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Different fumaric acid esters elicit distinct pharmacological responses

Brian T. Wipke, PhD*§, Robert Hoepner, MD*, Katrin Strassburger-Krogias, MD, Ankur M. Thomas, MS, Davide Gianni, PhD, Suzanne Szak, PhD, Melanie S. Brennan, PhD§, Maximilian Pistor, MD, Ralf Gold, MD, PhD, Andrew Chan, MD†, and Robert H. Scannevin, PhD†§

*These authors contributed equally to the manuscript.

†These authors contributed equally to the manuscript.

§employee of Biogen at the time the research was conducted

Brian T. Wipke, Biogen, Inc., Cambridge, MA; Robert Hoepner, Department of Neurology, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland; Katrin Strassburger-Krogias, Department of Neurology, St. Josef Hospital, Ruhr University Bochum, Bochum, Germany; Ankur M. Thomas, Biogen, Inc., Cambridge, MA; Davide Gianni, Biogen, Inc., Cambridge, MA; Suzanne Szak, Biogen, Inc., Cambridge, MA; Melanie S. Brennan, Biogen, Inc., Cambridge, MA; Maximilian Pistor, Department of Neurology, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland; Ralf Gold, Department of Neurology, St. Josef Hospital, Ruhr University Bochum, Bochum, Germany; Andrew Chan, Department of Neurology, Inselspital, Bern University Hospital,

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University of Bern, Bern, Switzerland; Robert H. Scannevin, Biogen, Inc., Cambridge, MA.

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Correspondence

Dr. Andrew Chan
Department of Neurology, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland
Telephone number: +41 31 632 76 95
Email: Andrew.Chan@insel.ch

Suzanne Szak
Biogen, Inc., Cambridge, MA, USA
Telephone number: +1-617-679-4923
Email: suzanne.szak@biogen.com

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Abstract (297 words)

Objective

In order to test the hypothesis that dimethyl fumarate (DMF, Tecfidera®) elicits different biological changes from DMF combined with monoethyl fumarate (MEF) (Fumaderm®, a psoriasis therapy), we investigated DMF and MEF in rodents and cynomolgus monkeys. Possible translatability of findings was explored with lymphocyte counts from a retrospective cohort of MS patients.

Methods

In rodents, we evaluated pharmacokinetic and pharmacodynamic effects induced by DMF and MEF monotherapies or in combination (DMF/MEF). Clinical implications were investigated in a retrospective, observational analysis of MS patients treated with DMF/MEF (n = 36).

Results

In rodents and cynomolgus monkeys, monomethyl fumarate (MMF, the primary metabolite of DMF) exhibited a higher brain penetration, whereas MEF was preferentially partitioned into the kidney. In mice, transcriptional profiling for DMF and MEF alone identified both common and distinct pharmacodynamic responses, with almost no overlap between DMF- and MEF-induced differentially expressed gene profiles in immune tissues. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-mediated oxidative stress response pathway was exclusively regulated by DMF, whereas MEF activated apoptosis pathways. DMF/MEF

treatment demonstrated that DMF and MEF functionally interact to modify DMF- and MEF-specific responses in unpredictable ways. In MS patients, DMF/MEF treatment led to early and pronounced lymphocyte suppression, predominantly $CD8^+$ T cells.

In a multivariate regression analysis, absolute lymphocyte count (ALC) was associated with age at therapy start, baseline ALC, and DMF/MEF dosage, but not with previous immunosuppressive medication and gender.

Further, ALC increased in a small cohort of MS patients (n = 6/7) after switching from DMF/MEF to DMF monotherapy.

Conclusions

Fumaric acid esters (FAEs) exhibit different biodistribution and may elicit different biological responses; furthermore, pharmacodynamic effects of combinations differ unpredictably from monotherapy. Strong potential to induce lymphopenia in MS patients may be a result of activation of apoptosis pathways by MEF compared with DMF.

Glossary

ALC = absolute lymphocyte count; **DEG** = differentially expressed gene; **DMF** = dimethyl fumarate; **FAE** = fumaric acid esters; **GAPDH** = glyceraldehyde 3-phosphate dehydrogenase; **GCRMA** = GC-content-based Robust Multi-Array Average; **GSH** = glutathione; **IACUC** = Institutional Animal Care and Use Committee; **ILN** = inguinal lymph node; **IPA** = Ingenuity Pathway Analysis; **IQR** =

130 interquartile range; **Keap1** = Kelch-like ECH-associated protein 1; **LI** =

131 lymphopenia index; **MEF** = monoethyl fumarate; **MLN** = mesenteric lymph node;

132 **MMF** = monomethyl fumarate; **MS** = multiple sclerosis; **Nrf2** = nuclear factor

133 (erythroid-derived 2)-like 2; **QC** = quality control; **RQS** = RNA Quality Score;

134 **RRMS** = relapsing remitting multiple sclerosis; **WBC** = white blood cell count.

135 **Introduction (≤250, currently 235)**

136 Multiple sclerosis (MS) is a chronic inflammatory, demyelinating, autoimmune
137 disease of the CNS.[1] During different MS disease stages, oxidative stress
138 precipitated by mitochondrial damage also may contribute to oligodendrocyte and
139 neuronal injury.[2] Fumaric acid esters (FAE) exhibit pleiotropic immunomodulatory
140 effects, as well as antioxidative properties. The FAE, dimethyl fumarate (DMF),
141 which has monomethyl fumarate (MMF) as its primary metabolite, is an oral
142 treatment approved for use in patients with relapsing-remitting MS (RRMS),[3, 4]
143 clinically isolated syndrome, and active secondary progressive MS.[3] Efficacy of
144 DMF and a combination of different salts of monoethyl fumarate (MEF) was
145 investigated in an early exploratory study in patients with RRMS[5] and is marketed
146 in Germany as an oral therapeutic to treat psoriasis (DMF/MEF, Fumaderm®).
147 It is unclear whether different FAEs are functionally equivalent and if a
148 combination treatment could alter pharmacological properties and clinical
149 parameters, although in vitro evidence shows that different FAEs may stimulate
150 distinct responses.[6-8] Both DMF and MEF treatment are associated with
151 lymphopenia in some patients; however, the underlying mechanisms and relative
152 contributions of each FAE are unknown.[9, 10]
153 We hypothesized that the standard clinical regimen of DMF and DMF/MEF
154 might have different pharmacokinetic distributions and provoke different
155 pharmacodynamic responses. We administered FAEs (DMF, MEF, DMF/MEF)
156 individually or at doses reflecting the Fumaderm® formulation and evaluated their

