66 publications on MS, we identified 26 articles reporting epidemiological information about lymphopenia in RRMS. The main data from the 22 articles are summarized in Table 1 in direct comparison to the above-mentioned phase 3 clinical trials. In addition, 5 MS studies included assessments of different immune cells as well as lymphocyte subsets during DMF therapy, an aspect that has been further evaluated and discussed in the 'Laboratory Findings in MS subjects' section. Finally, we found 1 additional paediatric study (Makhani and Schreiner, 2016), and 2 studies related to pharmacogenetics, which are discussed in the relevant sections. Among the 34 studies on psoriasis, we identified 15 articles reporting epidemiologic information about lymphopenia during FAEs or DMF treatment in psoriasis. Data are summarized in Table 2, including the results from the initial clinical trials on Fumaderm® in Germany. Based on reported clinical studies (Tables 1 and 2), we summarize what it is known about the epidemiology of DMF induced lymphopenia, including incidence and main risk factors. We also report on the impact of DMF on the immune cell population, including lymphocyte subsets, and explore the current understanding of DMF pharmacokinetics, pharmacodynamics and pharmacogenetics as well as available data on the emerging role of the gut microbiome in DMF pharmacology.

2.1.2. Overview of lymphopenia in MS.

In both the DEFINE and CONFIRM studies, mean lymphocyte counts in DMF-treated MS subjects decreased by approximately 30% in the first year, after which they plateaued (Gold et al., 2012; Fox et al., 2012). However, severe or grade 3 lymphopenia, that is ALCs < 0.5 x 10^9/L (Common terminology criteria for adverse events, CTCAE, version 5.0 (US Department of Health and Human Services, 2019)), was observed in 4-5% of DMF treated subjects regardless of the regimen adopted. In the ENDORSE study, with a longer use of the drug, the incidence of grade 3 lymphopenia increased to 6%-9% (Gold et al., 2017). There is evidence linking sustained moderate-severe lymphopenia to an increased risk of developing PML (Jordan et al., 2020; Gieselbach et al., 2017). Several cases have been reported in the literature with the majority in lymphopenic subjects with MS (Rosenkranz et al., 2015; Baharnoori et al., 2016; Lehmann-Horn et al., 2016). Two cases have been described in non-lymphopenic subjects, but these were confounded by previous treatment with mitoxantrone and concomitant intrathecal administration of triamcinolone in the first case (Motte et al., 2018) and by previous treatment with natalizumab in the second (Diebold et al., 2019). Currently, the incidence of PML due to DMF is estimated at around 0.002%. At the end of January 2020, a total of 9 cases were identified from the literature and Biogen medical information after treatment of more than 445,000 subjects. Thus, the risk of developing PML in DMF treated subjects remains low, specifically three times lower than what has been observed with fingolimod and 100 times lower than with natalizumab (Jordan et al., 2020). In addition, a case of herpes simplex encephalitis has been recently described in a lymphopenic MS subject (Perini et al., 2018) and two cases of tinea infections, one of which occurred in a lymphopenic MS subject (Greenstein, 2018). Interestingly, a case of acute omphalitis developed after 6 months of DMF therapy in a subject with normal lymphocyte counts. It was hypothesised that a change in the gut microbiome increased the risk of infection, but clearly with one case, it is difficult to be sure of the causal association (Lorefice et al., 2016).

Due to concern over the increased risk of PML, the Tecfidera® drug label includes the EMA recommendation for assessment of complete blood counts, including lymphocytes, before starting DMF treatment. It warns that DMF has not been studied in subjects with pre-existing low lymphocyte counts and advises caution in these cases. The label recommends monitoring complete blood counts, including lymphocytes, every 3 months after starting DMF. Drug interruption should be considered in subjects showing sustained and severe lymphopenia, i.e. ALC < 0.5 x 10^9/L persisting for more than 6 months. If treatment is continued despite persistent low lymphocyte counts, increased vigilance is recommended. In case of treatment interruption due to severe lymphopenia, the label advises to monitor lymphocyte counts until recovery before restarting Tecfidera®. Given that this may increase the risk of MS relapse, in clinical practice, alternative therapeutic options are often utilised in presence of sustained severe lymphopenia. In subjects with persisting low lymphocyte counts, 0.5 - 0.8 x 10^9/L for more than 6 months, an assessment of the benefit/risk ratio should be considered.

A meta-analysis of data from 2513 MS subjects treated with DMF in pre-licensing trials showed that the incidence of grade 1 lymphopenia was 9%, grade 2 was 21% and grade 3 was 7%, considering the worst post-baseline count. However, the incidence of sustained grade 3 lymphopenia, that is persistent more than 6 months, was limited to 2.2% (Fox et al., 2016). A subsequent integrated analysis carried out on the same subjects treated with DMF over 8 years confirmed these data, with incidence of sustained grade 2 and grade 3 lymphopenia of 9.4% and 2.1%, respectively (Mehta et al., 2019). A recent phase 3 clinical trial, NCT01838668, is exploring the efficacy and long-term safety profile of DMF in the Asian population. An interim analysis, carried out on subjects of Japanese ethnicity enrolled in this trial, showed a 17% reduction in the mean lymphocyte counts after 6 months of treatment. Grade 1 lymphopenia (ALCs < 0.9 x 10^9/L and > 0.8 x 10^9/L) was detected in 5.4% of the subjects. In the second part of this study, an open-label extension of DMF treatment, grade 2 lymphopenia was detected in 6.1% of subjects that switched from placebo to DMF and in 18.9% of subjects that continued on DMF. Data were analysed at 72 weeks of follow up from the beginning of the study, that is after 12 months since the start of the open-label extension. At this time, grade 3 lymphopenia was detected in one out of 53 subjects (1.9%) allocated to the DMF/DMF group (Ochi et al., 2018). A range of real-world studies have further evaluated the epidemiology of lymphopenia in MS post-marketing (Table 1). We identified 10 retrospective chart analyses of people with MS, including 5 single centre, 2 dual-centre and 3 multicentre studies, one of which evaluated more than 1000 subjects in Italy (Mirabella et al., 2018). A first analysis on 144 subjects showed an overall prevalence of grade 2-3 lymphopenia of 14% and grade 3 lymphopenia of 6%. However, the occurrence of grade 3 lymphopenia increased up to 28.6% for treatments longer than 12 months, that is 4 out of 14 DMF-treated subjects (Longbrake & Cross, 2015). Despite this initial observation, the remaining studies largely confirmed data from pre-licensing trials, showing a rate of grade 3 lymphopenia in real practice ranging between 1 and 7%. Similarly, the incidence was found to be between 0 and 6.5% in 7 prospective observational studies, with only one study reporting a higher value, 11% (Smoot et al., 2018). Notably between 7 and 30.8% of DMF treated subjects develop moderate or grade 2 lymphopenia (Table 1). Grade 4 lymphopenia, that is lymphocyte counts < 0.2 x 10^9/L was rarely observed (Table 1), with only one study reporting a rate of 0.2% (Baharnoori et al., 2018) and four others referring no cases (Ochi et al., 2018; Mallucci et al., 2018; Sejbaek et al., 2018; Briner et al., 2019).

Subjects at higher risk of severe and sustained lymphopenia appear to be those with a rapid decline of lymphocytes in the first 6 months of therapy (Fox et al., 2016). Severe lymphopenia occurs more

frequently in subjects with lower leukocyte counts at baseline (Baharnoori et al., 2018; Longbrake et al., 2015) and for treatments longer than 12 months (Longbrake & Cross, 2015; Longbrake et al., 2015). There is also an apparent association with age; lymphopenia is more frequently diagnosed in subjects aged 55 years and older (Longbrake & Cross, 2015, Longbrake et al., 2015, Baharnoori et al., 2018; Mallucci et al., 2018; Mirabella et al., 2018; Sainz de la Maza et al., 2019). Interestingly, older subjects and subjects with basal leukocyte counts < 3.5 x 10^9/L were excluded from phase 3 trials (Gold et al., 2012; Fox et al., 2012), which may explain the higher rates of lymphopenia reported in many real-world studies. Additionally, in clinical practice, DMF treated subjects are more frequently drug-experienced (Table 1) and it is possible that pre-exposure to other therapeutics increases the risk of lymphopenia. For example, severe lymphopenia seems to be more common after exposure to natalizumab (Longbrake et al., 2015). In early studies, ethnicity and gender did not emerge as risk factors for severe lymphopenia (Longbrake & Cross, 2015). More recent studies indicate that these factors may be relevant, however more work is needed. A large retrospective multi-centre study suggested that females were less likely to develop lymphopenia (Mirabella et al., 2018). Another study of 194 DMF-treated MS subjects confirmed a significant association with age and baseline ALCs, and in addition showed an association with Caucasian ethnicity and the overweight status, but not obesity (Sierra Morales et al., 2020). In this study, the use of tobacco appeared to be a protective factor. Moreover, there were no differences in the time to achieve various levels of lymphopenia. Interestingly, lymphopenia was not detected in a study of 13 children treated with DMF for RRMS or after a single episode of neurological symptoms compatible with a diagnosis of clinically isolated syndrome (Makhani & Schreiner, 2016). Moreover, in a recent phase 2 clinical trial, evaluating the efficacy and safety of DMF at 24 weeks in 22 paediatric RRMS subjects, reduced ALCs below the lower limit of normal were found in 5 subjects, with one showing transient grade 3 lymphopenia (Alroughani et al., 2018). In contrast to the sustained lymphopenia seen in most studies in RRMS, transient grade 3 lymphopenia was detected in 3.8% of people with progressive forms of MS (Strassburger-Krogias et al., 2014).

Several studies indicate that moderate-severe lymphopenia is sustained while people are under treatment but is reversible with drug discontinuation (Longbrake et al. 2015; Fox et al., 2016; Baharnoori et al., 2018). A recent study on lymphocyte repopulation showed that in subjects developing grade 3 lymphopenia (11/246), lymphocytes recovered to values ≥ 0.8 x 10^9/L within 0.5 years after DMF discontinuation. However, the age at withdrawal of DMF was identified as a predictive factor for delayed recovery of lymphocyte counts after drug discontinuation. Notably, five subjects were re-challenged with DMF after lymphocyte counts returned to within normal values, but the drug was discontinued again due to lymphopenia in two patients treated at full dose. The other three patients were treated at lower doses and did not develop lymphopenia, although one subject experienced disease relapse (Briner et al., 2019).

2.1.3. Overview of lymphopenia in psoriasis.

Fumaderm® was approved in Germany for psoriasis in 1994 following a long history of FAE clinical use. The clinical studies that led to Fumaderm® approval were small, ranging from 12 to 101 subjects, mostly limited to 4 months of follow up and not easily comparable to the standardized phase 3 clinical trials that led to DMF approval for MS (Table 2). However, these studies show that treatment with this mixture of FAEs is associated with lymphocyte reduction, possibly caused by DMF (Nieboer et al., 1989). The Fumaderm® trials compared the FAE mixture with DMF and MEF treatment. Lymphopenia,

defined as a 20% lymphocyte reduction, occurred in approximately 50% of psoriasis subjects treated with DMF at the dose of 240 mg daily from 4 months up to 9 months, combining all the subjects treated with DMF. Severe lymphopenia was diagnosed in 4 out of 78 subjects, equal to 5% (Nieboer et al., 1989). Lymphopenia was not observed in subjects treated with MEF for 4 months nor with the Fumaderm® mixture for a mean period of 9.7 months (Table 2). Similar to MS, lymphopenia due to DMF administration in psoriasis was reversible on discontinuation of treatment (Nieboer et al., 1989). Lymphopenia was observed in 4 out of 12 (33%) psoriasis subjects treated with the Fumaderm® mixture in a subsequent study (Nugteren-Huying et al., 1990). In this study, transient eosinophilia occurred in 5 out of 12 (42%) subjects. A larger randomized placebo-controlled trial confirmed the safety profile of Fumaderm®, with gastrointestinal complaints and flushing reported as the most common treatment related adverse effects (Altmeyer et al., 1994). Data were also replicated in an open-label multicentre perspective study enrolling 101 subjects (Mrowietz et al., 1998). In these two studies the drug was administered for 4 months. No significant reductions in total leukocyte counts nor lymphocyte counts were observed in one study, whereas reduced lymphocyte counts below 50% of normal values were detected in 9.9% of the treated subjects in the second study (Table 2). In contrast, prolonged treatments with Fumaderm® in clinical practice led to reduction in peripheral lymphocyte counts in 41-63% of treated subjects (Reich et al., 2009; Thaçi et al., 2013). Moreover, the clinical use of Fumaderm® in psoriasis has been also linked to PML; a 2017 study by Murk and co-workers identified 14 cases the majority of whom were lymphopenic (Gieselbach et al., 2017). Increased risk of developing Kaposi's sarcoma has been also associated with lymphopenia (Philipp et al., 2015).