157 distribution in various tissues and changes in transcriptional profiles. Finally, we
158 evaluated lymphopenia in patients with MS treated with DMF/MEF.
159

160 Materials and methods

161 Animals

162 All procedures involving animals were performed in accordance with standards
163 established in the Guide for the Care and Use of Laboratory Animals (US
164 National Institutes of Health). All rodent animal protocols were approved by the
165 Biogen Institutional Animal Care and Use Committee (IACUC). Animals used
166 included female C57BL/6 mice aged 8–10 weeks (Jackson Laboratories, Bar
167 Harbor, ME), male Sprague Dawley rats aged 12–14 weeks (Harlan
168 Laboratories, Indianapolis, IN or Charles River Laboratories, Wilmington, MA), or
169 female cynomolgus monkeys weighing 2–4 kg (dosing excretion studies were
170 conducted at Charles River Laboratories [Reno, NV] using protocols approved by
171 their IACUC).
172

173 Compound dosing

174 For transcriptional profiling and biodistribution studies, C57BL/6 mice or Sprague
175 Dawley rats were dosed with DMF, a mixture of MEF salts (Ca^{2+}, Mg^{2+}, and Zn^{2+}
176 in the ratio 91.5%:5.2%:3.2%), or a combination of DMF and MEF salts to mimic
177 the ratio of fumarates in Fumaderm®. DMF, MEF, and DMF/MEF were
178 formulated as fine suspensions in 0.8% hydroxypropyl methylcellulose (vehicle)
179 and stirred continuously throughout the studies. DMF was dosed at 100 mg/kg

180 (the efficacious dose in a mouse experimental autoimmune encephalomyelitis
181 model); MEF was dosed at 79.2 mg/kg (total MEF salts) representing the
182 proportional MEF dose in Fumaderm®; and DMF/MEF, which is reflective of the
183 ratio of DMF:MEF salts in Fumaderm® used in the clinic, was comprised of DMF
184 100 mg/kg and MEF 79.2 mg/kg. Mice received either a single dose (10 mL/kg
185 for PK) or 10 daily doses (10 mL/kg) of FAEs or vehicle-only control (0.8%
186 hydroxypropyl methylcellulose) via oral gavage. For urine excretion studies, rats
187 were dosed (30 mg/kg) with a mixture of DMF (55.5 %), Ca^{2+} MEF (39.8 %),
188 Mg^{2+} MEF (2.4%), Zn^{2+} MEF (1.49%), and fumaric acid (0.98%), reflective of
189 Fumaderm® dosing. Cynomolgus monkeys were dosed (50 mg/kg) with either
190 DMF or a mixture of MEF salts in the same proportions used in rats and mice.
191

192 **In vivo gene expression profiling**

193 Whole blood and, after perfusion, tissues were collected from naive C57Bl/6 mice
194 dosed with vehicle, DMF, a mixture of MEF salts, or DMF/MEF at 12 hours after
195 the final oral dose (10-day series), and snap frozen. RNA was prepared from
196 tissues and whole blood per standard practice. RNA integrity was assessed
197 using the HT RNA reagent kit (part number 760410, Caliper Life Sciences,
198 Hopkinton, MA) using a LabChip GX (PerkinElmer, Waltham, MA). RNA samples
199 with an RNA Quality Score (RQS) >8.0 were considered high quality for
200 microarray profiling. Sample labeling, hybridization, and scanning were
201 performed as described[11] using an Affymetrix chip HT-MG-430 PM (Affymetrix,
202 Santa Clara, CA). Affymetrix scans were subject to quality control (QC)

203 measures.[12] All sample scans that passed QC were included in the analysis;
204 these 204 CEL files (GEO accession number GSE63343) were either pooled all
205 together or segregated based on tissue and subjected to content-based GC-
206 Robust Multi-Array Average (GCRMA) normalization (version 2.20.0).[13, 14]
207 To identify genes that change uniquely in response to DMF or MEF
208 administration in each individual tissue, a linear modeling approach was used to
209 fit gene expression levels (log2 transformed) according to defined groups of
210 samples and Bayesian posterior error analysis as implemented by Smyth
211 (Bioconductor library limma, version 3.4.5).[15] Genes were considered
212 significantly different in DMF-vs-vehicle and MEF-vs-vehicle if they met the
213 following criteria: (1) average normalized signal intensity >4; (2) logarithm (base
214 10) of odds ("lods") score >0; and (3) fold change >1.5. All calculations and
215 analyses were carried out using R (version 2.11.1) and Bioconductor.[16]
216 Alternately, samples across all tissues and blood were pooled and
217 normalized together to avoid characterizing tissue-to-tissue variability in the
218 limited subset of tissues sampled, and to fully capture all differences in
219 DMF/MEF responses; this approach generalized the analysis and allowed us to
220 find those probe sets that were specifically changing due to DMF or MEF, as well
221 as those probe sets that exhibited a DMF:MEF interaction effect. The following
222 linear mixed model was applied to the normalized data set:
223 *Gene Expression ~ DMF + MEF + DMF:MEF + random(tissue)*
224 Interaction probe sets were defined as those with a Bonferroni-adjusted *p* value
225 <0.05 for the interaction term in this model. A simpler model (without the

226 interaction term) was fit to probe sets that exhibited no interaction effect.
227 Similarly, probe sets were considered significant and specific to DMF if the
228 Bonferroni-corrected *p* value was <0.05 for the DMF term and >0.05 for the MEF
229 term (and no interaction effect was found). MEF-specific probe sets were
230 identified by requiring the Bonferroni-corrected *p* value to be >0.05 for DMF and
231 <0.05 for MEF.
232 An in vivo MEF-DMF interaction was evaluated by analyzing the specific
233 differentially expressed genes (DEGs) modulated when these 2 compounds were
234 co-administered (DMF 100 mg/kg and MEF salts 79.2 mg/kg). The absolute
235 value of the difference between (DMF – vehicle) and (combination – vehicle) was
236 calculated for each of the identified interaction probe sets, and presented as the
237 log2 absolute difference for each probe set. In order to identify the most highly
238 enriched molecular pathways, the sets of DMF-specific, MEF-specific, and
239 DMF/MEF interaction probe sets were analyzed using Ingenuity Pathway
240 Analysis (IPA) software (Qiagen, Germantown, MD). The top 10 enriched
241 pathways for each were compared with each other for *p* value significance.
242
243 **Bioanalytical studies**
244 For biodistribution studies, immediately following blood collection, stabilizer
245 (sodium fluoride solution, 250 mg/mL NaF in water) was added to each blood
246 sample (10 mg/mL final) in a chilled lithium heparin blood collection tube (to
247 inhibit metabolism of MMF or MEF), and plasma was separated from whole blood
248 by centrifugation. Plasma was then snap frozen on dry ice and maintained

249 at -80°C until analyzed. MEF and MMF were measured in all experiments. MMF
250 represents the main metabolite of DMF, which itself cannot be detected in
251 systemic circulation after oral administration due to rapid pre-systemic
252 conversion in vivo. Sample extracts were evaluated by liquid chromatography
253 tandem mass spectrometry to determine MMF and MEF levels, using absolute
254 quantitation based on standard curves spiked in the appropriate biomatrix.
255 Results are expressed as absolute concentration (ng/g of tissue or ng/mL of
256 plasma) and relative concentration expressed as a percentage of plasma
257 concentration.

258 To measure the renal excretion of MMF and MEF, Sprague Dawley rats
259 received a single oral dose of 30 mg/kg DMF plus MEF salts in the Fumaderm®
260 ratio (DMF [55.5 %], Ca^{2+} MEF [39.8 %], Mg^{2+} MEF [2.4%], Zn^{2+} MEF [1.49%],
261 and fumaric acid [0.98%]). In a separate study, cynomolgus monkeys received a
262 single oral dose of 50 mg/kg DMF or MEF salts. In both studies, urine was
263 collected over a 24-hour period and analyzed for MMF and MEF levels.
264

265 **Patients with MS**

266 Patients were identified by retrospective analysis of medical records from a
267 single university hospital. Clinical characteristics (table e-1) of the majority of
268 patients (RRMS or relapsing progressive MS, n = 18; progressive MS, n = 17;
269 neuromyelitis optica, n = 1) treated with DMF/MEF (Fumaderm®, mean [SD] 285
270 [123] mg) in this retrospective, observational, cross-sectional study were
271 described previously.[17] Baseline values of white blood cell count (WBC) and

absolute lymphocyte count (ALC) of the DMF/MEF cohort were obtained 1 week (median and interquartile range [IQR]) before initiation of DMF/MEF and every 3 months thereafter. The 7 patients who switched from DMF/MEF to DMF switched within a mean (SD) of 0.9 (2.3) weeks (6/7 no treatment-free interval, 1 patient 6 weeks interval). In these patients, a lymphopenia index (LI) normalized for dosage of the DMF component was calculated using the following operator: (lymphocyte count during medication – baseline lymphocyte count)/mg of DMF. Statistical analyses including a multivariate regression analysis, Chi-square, and Spearman rho correlation were performed with SPSS 20 (IBM, Armonk, NY).

Standard protocol approvals, registrations, and patient consents

The retrospective observation was approved by the local ethics committee (Ruhr University Bochum; numbers 5408-15 and 4797-13) and conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation Guideline for Good Clinical Practice, and all applicable laws and regulations.

Data availability statement

Data supporting this article can be requested via the corresponding authors.

Results

293 **Biodistribution of DMF metabolite (MMF) and MEF in mice and rats**

294 Thirty minutes after DMF administration by oral gavage, MMF was broadly
295 distributed throughout the bodies of both rats and mice. MMF (dosed as DMF)
296 achieved higher brain penetration after oral administration compared with MEF,
297 by both absolute and relative concentration (mouse, figure 1, A vs B; rat, figure 1,
298 C vs D). In contrast, MEF preferentially partitioned to the kidney, leading to
299 higher absolute and relative concentration. These differences led to an increased
300 brain to plasma ratio for DMF ($p < 0.001$) (figure 1E) and conversely higher
301 kidney to plasma ratio for MEF compared with each other ($p < 0.01$) (figure 1F).
302 Differences in biodistribution remained similar after a 10-day dosing period (data
303 not shown).

304

305 **Renal excretion of MMF and MEF is significantly different in rats and**
306 **cynomolgus monkeys**

307 Consistent with pharmacokinetic and tissue distribution data, mean excretion of
308 intact MEF was significantly higher relative to MMF in rats (9-fold; $p < 0.05$) and
309 in cynomolgus monkeys (26-fold; $p < 0.001$) (data not shown). Thus, the kidney
310 experienced significantly greater exposure to MEF compared with MMF (after
311 DMF dosing), which might be expected as the kidney to plasma ratio was higher
312 for MEF.

313

314 **Interaction between DMF and MEF based on gene expression changes in**
315 **mice**
316 As determined by induced gene expression changes relative to vehicle, DMF,
317 MEF, and their combination exhibited varied pharmacodynamic activity based on
318 tissue type, with many gene expression changes unique to either DMF or MEF
319 exposure (figure e-1). All samples were normalized and analyzed together to
320 identify genes that exhibit a change in expression uniquely due to DMF or MEF,
321 as well as interaction effects between DMF and MEF. In the combined tissues
322 data set, 487 genes were found to change specifically from DMF treatment.
323 These genes were enriched for pathways for the nuclear factor (erythroid-derived
324 2)-like 2 (Nrf2)-mediated oxidative stress response, glutathione (GSH)-mediated
325 detoxification, and other environmental sensing pathways (e.g., aryl hydrocarbon
326 receptor signaling) (Table e-2). In total, 224 genes were identified with
327 expression changes specifically due to MEF; they were enriched for death
328 receptor signaling pathway, apoptosis signaling, and autophagy-related pathway.
329 The absolute mean value of each tissue for the DMF- and MEF-specific groups
330 was subjected to unsupervised hierarchical clustering (figure 2A). DMF specificity
331 was more pronounced in the mesenteric lymph node (MLN), inguinal lymph node
332 (ILN), spleen, and whole blood, whereas MEF specificity was found
333 predominantly in the kidney and MLN. After combination therapy, 132 DEGs
334 exhibited a significant interaction effect between DMF and MEF. The most
335 pronounced interactions between fumarates were found in tissues related to
336 immune function (whole blood, MLN, ILN, and spleen) (figure 2B and table e-3)