The incidence of severe lymphopenia seems to be higher among people with psoriasis than with MS. In four recent retrospective studies the incidence of severe lymphopenia was found to be higher than 10%, ranging between 11.4 and 25% (Table 2). A prospective study showed a rate of 8.75% for prolonged treatments, more than 36 months (Wain et al., 2010). DMF-treated psoriasis subjects included a greater percentage of males in comparison to MS. Moreover, they were treated with a mixture of FAEs at significantly higher doses of DMF and for longer periods of time. These differences may explain the apparent increased toxicity of DMF in the psoriasis clinical setting. Consistent with the findings in MS, lymphopenia in psoriasis subjects treated with Fumaderm® was more common in older people and in those with leuko- or lymphopenia when treatment commenced (Dickel et al., 2019). It is important to note that the rates of lymphopenia between MS and psoriasis patients are difficult to compare because of differences in clinical practice in these diseases (Mrowietz et al., 2018a). In general, however, the broad safety profile of DMF in psoriasis, as well as its efficacy, appear substantially similar to that of Fumaderm® (Mrowietz et al., 2017).

2.1.4. Relationship with efficacy.

MS and psoriasis and are immune mediated diseases. In both cases the beneficial effects of FAEs arise primarily from immune modulation (Mills et al., 2018; Mrowietz et al., 2018b). In addition, direct effects on the central nervous system as well as neuroprotective actions may contribute to the beneficial effects of DMF in MS (Mills et al., 2018). Initial studies showed that the efficacy of DMF was substantially similar in both lymphopenic and non-lymphopenic MS subjects (Longbrake et al., 2015; Fox et al., 2016). A more recent report analysing the lymphocyte levels and composition in 51 RRMS subjects treated with DMF showed that people with stable disease exhibited significantly lower ALCs compared to those with active disease under DMF treatment. The authors concluded that changes in

the cellular immune profiles (reduced ALCs, $CD8^+$ T cells and $CD19^+$ B cells) under DMF treatment might serve as a surrogate marker for treatment response (Fleischer et al., 2018). Another study reported a hazard ratio for disease relapse of 1.82 ($p < 0.001$) after 3 months of DMF treatment and of 1.73 ($p < 0.032$) at 6 months in subjects with higher lymphocyte counts (Wright et al., 2017). However, these data were not confirmed in a recently completed phase 3b study, PROCLAIM (EUDRA CT 2015-001973-42). PROCLAIM was a prospective, open-label, multicentre clinical trial that enrolled 218 MS subjects of which 27% were older than 50 years. The study showed that there was no correlation between the percentage change from baseline in ALCs and several efficacy endpoints, including the annualized relapse rate, changes in the Expanded Disability Status Scale (EDSS) score and the proportion of relapse-free subjects (Longbrake et al., 2020). Furthermore, a retrospective analysis carried out on 57 DMF treated subjects showed no significant correlation between ALCs and efficacy outcomes (Boffa et al., 2020). In contrast, lymphopenia has been suggested to be related to drug response in psoriasis (Harries et al., 2005). A retrospective observational sub-cohort study of 371 subjects showed a trend towards more rapid improvement in the severity of psoriatic skin lesions in subjects with low $CD4^+$ and $CD8^+$ T cell counts, below 0.2×10^9/L and 0.14×10^9/L respectively (Dickel et al., 2019). Similarly, in a retrospective analysis of 95 subjects, lymphocyte reductions were more pronounced in the group of subjects successfully treated with DMF (van Hezik & Bovenschen, 2019).

3. Laboratory findings

3.1. Laboratory findings in MS subjects.

Long term treatment with DMF is associated with changes in the cell composition and inflammatory activation status of the peripheral immune system. Several studies have explored this issue, both in MS and psoriasis, aiming to define the extent of DMF-induced changes and to identify immune cell populations mostly affected by the drug. In MS, most of these studies enrolled a small number of subjects, less than 50, except for one large retrospective analysis including 256 subjects at baseline (Khatri et al., 2015). There is enormous variation in data collection protocols and analyses between these studies. Different immune cell populations were studied in different studies, using a variety of surface biomarkers; different time points after initiation of therapy were considered and data were presented in numerous ways, including percentage of change from baseline or absolute counts. In Table 3, we have undertaken a comparative analysis of these studies by presenting outcomes as the percentage of change from baseline when absolute values were used, according to Zamvil and collaborators (Spencer et al., 2015). One study could not be included in the table because it did not provide baseline values for comparison (Chaves et al., 2017). Confirming observations from clinical trials, these studies showed that treatment with DMF in MS is associated with reduction in leucocyte and lymphocyte counts compared to baseline levels (Table 3). Unlike phase 3 trials, reduction in leukocytes ranged between 1.3 and 25% in the first 6 months of therapy and levels remained reduced by approximately 20% during long term treatments (Spencer et al., 2015; Wu et al., 2017). Reduction in lymphocyte counts were more pronounced than leucocytes; around 15-31% during the first 6 months of therapy with further reductions observed at later time points (Spencer et al., 2015; Khatri et al., 2015; Wu et al., 2017; Ghadiri et al., 2017; Marastoni et al., 2019). Reductions in lymphocyte counts were statistically significant at all time points analysed in comparison to baseline (Table 3). It is thus possible for DMF treated subjects to present with below-normal lymphocyte counts despite normal absolute leucocyte counts.

The peripheral $CD3^+$ T cell fraction appears to be progressively reduced during DMF treatment, with approximately 50% reduction observed after 12 months of therapy onwards (Spencer et al., 2015; Gross et al., 2015; Ghadiri et al., 2017; Marastoni et al., 2019). Within this immune cell population, several studies showed major reduction in the circulating levels of $CD8^+$ T cells compared to $CD4^+$ T cells and hence marked increase in $CD4^+/CD8^+$ ratios (Spencer et al., 2015; Khatri et al., 2015; Longbrake et al., 2016; Wu et al., 2017; Ghadiri et al., 2017; Marastoni et al., 2019). In addition, it has been found that DMF reduces the levels of activated/pro-inflammatory $CD69^+$ T cells (Wu et al., 2017) and consistently increases the levels of non-activated $CD62^{low}$ T cells (Longbrake et al., 2016). Likewise, the number of memory T cells, including both central and effector cells, was reduced compared with pre-treatment levels (Gross et al., 2015; Wu et al., 2017). In contrast, several studies reported an expansion of naïve T cell populations, encompassing both $CD4^+$ and $CD8^+$ cells, during DMF treatment (Gross et al., 2015; Longbrake et al., 2016; Wu et al., 2017). Interestingly, while the total T cell count decreases over time during DMF therapy, naïve T cells are proportionally increased in responders. The percentage of central memory T cells and effector memory T cells is reduced at 6 months in responders but unchanged in non-responders (Carlström et al., 2019). Thus, the therapeutic effects of the drug are likely to arise from both the reduction of activated T cells and the proportional increase in naïve populations.

The effects of DMF on T helper (Th) cells are not fully elucidated. Most studies showed reduction of Th1 and Th17 cells (Gross et al., 2015; Longbrake et al., 2016; Wu et al., 2017) and increases in Th2 cells (Wu et al., 2017; Marastoni et al., 2019), suggesting a shift toward a more anti-inflammatory environment. However, Marastoni and collaborators (2019) reported a 40% (albeit non-significant) reduction in the Th2 subpopulation at 24 months post therapy initiation. In the same study, 47-62% increases in the Th1 population were shown in the first 6-12 months of therapy, with 22.8% reductions observed at 24 months. The opposite results were found for Th17 cells, namely reduced values at 6 months and modestly increased values at 12-24 months (Marastoni et al., 2019). However, changes were not always significant largely because of the limited number of subjects studied. In addition, decreased levels of $IFN\gamma^+$ effector T cells (Ghadiri et al., 2017) and reduced fractions of T cells producing inflammatory cytokines, such as IFNγ, GM-CSF, IL-22 and TNFα, were also found during DMF treatment (Gross et al., 2015; Longbrake et al., 2018). No inhibitory effects were observed on T cell subsets releasing IL-17, IL-4 and IL-10 (Gross et al., 2015; Longbrake et al., 2018). The effects of DMF on regulatory T cells (Treg) also needs further evaluation; currently different studies report varying outcomes. Whilst many studies showed either between 20-50% reduction or no significant change in Treg levels (Longbrake et al., 2016; Wu et al., 2017; Marastoni et al., 2019) one study reported a 24.6% increase in Treg cells at 6 months of DMF treatment (Gross et al., 2015).

As shown in Table 3, other immune cell populations are also affected by DMF. For example, $CD19^+$ B cells appear to be significantly reduced during treatment, with 15.7-49.8% reductions observed within the first 6 months of therapy and 39.1-57% reductions after 12 months (Spencer et al., 2015; Lundy et al., 2016; Longbrake et al., 2016; Marastoni et al., 2019). However, one study reported a 25% reduction of the B cell fraction after 2 years of treatment. It is therefore possible that a certain degree of B cell recovery occurs while subjects are still under treatment; however, this observation is limited to 26 subjects (Marastoni et al., 2019). As for T cells, prolonged treatment with DMF significantly reduced the circulating levels of both activated $CD80^+$ (Longbrake et al., 2018) and memory B cells (Lundy et al., 2016; Longbrake et al., 2018). Conversely, an expansion of naïve B cells was reported at greater than 18 months post initiation of treatment (Longbrake et al., 2018). Data on $CD56^+$ NK cells

are conflicting. One study reported an approximately 20% reduction within the first year of therapy (Spencer et al., 2015), but in another study, no significant changes were found in the total NK cell population (Wu et al., 2017). In contrast, two other studies reported increased levels of NK cells (Longbrake et al., 2016; Marastoni et al., 2019). In particular, the study by Calabrese and co-workers showed significantly increased levels of NK cells in response to DMF treatment (85.9%, $p < 0.001$); however, although NK-levels were elevated at all timepoints post-initiation, the increase was non-linear over time and there was marked individual variability (Marastoni et al., 2019). In contrast, in a different study, a marked reduction in the $CD56^{dim}$ NK cell population with cytolytic activity was found after 6 months, particularly in lymphopenic subjects (Longbrake et al., 2016). A transient albeit not significant increase in the number of circulating monocytes was observed after 3 months of DMF therapy (Spencer et al., 2015). No significant changes were reported at 4-6 months (Wu et al., 2017) and 4-20% reductions in the number of circulating monocytes were observed at later time points (Spencer et al., 2015; Longbrake et al., 2016; Wu et al., 2017). Moreover, both plasmacytoid and myeloid dendritic cells (DCs) tended to be reduced by DMF (Longbrake et al., 2016). Interestingly, it has recently been shown that blood levels of isoprostane 8.12-iso-iPF2α-VI, a marker of increased oxidative stress, were significantly higher compared to baseline three months after DMF initiation and that the increase was sustained after 6 months. Accordingly, a significant upregulation of genes involved in the response to oxidative stress was detected in $CD14^+$ monocytes after 6 months of therapy in comparison to baseline (Carlström et al., 2019). These data would therefore suggest that $CD14^+$ monocytes are an early cellular target of DMF activity *in vivo*. At 3 months post DMF initiation, DMF responders had significantly higher counts compared with baseline of $CD14^+CD16^-$ cells, the main monocyte population, whereas other subsets remained unchanged. Non-responders displayed lower monocyte counts at 3 months and higher lymphocyte counts at 12 months compared to responders (Carlström et al., 2019), suggesting that reduction of lymphocytes is relevant to drug efficacy. As shown in Table 3, reduced eosinophil, basophil and neutrophil levels in response to DMF were observed at the majority of timepoints, with 4/13 measurements showing an increase (Spencer et al., 2015; Wu et al., 2017).

Recently, data from the PROCLAIM clinical study confirmed lymphocyte reductions of around 40% in comparison to baseline after 1 and 2 years of DMF treatment. Reductions appeared more marked, around 50%, in subjects older than 50 years after 2 years-treatment. Notably, 50% of the enrolled subjects never showed abnormal lymphocyte counts. The study also confirmed significant reductions ($p < 0.0001$) in the circulating levels of $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells and NK cells after 1-year of treatment that were sustained for up to 2 years. As discussed above, B cells tended to recover during DMF treatment, with a nadir (31% reduction) observed at week 24 and higher counts (13% reduction) reported after 2 years. Monocyte counts were stable during the whole study, with a modest decrease observed at 2 years. Finally, the study confirmed reductions in the memory component in comparison to naïve counterparts. Importantly, the study showed that lymphopenia is not directly linked to DMF efficacy (Longbrake et al., 2020). Taken together, these data suggest that DMF has differential effects on various immune cell populations. Both reduction and expansion of certain immune cell subsets have been observed during DMF therapy. However, there is a need for more comprehensive studies aiming to quantify the extent of change in larger cohorts, how changes in cell population are related to drug efficacy and toxicity and the mechanisms underlying these changes.

Although a mechanism for DMF toxicity has been postulated based on *in vitro* data (Ghadiri et al., 2017), as detailed in the 'Pharmacodynamics' section of this article, the determinants of cell death or

protection and proliferation remain unclear. Three studies have analysed the effects of DMF on T cell subpopulations in both lymphopenic and non-lymphopenic subjects. This work showed a more marked reduction in T cell levels and greater increase in $CD4^+/CD8^+$ ratios in lymphopenic subjects compared with the non-lymphopenic cohort (Longbrake et al., 2016; Nakhaei-Nejad et al., 2017; Chaves et al., 2017). Similarly, greater DMF-induced changes in B cell subsets were observed in lymphopenic subjects (Longbrake et al., 2018). In addition, it was recently shown that lymphopenic subjects have higher circulating levels of the chemokine CCL17 in comparison to non lymphopenic subjects and increased *in vitro* production of several pro-inflammatory and regulatory cytokines (Nakhei-Nejad et al., 2018). However, whether increased inflammation is a pathogenic mechanism leading to severe lymphopenia or a compensatory response to maintain immunity once lymphopenia occurs needs further investigation.