337 which is of interest for the relative amount of lymphocyte suppression by each
338 fumarate compound. The unfolded protein response (a stress response) and
339 neurodegenerative signaling (e.g., Huntington's disease, RNA polymerase III
340 assembly, and protein degradation) pathways were uniquely enriched for DMF
341 and MEF interaction. These biological trends were constant regardless of
342 whether the tissues were pooled or kept separate for the analysis.
343

344 **DMF/MEF combination induces fast and moderate-to-severe lymphopenia**
345 **in patients with MS**

346 To assess biological consequences in humans, effects on lymphocyte counts in
347 patients with MS treated with DMF/MEF were retrospectively analyzed.
348 DMF/MEF treatment led to a fast and profound reduction (44%) of ALC within the
349 first year of treatment (figure 3 and table 2). ALCs remained suppressed beyond
350 12 months until the end of the observation (24 months). Using a multivariate
351 linear regression analysis DMF/MEF dose (coef. -1.05, 95%CI -2.09 - -0.01,
352 p=0.047), age at treatment start (coef. -13.32, 95%CI -23.61 - -3.04, p=0.01),
353 time point of sampling (coef. -73.97, 95%CI -133.68 - -14.26, p=0.02) and
354 baseline ALC (coef. 0.51, 95%CI 0.33 – 0.70, p<0.001) influenced ALC, whereas
355 previous use of immunosuppressive treatments and sex did not.
356 Grade 2 or 3 lymphopenia was not present at baseline but occurred in
357 27.8% (grade 2) and 5.6% (grade 3) of patients at the second year of DMF/MEF
358 treatment (table 1).

359 In 17 of 21 patients with available lymphocyte subpopulation data, the
360 $CD4^+$:$CD8^+$ ratio correlated with ALC (Spearman rho correlation -0.52; p = 0.02;
361 n = 21) and increased 1.5-fold in the first year and 2.3-fold in the second year
362 (figure 4 and table 3). The increase in the $CD4^+$:$CD8^+$ ratio was driven by a 3.5-
363 fold higher suppression of $CD8^+$ compared with $CD4^+$ T cells (maximum
364 reduction of $CD4^+$ T cells 19% vs $CD8^+$ T cells 66%). Finally, we analyzed
365 lymphocyte data longitudinally from patients who switched from DMF/MEF to
366 DMF. In general, the LI normalized for dosage of the DMF component increased
367 in 6 of 7 patients, with an increase of median (IQR) LI from -4.33 (4.83) to -1.04
368 (4.33) (Mann–Whitney U test, p = 0.04) after switching from DMF/MEF to DMF.
369 In addition, when analyzing the ALC values without normalization to DMF
370 dosage, an ALC increase in 4 of 7 patients was observed despite an increase of
371 DMF dosage of 23%. One patient demonstrated stable ALCs, with a 100%
372 increase in DMF dose. In the remaining 2 patients, both experienced a further
373 decrease of ALCs, with a 78% increased DMF dose after withdrawal of MEF.
374

375 **Discussion**

376 Fumaderm® provided initial evidence of the potential therapeutic effects of
377 fumarates in patients with MS.[17, 18] The specific in vivo pharmacokinetic,
378 pharmacodynamic, and immunologic effects of DMF and MEF salts in
379 Fumaderm® have not been investigated.[7] In vitro studies have demonstrated
380 differential effects of DMF and MEF, which may provide insight to the in vivo
381 differences observed. Specifically, differential effects of DMF and MEF were

382 observed for a targeted set of biological properties, including Kelch-like ECH-
383 associated protein 1 (Keap1) modification, Nrf2 activation, and GSH consumption
384 and biosynthesis.[7] DMF and MMF could potentially inhibit the activation of
385 lymphoid and myeloid cells by downregulation of aerobic glycolysis via the
386 succination and inactivation of glyceraldehyde 3-phosphate dehydrogenase
387 (GAPDH).[19] In addition, DMF and MMF activate endogenous detoxifying and
388 antioxidant pathway genes through binding to Keap1, activating Nrf2
389 transcriptional activity, and modulating GSH levels and activating GSH
390 biosynthesis.[7, 20]

391 A primary goal of these studies was to determine whether
392 coadministration of DMF and MEF would provide an additive response or trigger
393 unique biological responses in vivo. An unbiased transcriptional approach was
394 used to characterize the differences between DMF, MEF, and DMF/MEF under
395 steady-state exposure in vivo. The individual contributions of DMF and MEF were
396 explored using doses that reflected the composition of Fumaderm®. Oral
397 administration of DMF and MEF showed significant differences in their
398 biodistribution and excretion profiles in mice, rats, and monkeys. MEF exhibited
399 10- to 20-fold higher compound exposure in the kidney relative to MMF.
400 Compared with systemic exposure, DMF levels were 4-fold higher than MEF
401 levels in the brain. This could indicate that DMF might be more potent in directly
402 targeting oxidative stress pathways in the CNS.

403 In mice, DMF showed preferential modulation of transcripts in tissues
404 related to immune function (spleen, MLN, ILN, and whole blood), whereas MEF

405 showed a preference for transcript modulation in the kidney and MLN. This
406 difference with MEF might be explained by its remarkably reduced concentration
407 and area under the curve compared with DMF, which are likely the result of the
408 combination of a lower relative dose and increased renal excretion. However,
409 these effects might also be associated with individual transcriptional effects of the
410 2 compounds, as the number of DEGs modulated by DMF are considerably
411 higher in organs with exposure similar to MEF, such as the kidney. It remains
412 uncertain whether the DMF-induced transcriptional changes are mediated by
413 MMF signaling through HCAR2[21] (expressed on myeloid cells), through Nrf2
414 (ubiquitously expressed in the body), or an additional pathway yet to be
415 described. DMF likely has multiple therapeutic targets as it functions through
416 both Nrf-2 dependent and independent pathways, indirect and/or direct inhibition
417 of NF- κB, and modulation of oxidative stress-sensitive transcription factors and
418 STATs through DMF-induced glutathione depletion and reactive oxygen species
419 induction. [18, 22, 23] These analyses did not identify differential effects of DMF/MEF
420 on Keap1 and GAPDH transcripts. In contrast, previous studies have shown
421 post-transcriptional regulation through direct modification of activity of proteins
422 such as Keap1 and GAPDH.[19, 24] Specifically, DMF modification of lipid metabolic
423 pathways and impairment of aerobic glycolysis and GAPDH activity by direct
424 modification of the GAPDH protein itself are both related to DMF-induced
425 immunological changes.[19, 24] There are legitimate questions about whether the
426 GAPDH preclinical data at high doses is relevant for human subjects that have
427 much lower C_{max} levels of MMF relative to mice, but the potential exists for it to