3.2. Laboratory findings in psoriasis subjects.

Data on peripheral immune cell modifications during FAE treatment in psoriasis are limited. Consistent with results in MS, cell immunotyping carried out during the initial clinical trials on 12 lymphopenic subjects with psoriasis showed marked reductions of T suppressor (Ts) cells leading to significant increases of Th/Ts ratio (that is equivalent to $CD4^+/CD8^+$ ratio). In 8 subjects, reduced levels of B lymphocytes were also detected (Nieboer et al., 1989). A subsequent study enrolling 10 subjects confirmed the reduction of leucocytes (20-30%) and lymphocytes (50-60%) in psoriasis subjects treated with FAEs (Höxtermann et al., 1998). This study showed reduction in the $CD3^+$ T cell component, with the $CD8^+$ T cell subset particularly affected. They also observed reduction of activated T cells in response to FAEs, as well as reductions of B cells and NK cells. A similar trend was reported by Sabat and collaborators, with reduced levels of NK cells apparently associated with the development of Kaposi's sarcoma in one HIV negative patient (Philipp et al., 2013).

4. DMF Pharmacokinetics.

Work to understand the mechanism through which DMF exerts its effects on the immune system is challenging since it is not completely clear whether DMF or a metabolite is the active and/or toxic species. DMF is rapidly hydrolysed to monomethyl fumarate (MMF, Mrowietz et al., 2018b), and thus MMF has been considered a strong candidate for the active species *in vivo* (Sheikh et al., 2013). However, as outlined in more detail in our discussion on DMF pharmacodynamics, MMF shows much lower *in vitro* activity than DMF. Several studies have shown that both DMF and MMF form glutathione (GSH) conjugates (Schmidt et al., 2007; Rostami-Yazdi et al., 2009; Dibbert et al., 2013) raising the possibility that these may be important in activity and/or toxicity.

The potential importance of MMF was identified in studies investigating the relative plasma concentrations of DMF and MMF. DMF is completely absorbed in the gastro-intestinal tract and is rapidly hydrolysed forming MMF (Mrowietz et al., 2018b). In a study on DMF pharmacokinetics in healthy subjects, peak plasma MMF concentrations were detected 2-4 h after the administration of Fumaderm®, whereas plasma levels of DMF remained below the limit of detection (Litjens et al., 2004; Rostami-Yazdi et al., 2010). The time to peak concentration for MMF was estimated around 210 min, with half-life between 38-56 min and maximal plasma concentration of 11.2 µM (1.46 mg/L) (Litjens et al., 2004; Rostami-Yazdi et al., 2010).

MMF is rapidly metabolized in the tricarboxylic acid cycle into water and carbon dioxide with excretion of metabolites occurring mainly through the lungs (Fig. 2). DMF does not bind to serum proteins while MMF shows a protein binding capacity of approximately 50% (Mrowietz et al., 1999). Thus, since DMF does not appear in the plasma, nor modify serum proteins, MMF has been considered by many to be the relevant species *in vivo*. Accordingly, the clinical development of DMF for MS was based on the pharmacokinetic profile of MMF in healthy volunteers (NCT01069913) as well as in people with RRMS (NCT00837785). However, results from these studies are not available through the clinicaltrials.gov database. In Table 4, we provide a synthetic view of MMF pharmacokinetic parameters, as reported in the Summary of Product Characteristics (SmPC) for Tecfidera®, and in subsequent studies. A randomized, double-blind, placebo-controlled study in healthy adult volunteers explored the pharmacokinetic profile of MMF during four-day treatment with DMF. The study explored the effect of giving DMF at different doses alone or in combination with aspirin, a combination proposed to prevent flushing (Sheikh et al., 2013). As shown in Table 4, results from healthy volunteers were comparable to those obtained in MS subjects. However, this study also highlighted that the plasma concentration profile of MMF is irregular with high inter-individual variability for all treatment groups. A phase 2, open-label, multicentre study evaluating the effect of DMF on radiological MS lesions and pharmacokinetics of DMF in paediatric RRMS subjects, aged 10 to 17 years (FOCUS, NCT02410200), largely confirmed the pharmacokinetic profile of MMF as consistent with data obtained in adult RRMS subjects (Alroughani et al., 2018). Collectively these data strongly support the hypothesis that the active compound *in vivo* is MMF.

However, an alternative hypothesis has been proposed based on the observed reactivity of DMF and MMF towards GSH (Fig. 2). GSH plays an important role in detoxifying electrophilic xenobiotics, either by spontaneous reaction or via the activity of glutathione-S-transferases (GST) (Gan et al., 2016). DMF readily reacts with GSH *in vitro* at physiological pH, leading to S-(1,2-dimethoxy-carbonylethyl) glutathione (GS-DMS). In contrast, *in vitro* MMF reacts slowly with GSH to give a mixture of adducts, collectively termed GS-MMS (Schmidt et al., 2007). There is strong evidence that these species are also relevant *in vivo*. Electrophilic drugs such as DMF and MMF undergo considerable phase 2 metabolism in the intestinal mucosa. It has been shown that DMF induces phase 2 detoxifying enzymes, including GST, in rat colon mucosa and liver after oral administration (Begleiter et al., 2003). More direct evidence of the role of GSH in DMF and MMF metabolism comes from a study of psoriasis subjects treated with Fumaderm®. In the kidney, GSH conjugates can be further metabolized to mercapturic acids which are then excreted in the urine (Gan et al., 2016). Rostami-Yazdi and co-workers showed that such mercapturic acids of DMF and MMF could be detected in urine 2-6 h after oral administration of Fumaderm® (Rostami-Yazdi et al., 2009). The detection of *N*-acetyl-*S*-(1,2-dimethoxycarbonylethyl)cysteine (NAC-DMS) is consistent with metabolism of GS-DMS. Thus, it is proposed that absorption of DMF is sufficiently rapid that some DMF escapes hydrolysis in the intestine and enters the portal vein blood. There it either reacts with GSH or is hydrolysed to MMF which can, in turn, react with GSH (Rostami-Yazdi et al., 2010). This is consistent with later work from the same authors showing that GSH adducts of DMF, including GS-DMF, could be detected in the rat portal vein two minutes after application of 2 ml of a DMF solution (5 mg/ml corresponding to 20 mg/kg DMF) into the small intestine (Dibbert et al., 2013). The level of free DMF at this, and all other, timepoints remained below the limit of detection; however MMF was detectable after two minutes and peaked at ~500 μM (65.05 mg/L, a concentration approximately 40 fold higher than the observed C_{max} in humans, Table 4) after 8.8 minutes. Finally, it has been shown that the only species detected

in plasma and in brain tissue 10 and 60 min after oral administration of DMF at a dose of 100 mg/kg in rats were MMF and DMF-GSH conjugate. In contrast, when the animals were treated with synthetic DMF-GSH conjugate via oral gavage at 100 mg/kg, DMF-GSH adducts could be detected in the plasma at 60 minutes post administration in only 2 out of 4 animals and at concentrations 40-fold lower than post DMF administration. DMF-GSH conjugates remained undetectable in brain at 10- and 60-minutes post dosing. Moreover, MMF was undetectable both in plasma and in brain when animals were treated with the synthetic conjugate, thus indicating that the DMF-GSH conjugate was stable and not significantly reversible to free DMF *in vivo* before absorption (Peng et al. 2016). Taken together, these strands of evidence suggest that a variable fraction of DMF may circulate in form of GSH adducts thus possibly exerting direct effects on both the immune system and the brain.

5. DMF Pharmacodynamics

Since there is strong evidence that MMF is the main active species *in vivo*, studies carried out *in vitro* using DMF should be interpreted with caution but are summarised here for completeness. DMF readily crosses the cellular membrane and reacts with many intracellular targets. In contrast, MMF does not readily diffuse across the plasma membrane due to its negative charge. Thus, it requires membrane receptors in order to produce intracellular effects (Mrowietz et al., 2018b). It has been shown that MMF can bind to the hydroxycarboxylic acid 2 (HCA2) receptor and act as a receptor agonist (Fig. 3A), with an EC_{50} of 9.4 µM, thus compatible with peripheral blood concentration measured in humans after oral administration of DMF or FAEs (Tang et al., 2008). The HCA2 receptors are abundantly expressed in adipocytes and selectively expressed in immune cells, including neutrophils, monocytes, macrophages, DCs and skin Langerhans cells (Offermanns, 2017). Activation of HCA2 receptors by MMF contributes to the anti-inflammatory and neuroprotective effects observed in DMF-treated MS patients, including a relevant reduction in neutrophil infiltration within the central nervous system (Chen et al., 2014, von Glehn et al., 2018). The activation of HCA2 receptors on Langerhans cells and keratinocytes by MMF can explain the occurrence of flushing during DMF therapy (Hanson et al., 2010; Sheikh S et al., 2013) (Fig. 3A). MMF interferes with monocyte and DC maturation as well as with macrophage polarization and proinflammatory cytokine production (Litjens et al., 2004; 2006). These anti-inflammatory effects of MMF are mediated by HCA2 receptor activation and subsequent inhibition of the nuclear transcription factor kappa B (NFκB) (Litjens et al., 2006, Digby et al., 2012). On the other hand, it is possible that a small fraction of MMF diffuses within the cells and interacts with other intracellular targets, thus mimicking the direct effects of DMF observed *in vitro* (Fig. 3B).

When studied *in vitro*, DMF appears to be a more potent immune modulator than MMF, reducing the production of several pro-inflammatory cytokines by activated human peripheral mononuclear cells and lymphocytes (Lehmann et al., 2007). In addition, DMF but not MMF exerted anti-proliferative effects on isolated human T cells (Lehmann et al., 2007) and significantly increased human T cell apoptosis *in vitro* (Treumer et al., 2003; Nicolay et al., 2016; Ghadiri et al., 2017). In a study evaluating the pro-apoptotic effects of DMF on purified human T cells from healthy donors, activated T cells underwent apoptosis at lower DMF levels than unstimulated cells. Other T cell subsets, including $CD8^+$, $CD4^+$ and $CD45RO^+$ memory T cells, behaved in a similar manner to the activated cells (Treumer et al., 2003). More recently, it has been shown that $CD8^+$ T cells and, in particular, memory T cells are more susceptible to DMF toxicity than $CD4^+$ cells *in vitro*. In contrast, MMF was largely non-toxic in these experiments (Ghadiri et al., 2017).

As described above, DMF reacts readily with intracellular GSH to produce GS-DMS reducing the availability of GSH (Fig. 4A). In immune cells, reduction in GSH levels was shown to trigger the expression of antioxidant enzymes including heme oxygenase-1 (HO-1) (Lehmann et al., 2007), a protein that can confer cellular protection against different stressors and mediate anti-inflammatory effects (Li et al., 2014). The induction of HO-1 in response to DMF was higher in monocytes than in lymphocytes, indicating a differential cellular ability to up-regulate protective mechanisms in response to similar stressors (Lehmann et al., 2007). Conversely, substantial depletion of GSH is known to increase the susceptibility of cells to apoptosis. Marked depletion of the intracellular GSH content, up to 2%, is rapidly induced by mM concentrations of DMF and GSH depleted cells are more susceptible to cell death (Held et al., 1988; 1991). However, it seems unlikely that such high concentrations of DMF would be present in blood after its administration *in vivo*, neither in rodents nor in humans. Tissue concentration of GSH is an important determinant of drug toxicity; tissues lacking GSH are more likely to experience damage due to oxidative stress. Enterocytes contain high levels of GSH levels, within the mM range (Moine et al., 2018), whereas lower amounts are detected in the immune cells (Park et al., 1998). Interestingly, both naïve and memory $CD8^+$ T cells are more significantly affected by oxidative stress than $CD4^+$ T cells, due to their lower intracellular levels of GSH (Gupta et al., 2007). Thus, it can be hypothesised that DMF exerts its toxic effects on immune cells through depletion of the cellular GSH pool.