428 be active in vivo. Pharmacodynamic data of DMF and MEF monotherapies
429 andcombined DMF/MEF treatment, as well as DEG data assessing compounds'
430 interactions, indicate that differential gene expression may be more complex than
431 increasing potency or total dosage. It is not known whether the fumarate tissue
432 distribution and gene-expression profiles shown in animals in this analysis differ
433 from that in humans.

434 Our analyses of lymphocyte kinetics in patients with MS support the
435 pharmacodynamic results. In patients who switched from DMF/MEF to DMF
436 monotherapy, ALC increased even after normalization for DMF dosage. A
437 pronounced and early reduction of ALCs during treatment with DMF/MEF was
438 shown over a follow up of 24 months. Treatment of patients with MS with
439 DMF/MEF led to an increase in the $CD4^+$:$CD8^+$ ratio, with a predominant
440 reduction of $CD8^+$ cells. Similar increases in $CD4^+$:$CD8^+$ ratios were observed in
441 DMF/MEF-treated patients with psoriasis,[9] yet this appears to be more
442 pronounced than in patients with MS receiving DMF monotherapy (1.4-fold).[25] In
443 a recent study, DMF monotherapy shifted the immunophenotype of circulating
444 lymphocyte subsets, and ALC closely correlated with $CD4^+$ and $CD8^+$ T-cell
445 counts.[26] No increased risk of serious infection was observed in patients with low
446 T-cell subset counts.[26]

447 Owing to the limited sample size,-data analyses were limited, especially
448 for T-cell subpopulations. Despite these limitations, multivariate regression
449 analysis demonstrated that ALC was significantly forecasted by age, baseline
450 ALC, DMF/MEF dose, as well as time point of sampling. Age and baseline ALC

451 are also known parameters predicting baseline ALC during DMF monotherapy,
452 further supporting our analysis.[27] Specifically, previous analyses found that age
453 ≥60 years and a baseline ALC <2 g/L are independent risk factors for the
454 development of a severe lymphopenia during DMF therapy.[27] The small
455 subpopulation of patients from our study that switched from DMF/MEF to DMF
456 and exhibited an increase in ALC had a mean (SD) age of 54.1 (14.9) years.[28, 29]
457 The retrospective design with intervals between testing not being well defined
458 might introduce bias in the results.

459 In conclusion, our experimental and clinical data provide evidence for
460 different immunological effector mechanisms of DMF compared with MEF. It is
461 not clear whether these different pathways are associated with lymphopenia
462 induced by FAEs, yet this study provides data on potential mechanisms for the
463 individual therapies. Although several mechanisms leading to lymphopenia have
464 been proposed (e.g., apoptosis, GSH depletion, oxidative stress, bone marrow
465 affection), exact pathomechanisms remain elusive.[6, 7, 20, 30] Prolonged severe and
466 moderate lymphopenia is considered a risk factor for very rare cases of
467 progressive multifocal leukoencephalopathy in patients treated with DMF;
468 therefore, identifying the differential effects of FAEs on lymphocyte counts is
469 relevant for MS patient management.[26, 30]

470 **Total 5 figures and Tables**

471 **Table 1.** Distribution of lymphopenia grade 1–4 in DMF/MEF-treated patients

Lymphopenia, n/N (%)	Before DMF/MEF	1st year of DMF/MEF	2nd year of DMF/MEF	No. of patients with lymphopenia (1st and 2nd year)
No lymphopenia, >900/µl	27/28 (96.4)	24/31 (77.4)	8/18 (44.4)	21/32 (65.6)
Grade 1, 800–900/µl	1/28 (3.6)	4/31 (12.9)	4/18 (22.2)	4/32 (12.5)
Grade 2, 500–799/µl	0/28(0)	1/31 (3.2)	5/18 (27.8)	5/32 (15.6)
Grade 3, 200–500/µl	0/28 (0)	2/31 (6.5)	1/18 (5.6)	2/32 (6.3)

472 Abbreviations: DMF = dimethyl fumarate; MEF = monoethyl fumarate.

473

474 **Table 2.** White blood cell data from DMF/MEF-treated patients

Month	Mean (SEM)	N
0	1.80 (0.11)	28
3	1.49 (0.12)	18
6	1.00 (0.12)	12
9	1.14 (0.11)	14
12	1.01 (0.17)	13
15	1.10 (0.26)	10
18	1.01 (0.15)	10
21	0.98 (0.12)	4
24	1.00 (0.19)	6

475

476 The table shows absolute lymphocyte counts in DMF/MEF-treated patients.
477 Mean (SEM) lymphocyte counts (× 10^9/L) over 3-month periods for patients
478 treated with DMF/MEF. ALC = absolute lymphocyte count; DMF = dimethyl
479 fumarate; MEF = monoethyl fumarate.