The roles of other modifying reactions in DMF activity/toxicity have also been considered. As a thiol reactive agent, DMF can covalently modify intracellular proteins containing reactive cysteine residues in a process termed succination. A wide range of human T cell proteins including enzymes, such as serine/threonine protein kinases and phosphatases, cytokines and DNA binding factors may contain DMF-sensitive cysteine residues (Blewett et al., 2016). These proteins could potentially contribute to the immunomodulatory and cytotoxic effects of DMF. One best-characterized intracellular targets for DMF modification is Keap1 (Fig. 4A). This protein interacts with the transcription factor Nrf2 promoting its constitutive ubiquitination and proteosomal degradation (Nguyen et al., 2009). Oxidative stress or exposure to electrophilic compounds, including DMF, irreversibly modifies Keap1 and hence reduces the levels of Nrf2 degradation (Fig. 4A). As a result, Nrf2 accumulates in the nucleus where it promotes the expression of antioxidant and detoxification genes thus conferring cellular protection (Nguyen et al., 2009; Linker et al., 2011). The activation of Nrf2 has been widely shown to downregulate innate immune responses, by inhibiting the expression of pro-inflammatory genes and increasing the activity of anti-inflammatory molecules (please refer to Mohan and Gupta, 2018, for a recent review). Moreover, the activation of Nrf2 and upregulation of antioxidant/detoxifying enzymes is thought to play a role in the direct neuroprotective effects of DMF as well as in the regulation of glial immune responses (Scannevin et al., 2012; Peng et al., 2016; Brennan et al., 2017). However, the ability to activate the Nrf2 pathway appears to vary between cell types and in the brain is mostly confined to astrocytes (Metz et al., 2015). Data from Nrf2 knock out mice suggest that additional targets also contribute to the beneficial effects of DMF in MS (Schulze-Topphoff et al., 2016).

Another target is the nuclear transcription factor NFκB (Fig. 4B), whose nuclear binding is inhibited by DMF but not MMF (Gerdes et al., 2007). NFκB is a crucial regulator of inflammation and immunity as well as cell survival and proliferation (Qu et al., 2018). DMF showed pro-apoptotic effects *in vitro* on patient-derived cutaneous T-cell lymphoma cells which are characterized by constitutive activation of NFκB (Nicolay et al., 2016). The B subunit of the inhibitory κB kinase, whose activity promotes NFκB

activation, contains a cysteine residue sensitive to DMF succination suggesting a potential mechanism of action for DMF (Blewett et al., 2016). Inhibition of the inhibitory κB protein kinases by DMF was shown to reduce the nuclear translocation of NFκB and hence its DNA binding capacity (Vandermeeren et al., 2001; Loewe et al., 2001, 2002). However, in the MCF-7 breast cancer cell line DMF inhibited the nuclear translocation and the transcriptional activity of the main NFκB family member, p65 (RelA). Treatment with DMF did not change the activity of IKK, the phosphorylation status of the inhibitory protein IκBα and/or p65, thus suggesting that DMF may exert direct activity toward an alternative/downstream target. Further experiments showed that DMF can covalently modify p65 at cys38, which is an important residue for nuclear translocation and DNA binding. Interestingly, in this research paper, DMF showed anti-proliferative activity on different breast cancer cells lines *in vitro* as well as on xenograft tumours *in vivo* when administered by oral gavage at 20 mg/kg (Kastrati et al., 2016). The inhibition of NFκB induced by DMF is not mediated by activation of the Nrf2 pathway (Gillard et al., 2015).

A third intracellular target for DMF is the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 3A) which can be significantly succinated at the active site cysteine resulting in permanent loss of catalytic activity (Kornberg et al., 2018). *In vitro*, inhibition of GAPDH by DMF significantly impaired glycolysis in activated mouse peritoneal macrophages (mPMs) whilst MMF was found to be less effective. At low glucose levels (0.5 mM), addition of DMF to mPMs resulted in the reduction of pro-inflammatory cytokines, including IL-1β, IL-6 and blocked LPS-induced expression of inducible nitric oxide synthase. Increased expression of the arginase-1 enzyme, regarded as a marker of alternative macrophage activation, was observed. The anti-inflammatory actions of DMF were reduced in the presence of high, 10 mM, extracellular glucose concentrations, GADPH overexpression and stimulation of the glycolytic pathway (Kornberg et al., 2018). Thus, the inhibition of GAPDH activity in the inflammatory cells by DMF can potentially contribute to its immunomodulatory actions. It is also notable that a small fraction of monomethyl-succinated GAPDH was detected in peripheral blood mononuclear cells isolated from DMF treated MS subjects (Kornberg et al., 2018), suggesting that MMF may also have direct intracellular effects (Fig. 3B). At the molecular level, DMF is the most reactive intracellular compound and *in vitro* data suggest that it can interfere with the activity of several intracellular proteins, leading to immuno-modulation as well as cell death. However, grade 1 lymphopenia was observed in 14% of psoriasis subjects treated with XP23829, (Gottlieb et al., 2017) and grade 2 lymphopenia sustained for more than 6 months in 7.3% of MS subjects treated with diroximel fumarate (DRF), a novel oral fumarate in clinical development for MS (Naismith et al., 2019). These are both MMF releasing compounds which suggests that the efficacy of DMF, as well as its toxicity *in vivo*, are principally through MMF. There are plausible roles for DMF and MMF in attenuating the immune response in MS and psoriasis. As yet, we do not know whether one of these has the dominant effect or whether the response is rather a synergy of multiple mechanisms. Further work is also needed to establish common features across the responses observed in MS and psoriasis.

6. DMF pharmacogenetics.

There are limited pharmacogenetic data on DMF induced lymphopenia. The role of GSH in detoxification of DMF suggested that polymorphisms in GST genes may be associated with treatment outcomes. The GST-θ class member 1 (GSTT1) shows three distinguishable phenotypes in humans: non-conjugator with homozygous deletion of GSTT1, the low-conjugator heterozygote and the high-

conjugator phenotype expressing two functional alleles. A study of FAE-treated psoriasis subjects investigating whether responder status and/or occurrence of side effects is associated with allelic variants and enzymatic activity of GSST1 showed that the non-conjugator GSTT1 *0/0 phenotype was associated with an increased risk of developing lymphopenia ($p = 0.036$, odds ratio: 6, 95% CI: 1.1 to 32) (Gambichler et al., 2014). No other significant associations were found between genetic variants and/or phenotypes and clinical parameters, including drug efficacy and other toxicities. A more recent study investigated the role of GSTM1 and GSTP1 polymorphisms on FAE response in psoriasis. The SNP rs1645 on exon 5 of the GSTP1 gene (105 Ile→Val) was characterized as a predictor of response to FAEs treatment in psoriasis, with the Val/Val GSTP1 genotype significantly associated with non-responders (Gambichler et al., 2016). Subjects with the Val/Val GSTP1 genotype show substrate-specific variation in GST activity. For example, subjects hetero- and homozygous for the GSTP1 Val alleles show greater activity against platin derivatives than the wild-type. It has been hypothesised that failure of FAEs is due to increased activity of GST towards the active compound(s). A recent study carried out in MS showed a significant association between the production of free oxygen radicals (ROS) by monocytes and subsequent lymphocyte reduction in response to DMF, with both effects linked to drug efficacy. Moreover, the NADPH Oxidase 3 rs6919626 minor G allele was significantly associated with reduced ROS generation in monocytes and reduced treatment efficacy (Carlstrom et al., 2019). Epigenetic analyses characterized '3 months' post treatment initiation as a critical time window to detect relevant variation of DNA methylation in monocytes, especially in pathways related to ROS production and oxidative stress. In contrast to monocytes, changes in DNA methylation were primarily detected in $CD4^+$ T cells at 3-6 months after DMF initiation. Hyper-methylation was detected in genes involved in regulation of T cell differentiation, in particular those determining Th17 and the Th17/Treg balance, migration, development and apoptosis. The data suggest that the extent of ROS production by monocytes influences the magnitude of lymphocyte depletion. Consistent with this hypothesis, the spontaneous generation of free oxygen radicals by monocytes, which is significantly high in MS subjects, was reduced by treatment with DMF independently of the responder status. However, DMF-responders showed increased ROS production *ex vivo* in response to the bacterial endotoxin LPS. Spontaneous ROS production was unaltered in granulocytes and lymphocytes (Carlstrom et al., 2019). This stresses the role of innate immunity as an early cellular target of DMF/MMF action *in vivo*. In fact, alterations of Nrf2, NFκB, hypoxia inducible factor 1α and fatty acid oxidation pathways occurred predominantly in $CD14^+$ peripheral monocytes in response to DMF (Carlstrom et al., 2019).

More recently, a genome-wide association study failed to confirm these findings or to detect common genetic variants, including imputed HLA variants, as predictors of prolonged severe and/or moderate lymphopenia in MS subjects treated with DMF (Sangurdekar et al., 2019). In this study, the GSTT1 gene deletion on chromosome 22 was not directly tested. The study however evaluated baseline expression level of GSTT1. This was similar across the different groups and it was not associated with development of lymphopenia. The study confirmed that lymphopenic subjects were on average older and had lower basal ALCs at the beginning of treatment (Sangurdekar et al., 2019). A similar case-control study is currently being undertaken at the Djavad Mowafaghian Centre for Brain Health MS clinic, located at the University of British Columbia, Vancouver, Canada (Kowalec K et al., 2017).

7. DMF and the gut microbiota.

People with MS have a distinct gut microbiota profile that potentially affects the aetiology of disease as well as its progression (Sauma & Casaccia, 2020). The gut is a site of important interactions between environmental factors and the immune system. Different drugs can modify the gut microbiota composition, favouring certain microbial species over others and thus modulating immune responses. Conversely, the gut microbiota can also contribute to drug metabolism, including inactivation of the parent compound or production of metabolites with different effects. Therefore, the gut microbiota has the potential to interfere with both drug efficacy and toxicity (Lam et al., 2019). With respect to DMF, bacteria or other microbial species that are able to metabolize fumaric acid may have a proliferative advantage in response to the drug (Jones et al., 2011; 2007; Eppinga H et al., 2017). On the other hand, DMF can exert antimicrobial effects *in vitro* (Wang et al., 2001). In a recent study, it was shown that DMF can inhibit *in vitro* the growth of the anaerobic gut bacterium, *Clostridium perfringens* (Rumah et al. 2017). Serotypes B and D of *Cl. Perfringens*, which are not normally present in the gut in physiological conditions, produce a bacterial neurotoxin, the epsilon toxin, characterized as a major player in MS demyelination, due to its tropism for the blood brain barrier and the myelin sheath (Rumah et al.,2013; Linden et al., 2015). In the human gut, DMF, at therapeutic doses, can reach intraluminal concentrations 10-20 times higher than the minimal inhibitory concentration (MIC_{95}) characterized *in vitro* for the inhibition of *Cl. Perfringens* growth (Rumah et al. 2017). Thus, the possibility exists that DMF interferes with the microbiota composition of MS treated subjects, and such modifications may contribute to its beneficial as well as toxic effects. However, data on this topic are still limited. One study, enrolling MS subjects that were stable on DMF treatment for at least 3 months, reported no significant changes in the overall microbial structure in response to DMF (Katz Sand et al., 2018). However, the relative abundance of certain species was altered by the treatment, with significant increases in *Bacteroidetes* and a reduction in *Firmucutes* observed in DMF treated subjects in comparison to MS drug naïve. The increase in the relative abundance of *Bacteroidetes* was mainly due to the genus *Bacteroides*, with almost statistic significant increases. *Bacteroides* are reduced in the gut microbiota of MS subjects (Miyake et al., 2015). Considering the immunomodulatory role of *Bacteroides* on innate immunity (Farrokhi V et al., 2013; Fujiwara M et al., 2018), this finding may be relevant for the anti-inflammatory actions of DMF. In addition, several *Clostridiales* species were also modified by DMF treatment, being mostly reduced (Katz Sand et al., 2018). In contrast, an increase in *Firmicutes* with a small reduction of *Bacteroidetes* was detected after 12 weeks of treatment with DMF in a prospective study (Storm-Larsen et al., 2019). DMF also increased basal levels of *Faecalibacterium*, a species frequently reduced in the MS microbiome (Cantarel et al., 2015; Miyake et al., 2015; Glenn et al., 2016; Cignarella et al., 2018; Zeng et al., 2019). A transient reduction of *Actinobacteria,* mainly driven by reduced levels of *Bifidobacterium,* was observed after two weeks of DMF treatment; this modification was not associated with development of gastrointestinal symptoms (Storm-Larsen et al., 2019). The main limitation of these studies is the small number of subjects. However, data suggest that alterations in the gut microbiota/microbiome can occur in response to DMF. Further investigations are needed characterize these changes and to address whether they are indeed important for the immune modulatory action of DMF as well as for the development of lymphopenia.

8. Conclusions

Taken together these data suggest that DMF exerts complex immunomodulatory actions including, at least in part, a reduction of different components of the immune system. Collectively the depletion of memory T cells, which are the major autoreactive T cells in MS, a decrease in activated T cells, which

sustain inflammation, and the expansion of naïve T cells can contribute to the therapeutic effects of DMF. Similar changes in the B cell component may also have a role in the beneficial effects of DMF. However, it is not currently clear why a small number of subjects develop more severe and prolonged lymphopenia, nor whether this depends on higher levels of circulating DMF or its GSH conjugates. The latter seem to be quite stable compounds, although few studies have investigated their pharmacological role. Lymphopenic subjects are usually older and have lower baseline leukocyte counts than non-lymphopenic subjects, suggesting failure in lymphopoiesis as a trigger factor for severe lymphopenia during DMF treatment. This hypothesis is in part supported by the fact that toxicity seems to be cumulative over time and is dose dependent, with more severe reductions observed for prolonged treatments and in psoriasis subjects treated with higher doses of FAEs. The $CD8^+$ T cells appear to be the subset of immune cells most susceptible to DMF induced cell death, with marked reductions in $CD8^+$ counts observed in lymphopenic subjects. However, the reason(s) underlying such increased sensitivity are not known, and nor is it known why there is an expansion of other lymphocyte subsets.