480

481 **Table 3.** $CD4^+$:$CD8^+$ ratio correlated with lymphocyte count

DMF/MEF	CD4		CD8		CD4/CD8 Ratio
	Median (IQR)	Percent change	Median (IQR)	Percent change	
Before DMF/MEF (n=5)	468 (434)		301 (194)		1.56
1st year of treatment (n=6)	374 (203)	-20%	161 (219)	-47%	2.32
2nd year of treatment (n=10)	378 (399)	-19%	103 (199)	-66%	3.69

482

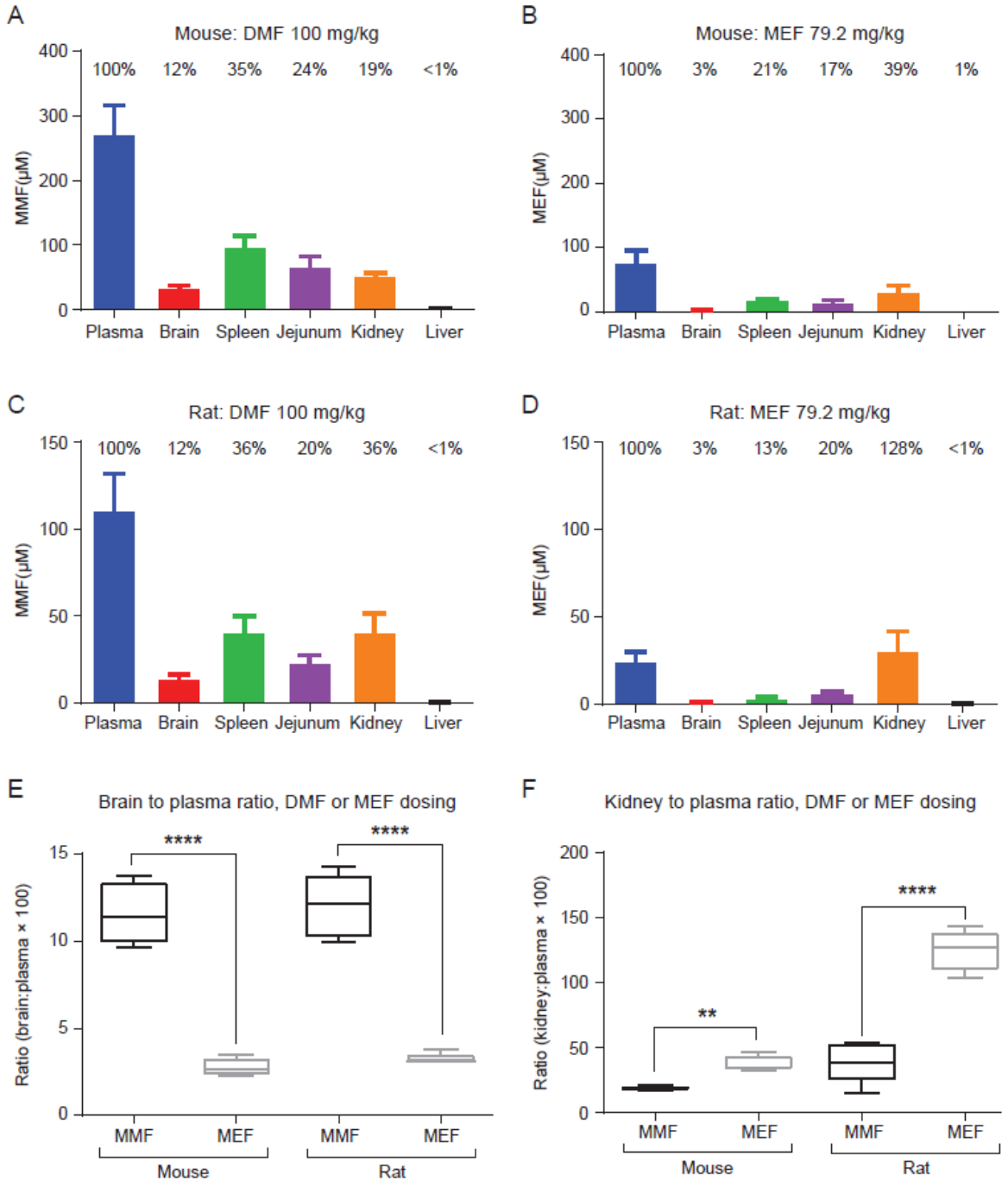
483 The median and percentage change for $CD4^+$ and $CD8^+$ T cells are shown below

484 the figure. DMF = dimethyl fumarate; IQR = interquartile range; MEF = monoethyl

485 fumarate.

486

487 **Figure 1** Tissue distribution of MEF and DMF metabolite (MMF) in mice and rats



488

489 **Figure 1 legend** (A–D) Mice and rats were administered a single dose of DMF

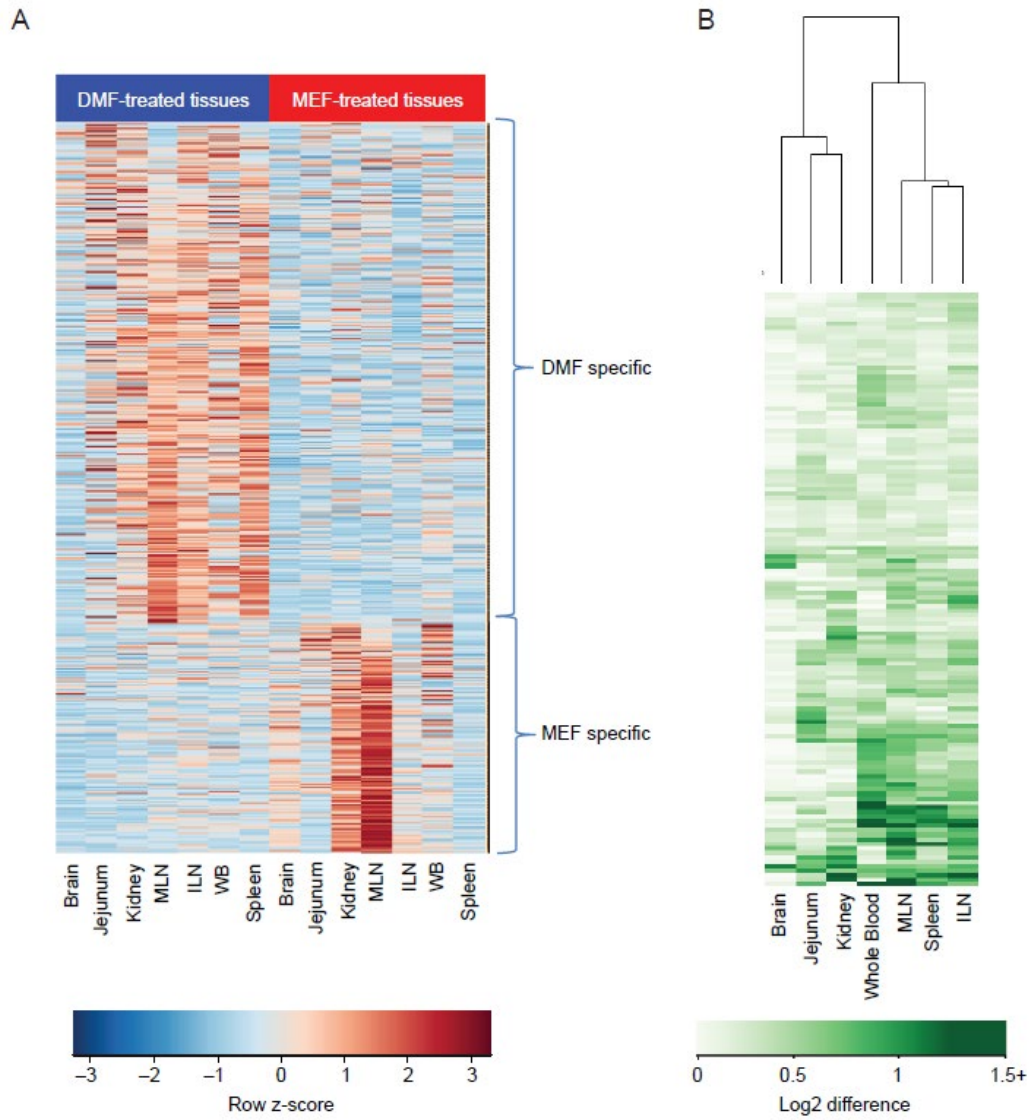
490 (100 mg/kg) (A and C) or MEF (79 mg/kg) (B and D). Plasma and tissues levels

491 (brain, spleen, jejunum, kidney, and liver) of MEF and MMF were determined 30

492 minutes after dosing. Percentages above each bar represent the percent tissue
493 penetration relative to plasma concentration. (E) Plasma to brain ratios for DMF
494 and MEF treatment in mice and rats highlight significantly higher DMF (MMF)
495 brain exposure ($p < 0.001$ for both species). (F) Plasma to kidney ratios for DMF
496 and MEF treatment in mice and rats indicate significantly lower kidney exposure
497 for DMF treatment compared with MEF ($^{**}p < 0.01$ and $^{****}p < 0.001$ in mice and
498 rats, respectively). DMF = dimethyl fumarate; MEF = monoethyl fumarate; MMF =
499 monomethyl fumarate.
500

501 **Figure 2** (A) DMF and MEF specificity across tissues and blood and (B)

502 magnitude of interaction effect in mice



503

504 **Figure 2 legend** (A) After pooling all tissues, the absolute value in each tissue of

505 the group averages (DMF – vehicle) and (MEF – vehicle) were subjected to

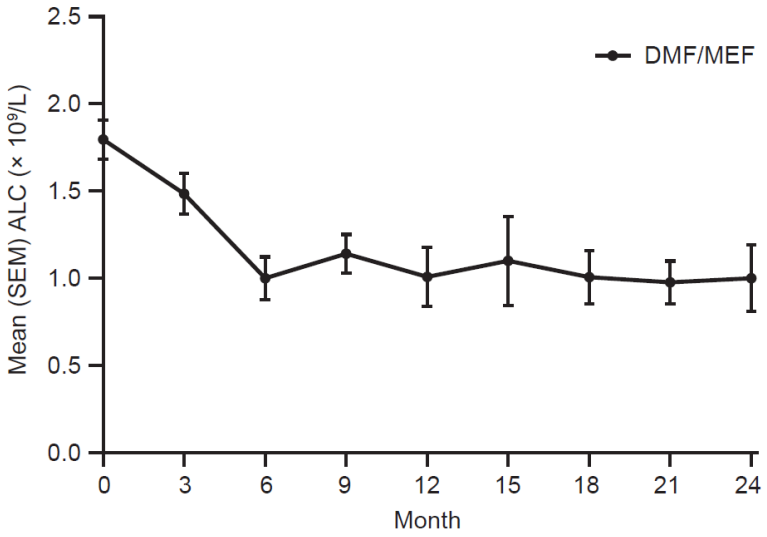
506 unsupervised hierarchical clustering (n = 7 biological sample sets each) for the

507 487 DMF-specific and 224 MEF-specific probe sets. The relative magnitude of

508 the degree of specificity in each tissue is shown. DMF specificity is most

509 pronounced in MLN, ILN, spleen, and whole blood, whereas MEF specificity is
510 most evident in the kidney and MLN. (B) For each of the 132 interaction probe
511 sets, the absolute value of the difference of (DMF – vehicle) and (combination –
512 MEF) was subjected to unsupervised hierarchical clustering. The interaction
513 effect in each tissue is shown. An interaction between DMF and MEF is most
514 pronounced in the immunological tissues: whole blood, MLN, ILN, and spleen.
515 DMF = dimethyl fumarate; ILN = inguinal lymph node; MEF = monoethyl
516 fumarate; MLN = mesenteric lymph node; WBC = white blood cell.

517 **Figure 3** White blood cell data from DMF/MEF-treated patients



518

519

520

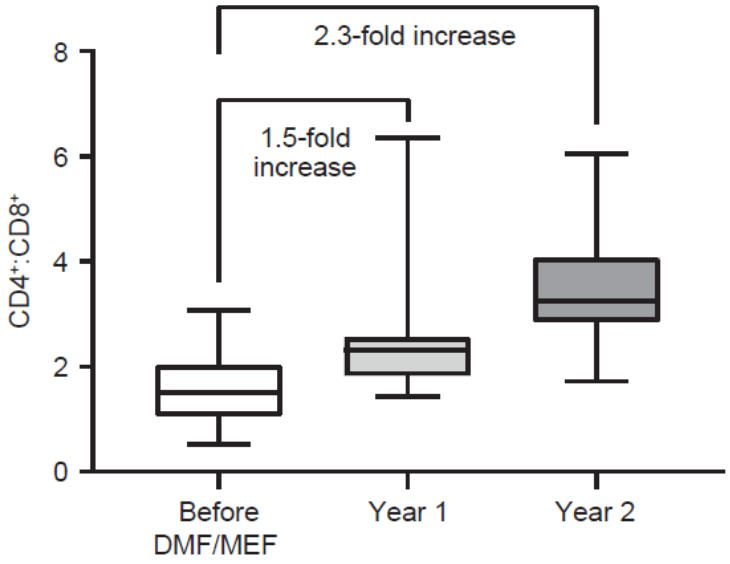
521 **Figure 3 legend** The figure shows absolute lymphocyte counts ƒ in DMF/MEF-

522 treated patients. Mean (SEM) lymphocyte counts (× 10^9/L) over 3-month periods

523 for patients treated with DMF/MEF. ALC = absolute lymphocyte count; DMF =

524 dimethyl fumarate; MEF = monoethyl fumarate.