Pharmacokinetic studies showed that DMF is rapidly metabolized to MMF *in vivo*, albeit both DMF and MMF can circulate as GSH-conjugates. Whether in this form they can be targeted to the immune system as well as the brain is matter of speculation. The relative importance of DMF and MMF in toxicity remains unclear – there is evidence for each of them being the toxic species. Most of the physiological actions of MMF are mediated by membrane HCA2 receptors. Lymphocytes do not express HCA2 receptors (Offermanns, 2017), supporting the hypothesis that lymphopenia may be a direct toxic effect of DMF or a consequence of increased ROS production by monocytes, emerging as a first cellular target. Detection of monosuccinate GAPDH in peripheral mononuclear cells purified from DMF treated MS subjects also suggests that a proportion of MMF may enter the cells. Pharmacogenetic studies are limited; while a small gene candidate study indicated GSTT1 genotype was a predictor for developing lymphopenia in psoriasis, a more recent study, carried out in MS, showed a significant association between the production of ROS by monocytes and subsequent lymphocyte reductions in response to DMF, with both effects linked to drug efficacy. However, these data were not confirmed in a larger genome wide association study, although the numbers studied in this study were small.

In conclusion, the mechanisms of DMF therapeutic action and toxicity are not clear. We have identified five major areas of research that need further investigation in order to understand the molecular basis of DMF induced lymphopenia and devise preventive measures (Box. 1). At the present, it is indeed possible to identify subjects at higher risk of developing severe lymphopenia through monitoring of baseline blood counts but not stratify treatment based on a specific molecular marker. Thus, monitoring of lymphocyte counts at present remains the most feasible strategy to limit drug toxicity.

Figures and Figure legends

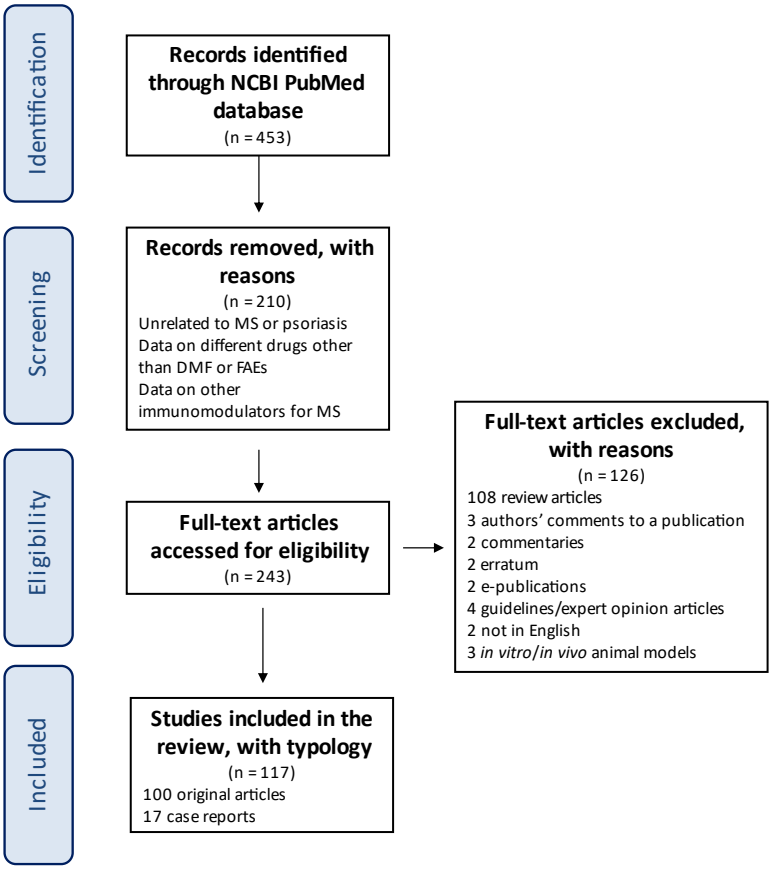

Figure 1

Fig. 1. Search strategy and flow diagram followed for the literature review. The diagram shows the search strategy adopted to select papers related to lymphopenia in MS and psoriasis. Briefly, 453 papers were retrieved in the NCBI PubMed database, accessed on 9 January 2020, using ((dimethyl fumarate) OR (dimethylfumarate) OR (fumaric acid esters) OR (fumaderm)) AND ((lymphopenia) OR (lymphocytopenia) OR (safety)) as keywords. From screening of the abstracts or the papers (when abstracts were not available), 210 papers were excluded. Among the full-text articles accessed for eligibility, 108 review articles and 18 other papers were excluded as detailed in the diagram. This leaves 100 original articles and 17 case reports. Of the remaining 100 original articles, 66 focused on the use of DMF in MS and 34 in the psoriasis setting.

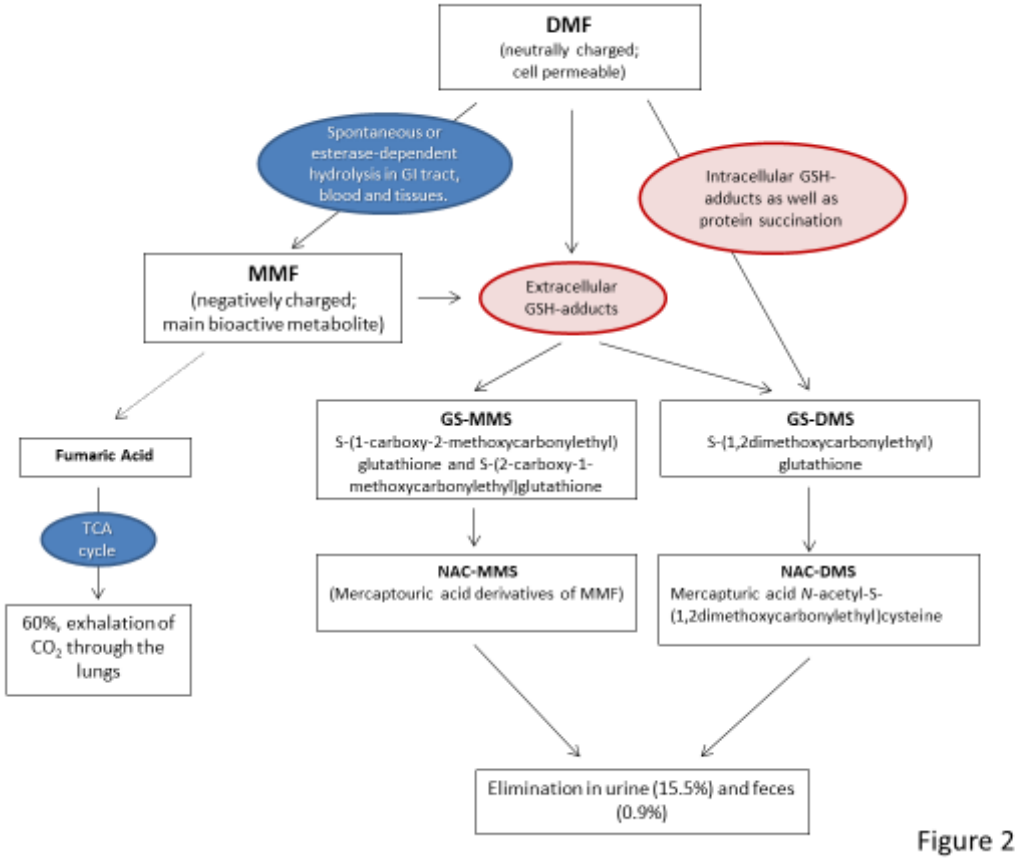

Figure 2

Fig. 2 Main metabolic pathways involved in DMF elimination. *In vivo*, DMF is rapidly hydrolysed forming MMF that is further metabolized in the citrate cycle (TCA cycle) into water and carbon dioxide (CO_2) with excretion of metabolites occurring mainly through the lungs. DMF can also reacts with GSH forming both intracellular and extracellular adducts, namely S-(1,2-dimethoxy-carbonylethyl)glutathione (GS-DMS). In contrast, MMF slowly reacts with GSH to give a mixture of adducts, collectively termed GS-MMS. GSH conjugates can be further metabolized to mercapturic acids which are then excreted in the urine and faeces. The percentages of drug eliminated by different routes are derived from the Summary of Product Characteristics of Tecfidera®. GI, gastrointestinal tract; GSH, glutathione.

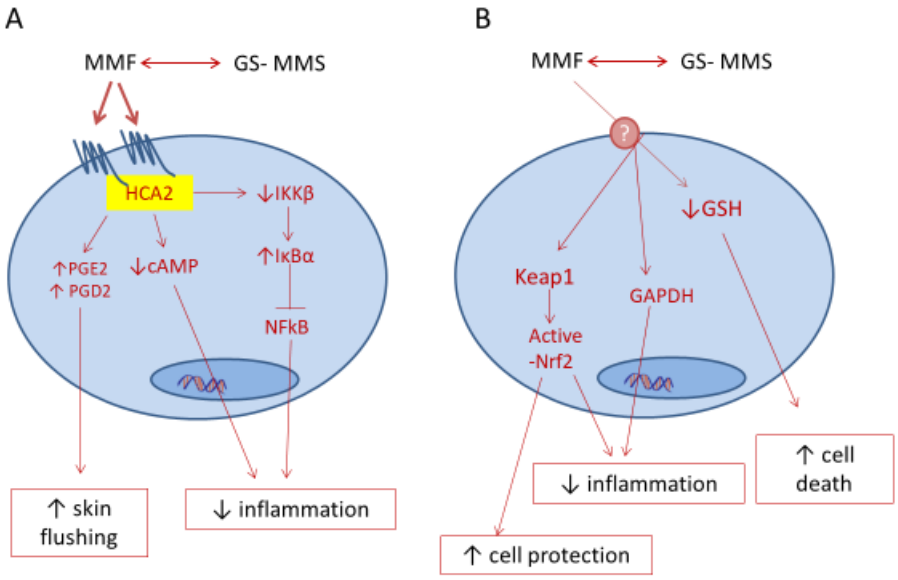

Figure 3

Fig. 3 Main receptors and intracellular pathways modulated by MMF. MMF does not readily diffuse across plasma membranes due to its negative charge. **A.** MMF binds to the hydroxycarboxylic acid receptor 2 (HCA2) and acts as a receptor agonist, with an EC_{50} of 9.4 µM. The HCA2 receptor is a Gi/Go protein coupled receptor, also known as GPR109A. Activation of these receptors is associated with inhibition of adenylate cyclase and reduced level of intracellular cyclic-AMP (cAMP). In addition, MMF inhibits the activation of nuclear factor kB, NFkB, thus explaining its anti-inflammatory actions. On the other hand, activation of HCA2 receptors on keratinocytes and Langerhans cells, is associated with increased local production of prostaglandin (PG)E2 and PGD2, dermal vasodilation and flushing. **B.** Minimal diffusion of MMF has been postulated, possibly facilitated by as yet unknown transporter. Intracellular MMF can lead to protein succination and GSH depletion, thus eliciting similar intracellular responses to DMF (see Fig. 4).

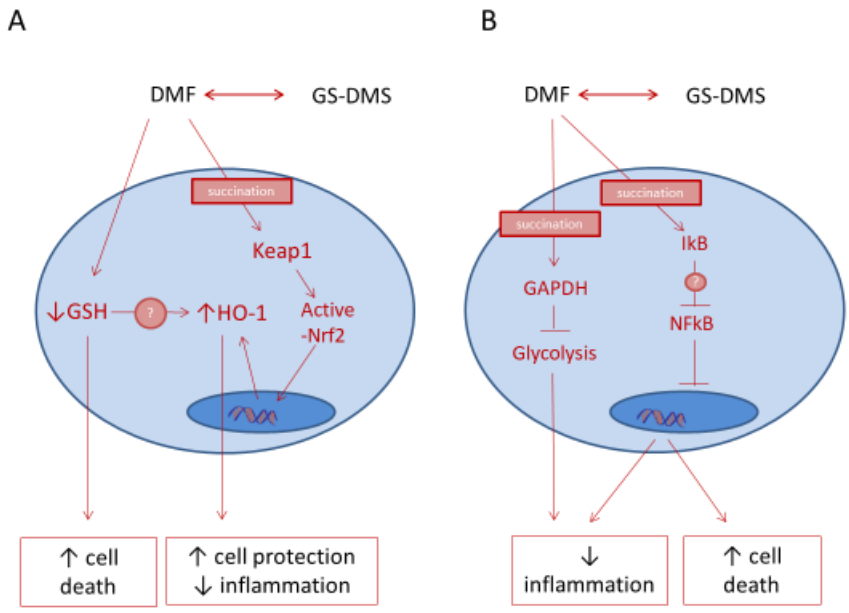

Figure 4

Fig. 4 Main intracellular targets and signalling pathways modulated by DMF. DMF readily crosses the cellular membrane, interacting with many intracellular targets, some of which are reported in the Figure. **A.** DMF readily reacts with intracellular GSH, thus reducing its availability. In immune cells, reduction in GSH levels can trigger the expression of antioxidant enzymes including heme oxygenase-1 (HO-1), thus conferring cell protection. Increased HO-1 expression can also mediate anti-inflammatory effects. On the other hand, marked depletion of the intracellular GSH content can increase cell death. In addition, as a thiol reactive agent, DMF can covalently modify intracellular proteins containing reactive cysteine residues in a process called succination. One intracellular target is Keap1, a protein that normally promotes the proteosomal degradation of the transcription factor Nrf2. As a result of DMF dependent Keap1 modifications, Nrf2 accumulates in the nucleus where it promotes the expression of antioxidant and detoxification genes thus conferring cell protection. Activation of Nrf2 can also downregulate the innate immune responses, by inhibiting the expression of pro-inflammatory genes and increasing the activity of anti-inflammatory molecules**. B.** Another target of DMF succination is the nuclear transcription factor NFκB, whose activation is inhibited for example by succination of the B subunit of the inhibitory kB kinase (IkB). In addition, DMF can succinate the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with permanent loss of catalytic activity. Inhibition of GAPDH in the inflammatory cells can in part explain the anti-inflammatory actions of DMF.

Table 1. Epidemiology of DMF induced lymphopenia in MS treated subjects. Lymphopenia is defined according to the Common Terminology Criteria for Adverse Events (CTCAE v5.0) as it follows: Grade 1: absolute lymphocyte count (ALC) 0.800-0.999 x 10^9/L; grade 2: ALC 0.500-0.799 x 10^9/L; grade 3: ALC 0.200-0.499 x 10^9/L; and grade 4: ALC < 200 x 10^9/L. DMF dose refers to the maintenance regimen.

Relevant aspects of the study	DMF Dose (mg /day)	Subjects (n)	Female Sex (%)	Mean age (years, ± SD)	Drug naïve (%)	Mean EDSS (± SD)	Mean length of treatment/ observation time (months, ± SD)	Lymphopenia				References
								Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	
RANDOMIZED CLINICAL TRIALS												
Phase 3 (DEFINE)	480	410	72	38.1 ± 9.1	60	2.40 ± 1.29	24	NR	NR	4	NR	Gold et al. 2012
Phase 3 (DEFINE)	720	416	74	38.8 ± 8.8	60	2.36 ± 1.19	24	NR	NR	4	NR	Gold et al. 2012
Phase 3 (CONFIRM)	480	359	68	37.8 ± 9.4	72	2.6 ± 1.2	24	NR	NR	5	NR	Fox et al., 2012
Phase 3 (CONFIRM)	720	345	72	37.8 ± 9.4	71	2.5 ± 1.2	24	NR	NR	4	NR	Fox et al., 2012
Long- term extension (ENDORSE)	480-720	1736[a]	69.33	39.63 ± 9.1	NR	2.53 ± 1.2	60	NR	NR	6-9	NR	Gold et al., 2017
Phase 3 (APEX)	480	56[b]	79	38.4 ± 8.16	44.6	1.9 ± 1.3	6	5.4	0	0	0	Ochi et al., 2018
Long-Term extension (APEX)	480	PBO/DMF 53[b] DMF/DMF 53[b]	PBO/DMF 79 DMF/DMF 77	PBO/DMF 36.1 ± 7.3 DMF/DMF 38.3 ± 8.2	PBO/DMF 41.5 DMF/DMF 43.4	PBO/DMF 1.8 ± 1.4 DMF/DMF 1.9 ± 1.4	18	PBO/DMF 10.2 DMF/DMF 1.9	PBO/DMF 6.1 DMF/DMF 18.9	PBO/DMF 0 DMF/DMF 1.9	0	Ochi et al., 2018

Relevant aspects of the study	DMF Dose (mg /day)	Subjects (n)	Female Sex (%)	Mean age (years, ± SD)	Drug naïve (%)	Mean EDSS (± SD)	Mean length of treatment/ observation time (months, ± SD)	Lymphopenia				References
								Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	
OBSERVATIONAL PROSPECTIVE STUDIES												
National MS registry (Kuwait)	480	119	59.7	33.5 ± 11.1	24.4	2.8 ± 1.8	20.5 ± 9.5	8.4		2.5	NR	Alroughani, et al., 2017
Single centre	NR	25[c]	73.5	42 ± 9	50	2.25	> 18	16	20	4	NR	Nakhaei-Nejad et al., 2017
Multicentre	NR	405[d]	72.8	45.3 ± 11.3	34.5	2.1 ± 1.9	28.8 ± 7.2	57.7		1.7[e]		Baharnoori et al., 2018
								NR	17	NR	0.2	
									11			
Multicentre	Standard approved dose	234	63.3	45.7 ± 9.9	10.3	2.5[f] (IQR, 1.5-3.5)	12	1.2		0.9	NR	D'Amico et al., 2018
Multicentre	Standard approved dose	720	66.4	38.8 ± 10	45.4[g]	2[f] (min 0 – max 6.5)	17[f] (min 0 – max 33)	18.7 (mostly mild)		0	0	Mallucci et al., 2018
Single centre	480[h]	412	77	49.4 ± 12.0	30	NR	18.3[f] (IQR 6.1–24.3)	39		11	NR	Smoot et al., 2018
Single centre	NR	106 [c]	75.5	41.5 ± 8.9	30.2	1.5[f] (range 0-7)	24.67[f] (range 6.03-34.63)	17.9	13.2	5.7	NR	Sainz de la Maza et al., 2019

Relevant aspects of the study	DMF Dose (mg /day)	Subjects (n)	Female Sex (%)	Mean age (years, ± SD)	Drug naïve (%)	Mean EDSS (± SD)	Mean length of treatment/ observation time (months, ± SD)	Lymphopenia				References
								Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	
Single centre	Standard approved dose	38	60.5	38.0 ± 8.7	65.8	NR	12[i] 24[j]	6.5[i] NR[j]	12.9[i] 30.8[j]	6.5[i] 3.8[j]	NR	Marastoni et al., 2019
Single centre	NR	18	72.2	41.3 ± 10	33.3	2[f] (IQR 1.5)	6	11.1	27.8	5.6	NR	Bhargava et al., 2019
Single centre (Cross Sectional/ Longitudinal)	480	51	68.6	30.1 ± 10.5	NR	1.5[f] (range 0-6)	6	23 (ALCs < 1x10^9/L)				Fleischer et al., 2018
RETROSPECTIVE CHART ANALYSES												
Single centre	NR	144[k] 40[l] 14[m]	NR	NR	NR	NR	- 4-6[l] >12[m]	NR	14[k] NR[l] NR[m]	6[k] 10[l] 28.6[m]	NR	Longbrake & Cross, 2015
Single centre	480	23	52	44.5 ± 14.1	0	3.5 ± 1.7	3.9 ± 1.2	NR	24	5	NR	Berkovich & Weiner, 2015
Single centre	NR	221	73	45[f] (range 18-76)	14	NR	11[f] (range 1-23)	NR	9.1 2.7[n]	5.9	NR	Longbrake et al., 2015
Dual-centre	474 ± SD, 40	644[o]	70	39 ± 10.7	45.2	3.0 ± 1.6[p]	6	34.9 (ALC < 1x10^9/L)				Miclea et al., 2016

Relevant aspects of the study	DMF Dose (mg /day)	Subjects (n)	Female Sex (%)	Mean age (years, ± SD)	Drug naïve (%)	Mean EDSS (± SD)	Mean length of treatment/ observation time (months, ± SD)	Lymphopenia				References
								Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	
Dual-centre[q]	NR	103	76.7	40.2 ± 10.7	17.5	NR	NR	23.3		2.4[q]	0	Sejbaek et al., 2018
		150	64.7	40.9 ± 10.9	37.3			12.7	17.3			
Multicentre	NR	1089	70.2	38.9 ± 10.2	30.4	1.95[f] (interval 0-6.0)	17.1[f] ± 8.2	12[r]		4.5[r]	NR	Mirabella et al., 2018
Single centre	480	246	NR	49.8 (95% CI, 40.4-59.3)	NR	NR	16.7 (range 5.7-35.5)	NR	NR	4.5	0	Briner et al., 2019
Multicentre	NR	189	78.3	43.2 ± 0.9	31.2	1.7 ± 0.1	25.0 ± 0.7	NR[s]	13.2[s]	3.2[s]	NR	Condè et al., 2019
Single centre	NR	194	69.6	> 40 (70%)	10.3	NR	17[f] (range 1-32)	16	14	7	NR	Sierra Morales et al., 2020
Multicentre	Standard approved dose	456[t]	67.11	40.4 ± 11.8	32.7	2.5[f] (range 0-8)	18.2 ± 11	13	7	1	NR	Lanzillo et al., 2020

A total of 26 articles were identified in the literature, reporting epidemiological information on DMF-induced lymphopenia. The table summarizes data from 22 articles in direct comparison to the phase 3 clinical trials that led to drug approval, including one publication related to the long term extension randomized clinical trial ENDORSE (Gold et al., 2017), one phase 3 clinical study (APEX, NCT01838668), 10 observational prospective studies, 10 retrospective chart analyses of DMF treated subjects. Four studies were not included in the table for different reasons: one is a phase 2 trial in paediatric MS (Alroughani et al., 2018), one is a retrospective analysis in the progressive form of MS (Strassburger-Krogias et al., 2014) and two are integrated analyses of all pre-licensing clinical trials (Fox et al., 2016; Mehta et al., 2019). However, data from these studies are discussed in the text.

[a]: in the study by Gold and collaborators (2017), baseline demographic data were reported separately per each group of treatment, including 2 groups that continued DMF (1003 subjects) and 4 groups that switched to DMF either from placebo (496 subjects) or from GA (236 subjects). Female sex was reported as % for each group and averaged in a single value. Age and EDSS score were reported as mean ± SD per each group, both averaged to provide a single value for all subjects included in the study. This study presents an interim analysis at 5 years (60 months), which include the first 2 years in the DEFINE/CONFIRM studies.

[b]: data refer to MS subjects of Japanese ethnicity (Ochi et al., 2018). For the long-term extension, part II, of the APEX study, we reported separately data about two groups of treatment: PBO/DMF refers to subjects that switched from placebo to DMF whereas DMF/DMF refers to subjects that continued DMF treatment. The second part of the study presents an interim analysis at 72 weeks (18 months), which include the first 24 weeks in part I of the study.

[c]: DMF treated subjects were divided in two groups, i.e. lymphopenic and non-lymphopenic subjects. Demographic data reported in the table are the average among the two groups.

[d]: RRMS, 76.5%.

[e]: grade 3b-4 lymphopenia, that is ALC < 0.35 x 10^9/L.

[f]: median.

[g]: In this study, 186 (25.83%) subjects were effectively drug naïve, whereas 141 (19.58%) discontinued a previous DMT therapy more than 12 months before starting on DMF (extended naïve group).

[h]: 38 subjects (7.9%) were treated at lower dose.

[i]: epidemiologic data on lymphopenia refer to the first 12 months of DMF therapy.

[j]: epidemiologic data on lymphopenia refer to the first 24 months of therapy. It was reported that 2 subjects developed persistent grade 3 lymphopenia within 12 months of therapy and discontinued therapy. Therefore, if we include these patients in the estimate of grade 3 lymphopenia after 24 months of treatment, the total would be 3 out of 38 subjects and the rate of grade 3 lymphopenia would be 7.9% after 24 months of treatment.

[k]: data refer to the whole cohort. Specifically, it was reported that 20/144 (14%) developed grade 2 or grade 3 lymphopenia.

[l]: data refer to a time frame of 4-6 months, considering 40 subjects in the analysis.

[m]: data refer to a time frame longer than 12 months, considering 14 patients in the analysis.

[n]: in 6/221 (2.7%) subjects, ALCs were equal to 0.5 x 10^9/L, that is grade 2-3 lymphopenia.

[o]: subjects included in the analysis were diagnosed with RRMS (87.6%), with progressive-relapsing MS (10.2%) and centrally isolated syndrome (2.2%).

[p]: EDSS refer to 623/644 subjects

[q]: in this study, data were collected in two different clinical centres and are reported in the table separately. The rate of grade 3 lymphopenia in the second centre was 4%.

[r]: the rate of lymphopenia refers to a cohort of 1005 subjects.

[s]: Data refer to lymphocyte counts at any time-point. A total of 96 subjects (50.8%) had lymphocyte counts < 1.5 x 10^9/L.

[t]: subjects included in the analysis were diagnosed with RRMS (92.76%) and with progressive-relapsing MS (7.24%). Data on lymphopenia were collected for 393 subjects (Lanzillo et al., 2020).

Abbreviations: PBO, placebo; SD, standard deviation; IQR, interquartile range; EDSS, Expanded Disability Status Scale; 95% CI, 95% confidence interval; NR, not reported.

Table 2. Epidemiology of DMF induced lymphopenia in psoriasis treated patients. Lymphopenia is defined according to the Common Terminology Criteria for Adverse Events (CTCAE v5.0) as it follows Grade 1: absolute lymphocyte count (ALC) 0.800-0.999 x 10^9/L; grade 2: ALC 0.500-0.799 x 10^9/L; grade 3: ALC 0.200-0.499 x 10^9/L; and grade 4: ALC < 200 x 10^9/L. DMF dose refers to the maintenance regimen. When indicated, it takes into consideration the amount of DMF contained in the Fumaderm® formulation.

Relevant aspects of the study	DMF Dose (mg /day)	Patients	Female Sex (%)	Mean age (years, ± SD)	Drug naïve (%)	Mean PASI (± SD)	Mean length of treatment/ observation time (months)	Lymphopenia				References
								Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	
PRE-LICENSING CLINICAL TRIALS												
Open label prospective	120-360 (Fumaderm®)	36	42	NR	NR	NR	9.7 (range 1-32)	0	NR	0	NR	Nieboer et al., 1989
Double blind placebo controlled	0 (240 MEF)	19	58[a]	NR	NR	NR	4	0	NR	0	NR	Nieboer et al., 1989
Double blind placebo controlled	240	22	45[a]	NR	NR	NR	4	64 (ALCs<20%)	NR	4.5	NR	Nieboer et al., 1989
Open label prospective	60-240	56	NR	NR	NR	NR	4-9	45 (ALCs<20%)	NR	5.4	NR	Nieboer et al., 1989
Double blind placebo controlled	120-720 (Fumaderm®)	12	31[a]	44[a] (range 20-73)	NR	NR	4	33 (lymphopenia - parameters not specified)	NR	NR	NR	Nugteren-Huying et a., 1990
Double blind placebo controlled	120-720 (Fumaderm®)	50	Mostly males	41.1 (range 21-69)	NR	21.57	4	Not significant reduction	NR	NR	NR	Altmeyer et al., 1994

Relevant aspects of the study	DMF Dose (mg /day)	Patients	Female Sex (%)	Mean age (years, ± SD)	Drug naïve (%)	Mean PASI (± SD)	Mean length of treatment/ observation time (months)	Lymphopenia				References
								Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	
Open label multicentre prospective	120-720 (Fumaderm®)	101	32.7	43.4 (range 21-69)	NR	20.04	4	NR	9.9 (ALCs<50%)		NR	Mrowietz et al., 1998
OBSERVATIONAL PROSPECTIVE STUDIES												
Single centre	120-720 (Fumaderm®)	80	27	44 ± 12	1.25	13.9 ± 9.0	3-36	33 (after 3 months; ALCs <1.2x10^9/L)	NR	1.25 (after 3 months) 8.75 (after 36 months)	NR	Wain et al., 2010
Dual centre	120-720 (Fumaderm®)	27[b]	26	45 ± 13	NR[c]	11[d] (IQR 8–15)	4	44 (mild)	15 (moderate)	4 (severe)	NR	Schmieder et al., 2015
Single centre	480[d] (IQR 270-960; DMF monotherapy)	176	42	47 ± 14	18	49.4%[e] (moderate-severe) 7.95%[e] (very severe)	9[d,f] (IQR 6-18)	34				Lijnen et al., 2016
RETROSPECTIVE CHART ANALYSES												
Single centre	120-720 (Fumaderm®)	66	38	52 ± 14.7	65	NR	> 12[g]	~ 76[h] (ALCs <20%)	NR	NR	NR	Hoefnagel et al., 2003
Single centre	120-720[i] (Fumaderm®)	58	43	47.2 ± 14.9	5	NR	> 12[j]	57 (ALCs <1.5x10^9/L)	NR	3.4	NR	Harries et al., 2005
Single centre	120-720 (Fumaderm®)	31	32.3	46.8 ± 13.9	0	NR	> 6	61.3 (ALC <1.5x10^9/L)	NR	NR	NR	Brewer& Roger, 2007
Multicentre Cross-sectional	Standard (Fumaderm®)	984	41.8	50.5 ± 13.18	80.6	21.1[k]	44.1 (continuously)	41 (after 24 months)	NR	NR	NR	Reich et al., 2009

Relevant aspects of the study	DMF Dose (mg /day)	Patients	Female Sex (%)	Mean age (years, ± SD)	Drug naïve (%)	Mean PASI (± SD)	Mean length of treatment/ observation time (months)	Lymphopenia				References
								Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	
							46.6 (intermittently)					
Multicentre Cross-Sectional	Standard (Fumaderm®)	69	25	57.8 ± 12.6	NR	89.5%[e] (moderate-severe) 7.5%[e] (very severe)	27.4	63 (after 12 months)	NR	NR	NR	Thaçi et al., 2013
Single centre	FAEs	151	39.7	48 ± 13	60	NR	last 12[l]	NR	17	7	NR	Foley et al., 2017
Single centre	Standard[m] (Fumaderm®)	105	38.1	43.3 (range 16-73)	64.8	18.3 ± 12.5	26.3 (range 1 -112)	46.7 (ALCs <1.0x10^9/L)	NR	11.4	NR	Sondermann et al., 2017
Single centre	Standard (Fumaderm®)	62	55	47 (range 18-83)	NR	NR	50.4[d,n] (range 7-120)	NR	43	25	NR	Roche et al., 2018
Single centre	373.7 ± SD, 182.1 (Fumaderm®)	626[o]	38	45.9 ± 14.6	90.6[p]	22.3 ± 8.3[q]	42 ± 50.4 (continuously)	NR	NR	16.8		Dickel et al., 2018
Single centre	345.8 ± SD, 167.0[r] (Fumaderm®)	371	36.7	47.8 ± 14.6	88.1[p]	22.3 ± 8.1[s]	34.8 ± 32.4 (continuously)	60.9 (ALC <1.0x10^9/L)	35.9 (ALC <0.7 x 10^9/L)	14.3		Dickel et al., 2019

[a]: referred to all the enrolled subjects

[b]: referred to patients that completed the two scheduled visits, V0 and V1 after 4 months.

[c]: A total of 26 subjects (96.3%) had previous topical therapy and 23 (85.2%) phototherapy. One subject was previously treated with FAEs, 2 with methotrexate and 1 with cyclosporin.

[d]: median

[e]: PGA score, Static Physician Global Assessment score, measured on digital photographs.

[f]: median time at which lymphopenia was detected.

[g]: A total of 41 subjects (62%) were treated for more than 1 year, up to 14 years in 12 subjects.

[h]: The rate of lymphopenia refers to 41 patients without relative lymphopenia at the start of treatment.

[i]: 40 % of subjects required the maximum dose of Fumaderm®.

[j]: A total of 32 subjects (55%) discontinued FAE treatment after a mean period of 4.7 months (range 1 day to 2 years). A total of 7 subjects (12%) had been continuously on treatment for more than 12 months.

[k] the PASI score refers to 10.9% of enrolled subjects. According to the PGA score, the disease was moderate-severe in 93% and very severe in 4% of the subjects.

[l]: In this study, 113/151 (75%) subjects remained on treatment for an average of 4.3 ± 3.5 (SD) years. Lymphopenia (ALC < 0.7 x 10^9/L) was detected within the last 12 months in 24% of the subjects.

[m]: For a total of 48 subjects, mean DMF dosage at the time of lymphopenia was 468 ± 229.6 (SD) mg.

[n]: median time to onset of lymphopenia was 3.5 months (range 1-20).

[o]: cohort of subjects treated with Fumaderm® as monotherapy.

[p]: the percentage refers to subjects naïve to prior systemic therapies.

[q]: the score refers to 79.4% of the studied subjects.

[r]: The mean dose refers to 72% of subjects treated with FAE monotherapy. In 53 subjects FAEs were associated to methotrexate (mean average daily DMF dose, 416.8 mg ± 196.2 mg) or in 51 subjects with phototherapy (mean average daily DMF dose, 362.4 ± 151.9 mg)

[s]: the score refers to 83.3% of the studied subjects.

Abbreviations: IQR, interquartile range; PASI, Psoriasis Area Severity Index; 95% CI, 95% confidence interval; NR, not reported.

Table 3. Main laboratory findings in MS patients after DMF administration.

Immune cell type	Laboratory Assessments carried out after different times from initiation of DMF therapy % change (number of patients)					References
	3 months	**4-6 months**	**6 months**	**≥ 12 months**	**≥ 17 months**	
Leucocytes	-1.3 (35)	-25.0 (20)*	-4.8 (35)	-23.4 (35)**	-22.2 (18)*	Spencer et al., 2015; Wu et al., 2017
Lymphocytes	-23.1 (35)** -15.0 (23)*	-29.7 (191)****,a -27.8 (20)**	-27.8 (35)* -41.2 (139) a,b -31.0 (29)***	-50.1 (35)**** -36.8 (69)****,a -48 (13)** -35.0 (31)***	-38.9 (18)** -33.7 (26)***	Spencer et al., 2015; Khatri et al., 2015; Wu et al., 2017; Ghadiri et al., 2017; Marastoni et al., 2019
$CD3^+$ T cells	-5.7 (35) -16.7 (23)*		-23.0 (35)* Unchanged (12) -35.0 (29)***	-44.2 (35)**** -54 (13)** -47.5 (31)***	-47.7 (26)***	Spencer et al., 2015; Gross et al., 2015; Ghadiri et al., 2017; Marastoni et al., 2019
$CD4^+$ T cells	-8.8 (35)	-30.3 (135) ****,a ↓ (20)**	-21.1 (35)* -44.8 (94) a,b -49.6 (41)c -18.8 (13)	-39.2 (35)*** -37.7 (42)***,a -44 (13)** -16.2 (13)	↓ (18)** -19.3 (13)	Spencer et al., 2015; Khatri et al., 2015; Ghadiri et al., 2017; Wu et al., 2017; Longbrake et al., 2016; Marastoni et al., 2019
$CD8^+$ T cells	+0.7 (35)	-36.9 (132)****,a -45 (20)*	-33.3 (35)* -57.5 (90) a,b -19.4 (12)* -55.36 (41)c -35.6 (13)*	-54.6 (35)**** -47.6 (39)***,a -77 (13)* -59.1 (13)**	-57.5 (18)*** -46.8 (13)**	Spencer et al., 2015; Khatri et al., 2015; Ghadiri et al., 2017; Gross et al., 2015; Longbrake et al., 2016; Wu et al., 2017; Marastoni et al., 2019
$CD4^+/CD8^+$ ratio	-17.1 (35)	+38.5 (132)***,a	+17.5 (35) +42.3 (90)a,b	+35.5 (35)** +38.5 (39)****,a +53.1 (13)**		Spencer et al., 2015; Khatri et al., 2015; Ghadiri et al., 2017;

Immune cell type	Laboratory Assessments carried out after different times from initiation of DMF therapy % change (number of patients)					References
	3 months	4-6 months	6 months	≥ 12 months	≥ 17 months	
		↑ (20)	+43.6 (41) [c] +9.1 (13)	+72.7 (13)**	+61.5 (18)* +22.7 (13)*	Longbrake et al., 2016; Wu et al., 2017; Marastoni et al., 2019
Activated T cells: $CD69^+$ $CD4^{+/-}$ $CD62^{low}CD4^+$ $CD62^{low}CD8^+$		↓ (20)	+9.1 (41)[c] +14.7 (41)[c]		↓ (18)**/*[,d]	Wu et al., 2017; Longbrake et al., 2016; Longbrake et al., 2016
$CD45RO^+$ $CD4^+$ memory T cells		-37.2 (20)***[,e]	-31.13 (12)***		-45.4 (18)***[,e]	Gross et al., 2015; Wu et al., 2017
$CD45RO^+$ $CD8^+$ memory T cells		-49.1 (20)**[,e]	-29.90 (12)**		-53.6 (18)***[,e]	Gross et al., 2015; Wu et al., 2017
Naïve ($CD45RO^-$ $CD27^+$) $CD4^+$ T cells		↑ (20)***[,f]	+19.26 (12)** +81.43 (41) [c]		↑ (18)***[,f]	Gross et al., 2015; Longbrake et al., 2016; Wu Q et al., 2017
Naïve ($CD45RO^-$ $CD27^+$) $CD8^+$ T cells		↑ (20)*[,f]	+18.26 (12)** +44.44 (41)[c]		↑ (18)**[,f]	Gross et al., 2015; Longbrake et al., 2016; Wu Q et al., 2017
$CD4^+$ Th1		↓ (20)	-36.77 (12)** -38.5 (41)[c] +47.0 (13)	+61.7 (13)*	↓ (18)* -22.8 (13)	Gross et al., 2015; Longbrake et al., 2016; Wu Q et al., 2017; Marastoni et al., 2019
$CD4^+$ Th17		↓ (20)	-32.10 (12)** -45.5 (41) [c] -21.4 (13)	+5.3 (13)	↓ (18) +4.1 (13)	Gross et al., 2015; Longbrake et al., 2016; Wu Q et al., 2017; Marastoni et al., 2019
$CD4^+$ Th2		↑ (20)	Unchanged (12) +21.1 (13)	+37.5 (13)	↑ (18)*** -40.6 (13)	Gross et al., 2015; Wu et al., 2017; Marastoni et al., 2019
T_{reg} cells			+24.6 (12)**			Gross et al., 2015;

Immune cell type	Laboratory Assessments carried out after different times from initiation of DMF therapy % change (number of patients)					References
	3 months	4-6 months	6 months	≥ 12 months	≥ 17 months	
		Unchanged (20)	-52.7 (41) [c] -19.6 (13)	-50.0 (13)*	Unchanged (18) -0.7 (13)	Longbrake et al., 2016; Wu et al., 2017; Marastoni et al., 2019
$CD19^{+}$B cells	-15.7 (35) -34.1 (23)***	-49.8 (12)** Unchanged (20)	-32.2 (35) -20.6 (41) [c] -33.0 (29)***	-37.5 (35)* -57 (8) -39.1 (31)***	↓ (18)* -24.8 (26)*	Spencer et al., 2015; Lundy et al., 2016; Longbrake et al., 2016; Wu et al., 2017 Marastoni et al., 2019
Activated $CD80^{+}$ B cells					Significant ↓ (66)[g]	Longbrake et al., 2018
Memory B cells ($CD27^{+}$)		-52.6 (12)**		-59.6 (8)	Significant ↓ (66)[g]	Lundy et al., 2016; Longbrake et al., 2018
Naïve B cells					Significant ↑ (66)[g]	Longbrake et al., 2018
$CD56^{+}$ NK cells	-19.5 (35) +41.2 (23)	Unchanged (20)[h]	-19 (35) +41.7 (41) [c] +18.9 (29)	-24.6 (35) +75.3 (31)*	Unchanged (18)[h] +85.9 (26)***	Spencer et al., 2015; Longbrake et al., 2016; Wu et al., 2017; Marastoni et al., 2019
$CD56^{dim}$ NK cells			-44.9 (41) [c]			Longbrake et al., 2016;
Monocytes	+8.2 (35)	Unchanged (20)	-4.1 (35) -20.1 (41) [c]	-18.4 (35)	-8.3 (18)	Spencer et al., 2015; Longbrake et al., 2016; Wu et al., 2017
Eosinophils	-11.8 (35)	-50.0 (20)	+15.3 (35)	-54.1 (35)**	-50.0 (18)	Spencer et al., 2015; Wu et al., 2017
Basophils	+18.4 (35)		-39.5 (35)	-21.1 (35)		Spencer et al., 2015
Neutrophils	+9.0 (35)	-15.9 (20)	+10.6 (35)	-10.1 (35)	-18.2 (18)	Spencer et al., 2015; Wu et al, 2017

According to Spencer et al., 2015, data are expressed as percentage of change from baseline when absolute values were provided in the original papers, to allow for comparison among different studies. The number of patients analysed in each study, in relation to the respective change from baseline, is included. This approach has some important limitations. Some studies reported absolute values as the mean of absolute counts whilst others reported the median values. Therefore, percentage of changes from the baseline were calculated, taking into account either mean or median absolute values provided in the papers. In some studies, comparisons between DMF treated subjects were carried out versus healthy volunteers or untreated MS subjects, further increasing variability (Longbrake et al., 2016; 2018; Wu et al., 2017). In addition, in two studies, subjects were divided into two subgroups, lymphopenic and non-lymphopenic DMF treated subjects (Longbrake et al., 2016; 2018). Data from these two groups were averaged and compared to the untreated MS subjects when absolute values were provided (Longbrake et al., 2016). Data were sometimes available only in graphs; thus, it was not possible to estimate a percentage of change from baseline, then a comment is provided (Wu et al., 2017; Longbrake et al., 2018). Statistical significance is reported considering the original papers and refers to values at specific time points significantly different *versus* baseline, *p < 0.05; **p < 0.01; ***p < 0.001; **** p < 0.0001.

[a]: In Khatri et al., 2015, blood assessments were performed according to the following time points: at 4.8 ± 3.6 months with data reported in the column '4-6 months'; at 8.5 ± 3.9 months with data reported in the column '6 months'; and at 10.4 ± 4.9 months with data reported in column '≥ 12 months'.

[b]: In the original paper, the significance at this time point (visit 3) was referred to changes from the previous time point (visit 2): lymphocytes, $CD4^+$, $CD8^+$, p < 0.0001 and $CD4^+/CD8^+$ ratio, p = 0.0006.

[c]: data on DMF treated subjects were reported separately for grade 2-3 lymphopenic (n=17) and non-lymphopenic (n=24) subjects and compared either versus healthy volunteers (n=23) or versus untreated MS subjects (n=17). Laboratory values were averaged for all DMF treated individuals (n=41) and percentage of changes was calculated considering as baseline values those provided for the untreated MS group. It is therefore not possible to include significance values for this study (Longbrake et al., 2016).The study also shows relevant reduction in the $CD4^+$ and $CD8^+$ T cell memory component, including both central memory and effector memory T cells.

[d]: both $CD69^+CD4^+$ and $CD69^+CD4^-$ activated T cells were significantly reduced after 18 months of treatment as compared with the untreated MS group, p = 0.004 and p = 0.03 respectively.

[e]: original values were expressed as median % of CD4+ or CD4- T cells.

[f]: the naïve T cell fraction was identified as $CD3^+CD4^{+/-}CD45RA^+CD45RO^-CCR7^+$ T cells (Wu et al., 2017, Fig. 2C in the original paper).

[g]: data on DMF treated subjects were reported separately for grade 2-3 lymphopenic (n=23) and non-lymphopenic (n=43) subjects and compared either versus healthy volunteers (n=27) or versus untreated MS subjects (n=50). Absolute values are not available for this study, thus a general comment is included considering the results for all DMF treated patients (n=66). For the same reason, it was not possible to provide exact significance values (Longbrake et al., 2018).

[h]: Total NK cells were identified as $CD14^-$ $TCR\alpha\beta^-$ $CD56^+$ cells, whereas NK T cells (NKT cells) were $CD14^-$ $TCR\alpha\beta^+$ $CD56^+$ cells. NKT cells tended to be reduced in short term and were significantly reduced with long-term treatment (Wu et al., 2017 Fig. 1B in the original paper).
Symbols: ↑, increase versus baseline; ↓ decrease versus baseline.

Table 4. Pharmacokinetics parameters of MMF.

Subjects	DMF dosing	Tmax (hours)	Cmax (mg/L)	AUC (mg*h/L)	Vz/F (L)	Human plasma protein binding (%)	CL/F (L/h)	$T_{1/2}$ (h)	Ref.
RRMS adult subjects	240 mg twice daily	2-2.5	1.72	8.02	60-90	27-40		~ 1	Tecfidera® SmPC
Healthy volunteers (n=8)	240 mg twice daily (day 1)	4.0 (2-8)	1.34 (1.03- 2.00)	$AUC_{0\text{-}10hr}$: 2.80 (2.19 - 4.45)				0.81 (0.5–3.3)	Sheikh et al., 2013
Healthy volunteers (n=8)	240 mg twice daily (day 4)	3.0 (1-5)	1.73 (1.11-2.80)	$AUC_{0\text{-}10hr}$: 2.87 (2.45 – 4.29)				0.63 (0.5–1.4)	Sheikh et al., 2013
RRMS paediatric patients (n=14-21)	240 mg twice daily (day 8)	4.2 (Coefficient of variation, 37%)	2.00 ± 1.29 (Coefficient of variation, 64%)	$AUC_{0–12h}$: 3.62 ± 1.16 (Coefficient of variation, 32%)	98.2 (Coefficient of variation, 93%)		74.5 (Coefficient of variation, 41%)		Tecfidera® SmPC; Alroughani et al., 2018

Exhalation of CO_2 is the primary route of DMF elimination accounting for 60% of the dose. Renal and faecal elimination are secondary routes of elimination, accounting for 15.5% and 0.9% of the dose respectively.

Abbreviations: RRMS, relapsing -remitting multiple sclerosis; SmPC, summary of product characteristics; Tmax, time to peak concentration; Cmax, maximum observed plasma concentration; AUC, area under the concentration-time curve; Vz/F, apparent volume of distribution; CL/F, apparent clearance; $T_{1/2}$, terminal half-life.

Box 1. Further avenues for research.

Effects on lymphocyte subpopulations.

In this area, there is a need for more comprehensive and standardised studies on lymphocyte subsets, aiming i) to quantify the extent of changes among different lymphocyte subpopulations in larger cohorts, ii) to characterize how changes in cell subsets are related to drug efficacy and toxicity and iii) to define the molecular determinant(s) of cell death *versus* cell protection/proliferation leading to expansion of specific subpopulations.

Role of MMF in mediating the pharmacological as well as toxic effects of DMF in vivo.

MMF has been considered a strong candidate for being the active species *in vivo*; however, it shows much lower immune modulatory activity as well as less toxicity *in vitro* than DMF. Thus, there is a need for studies aiming i) to better characterize the pharmacological actions of MMF, including potential toxicity *in vitro* and *in vivo*; ii) to expand emerging observations that MMF may have direct intracellular effects *in vivo*; iii) to characterize the HCA2 receptor independent effects of MMF, including the mechanisms that may facilitate its cellular uptake, for example the presence of a specific transporter.

Role of GSH adducts of both MMF and DMF in mediating the pharmacological as well as toxic effects of DMF in vivo.

Several studies have shown that both DMF and MMF form glutathione (GSH) conjugates raising the possibility that these may be important species in the activity and/or toxicity of DMF. However, pharmacokinetic studies in humans are limited to evaluation of such adducts in the urine, thus leaving unexplored their kinetics in the systemic circulation, the assessment of intra- and interindividual variability and the association with efficacy and toxicity. There is also a need to characterize the role of different GST isoforms in the detoxification of the drug and to explore whether genetic variants can impact on this function, and thus on drug efficacy and toxicity. Finally, it would be useful to address if differences in the intracellular GSH content affect the susceptibility to DMF/MMF dependent cell death. The latter can be extended to the study of different immune cells as well as lymphocyte subsets.

Role of the innate immunity as a first cellular target for DMF/MMF action.

Recent data showed a significant association between the production of free oxygen radicals by monocytes and subsequent lymphocyte reduction in response to DMF, with both effects linked to drug efficacy. Moreover, the NADPH Oxidase 3 rs6919626 minor G allele was significantly associated with reduced ROS generation in monocytes and reduced treatment efficacy. There is a need to further understand the interplay between the innate and adaptive immune system in mediating both the pharmacological actions of DMF as well as its toxicity.

Role of the gut microbiome in DMF pharmacology.

Emerging data suggest that alterations in the microbiome can contribute to the pharmacological responses to DMF. Further investigations are needed to characterize these changes and to address whether they are indeed important for the immune modulatory actions of DMF, as well as for the development of lymphopenia.

List of abbreviations

95% CI, 95% confidence interval;

ALCs, absolute lymphocyte counts;

DCs, dendritic cells;

DMF, Dimethyl Fumarate;

DRF, Diroximel Fumarate;

EDSS, Expanded Disability Status Scale;

FAE, fumaric acid ester;

GA, glatiramer acetate;

GAPDH, glyceraldehyde 3-phosphate dehydrogenase;

GM-CSF, granulocyte/monocyte-colony stimulating factor;

GS-DMS, S-(1,2-dimethoxy-carbonylethyl)glutathione;

GSH, glutathione;

GS-MMS, mixture of MMF-GSH adducts;

GST, glutathione-S-transferases;

GSTM1, glutathione-S-transferases μ;

GSTP1, glutathione-S-transferases π;

GSTT1, glutathione-S-transferases θ;

HCA2, hydroxycarboxylic acid 2 receptors;

HP-1, heme oxygenase-1;

IFN, interferon;

IkB, inhibitory kB kinase;

IL, interleukin;

IQR, interquartile range;

LPS, lipopolysaccharide (bacterial endotoxin);

MEF, monoethylfumarate;

mPMs, mouse peritoneal macrophages;

MS, multiple sclerosis;

NAC-DMS, *N*-acetyl-*S*-(1,2-dimethoxycarbonylethyl)cysteine;

NFkB, nuclear transcription factor kappa B;

NR, not reported;

PBO, placebo;

PML, progressive multifocal leukoencephalopathy;

ROS, free oxygen species;

RRMS, relapsing-remitting multiple sclerosis;

SmPC, Summary of Product Characteristics;

Th, T helper cell;

TNFα, tumor necrosis factor alfa;

T_{reg}, regulatory T cells;

Ts, T suppressor.

Funding information

The authors wish to thank the MRC Centre for Drug Safety Science and the Wolfson Centre for Personalised Medicine for infrastructure support. No specific funding was received for this article.

Declaration of Competing Interest

MP receives research funding from various organisations including the MRC, NIHR, EU Commission and Health Education England. He has also received partnership funding for the following: MRC Clinical Pharmacology Training Scheme (co-funded by MRC and Roche, UCB, Eli Lilly and Novartis); a PhD studentship jointly funded by EPSRC and Astra Zeneca; and grant funding from Vistagen Therapeutics. He has also unrestricted educational grant support for the UK Pharmacogenetics and Stratified Medicine Network from Bristol-Myers Squibb and UCB. He has developed an HLA genotyping panel with MC Diagnostics, but does not benefit financially from this. None of the funding declared above has been used for the current paper.

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