525 **Figure 4** $CD4^+$:$CD8^+$ ratio correlated with lymphocyte count



526

527 **Figure 4 legend** $CD4^+$ and $CD8^+$ T cells in patients before DMF/MEF and 1 and

528 2 years after DMF/MEF treatment. The box and whiskers plot shows median,

529 IQR, and minimum/maximum for the $CD4^+$:$CD8^+$ ratio. DMF = dimethyl fumarate;

530 IQR = interquartile range; MEF = monoethyl fumarate.

531

532 ***Supplementary tables and figures = limited to 3 figures / tables***

533 **Table e-1** Characteristics of DMF/MEF-treated patients with MS

Characteristic	Patients (N = 36)
MS disease course, n/N	
RRMS or relapsing progressive MS	18/36
Progressive MS	17/36
Neuromyelitis optica	1/36
Any previous MS medication, n/N	28/36
MS therapy within 3 months before switch, n/N	
None	26/36
Interferon-beta formulations	5/36
Fingolimod	2/36
Mitoxantrone	2/36
Azathioprine	1/36
Mean (SD) age at switch to MEF/DMF, y	56 (10.6)
Female, n/N	24/36
MS duration (SD) at switch to MEF/DMF, y	13.1 (7.8)
IV steroids at baseline (within 2 weeks), n/N	3/36
Mean (SD) IV steroids dose, mg	1167 (577)
Immunosuppressive drug in medical history, n/N	16/36
Mitoxantrone, n/N	14/36
Mean (SD) cumulative dose of mitoxantrone, mg/m^2 body surface area	73 (31.6)

Mean (SD) interval between mitoxantrone and Fumaderm©, y	2.4 (1.9)
Azathioprine, n/N	3/36
Mean (SD) interval between azathioprine and Fumaderm©, y	7.7 (6.8)
Methotrexate, n/N	2/36
Mean (SD) interval between methotrexate and Fumaderm©, y	2 (1.4)
Switch MEF/DMF to DMF	
Mean (SD) therapy durations MEF/DMF, mo	12 (8)
Mean (SD) follow-up during DMF, mo	7.7 (4.1)
No therapy-free interval, n/N	6/7
Therapy-free interval, wk (n)	6 (1)

534 Abbreviations: DMF = dimethyl fumarate; MEF = monoethyl fumarate; MS =
535 multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis.
536

537 **(NEW) Table e-2 Specific genes/pathways in mice most impacted by DMF and MEF**

Pathways	Gene Symbols	-log (P-value)
Interaction Pathways		
Aldosterone Signaling in Epithelial Cells	DNAJA1, DNAJB1, HSPA8, HSPH1, SOS1	3.13E+00
Assembly of RNA Polymerase III Complex	GTF3C4, GTF3C2	2.79E+00
Unfolded protein response	Hspa1b, HSPA8, HSPH1	2.68E+00
Huntington's Disease Signaling	Hspa1b, DNAJB1, HSPA8, NCOR1, SOS1	2.34E+00
DMF-specific Pathways		
NRF2-mediated Oxidative Stress Response	SQSTM1, GSTA3, GSTA5, GCLC, CBR1, TXN, NQO1, GSTK1, MGST1, PRDX1, GSTM1, GSTM5, CAT, AOX1, MAFG, FTL, GSTP1, FTH1	9.27E+00
Xenobiotic Metabolism Signaling	GSTA3, GSTA5, GCLC, UGT2B7, UGT1A9 (includes others), CAMK2D, Ces1g, NQO1, GSTK1, MGST1, ESD, GSTM1, GSTM5, CAT, UGT2B28, FTL, NDST1, GSTP1, ABCC3, UGT1A6	7.92E+00
Glutathione-mediated Detoxification	GSTA3, GSTA5, GSTM1, GSTM5, GSTP1, GSTK1, MGST1	6.48E+00
Aryl Hydrocarbon Receptor Signaling	GSTA3, GSTA5, GSTM1, GSTM5, RBL1, NQO1, GSTP1, GSTK1, CTSD, MGST1	4.13E+00
Nicotine Degradation III	UGT2B7, UGT1A9 (includes others), AOX1, UGT2B28, Aox3, UGT1A6	3.71E+00
Formaldehyde Oxidation II (Glutathione-dependent)	ADH5, ESD	3.61E+00
Nicotine Degradation II	UGT2B7, UGT1A9 (includes others), AOX1, UGT2B28, Aox3, UGT1A6	3.34E+00
Serotonin Degradation	UGT2B7, UGT1A9 (includes others), ADH5, ALDH2, UGT2B28, UGT1A6	3.30E+00
LPS/IL-1 Mediated Inhibition of RXR Function	GSTA3, GSTA5, GSTM1, GSTM5, CAT, APOE, NDST1, GSTP1, GSTK1, MGST1, ABCC3	3.14E+00
Thyroid Hormone Metabolism II (via Conjugation and/or Degradation)	UGT2B7, UGT1A9 (includes others), UGT2B28, UGT1A6	2.67E+00
Pentose Phosphate Pathway (Oxidative Branch)	PGD, G6PD	2.62E+00

Pathways	Gene Symbols	-log (P-value)
Glutathione Redox Reactions I	PRDX6, GSTK1, MGST1	2.51E+00
Superoxide Radicals Degradation	CAT, NQO1	2.31E+00
Estrogen-mediated S-phase Entry	E2F6, SKP2, RBL1	2.22E+00
Role of BRCA1 in DNA Damage Response	E2F6, RFC1, FAM175A, SMARCA2, RBL1	2.12E+00
MEF-specific Pathways		
RhoA Signaling	MYL12B, PIP5K1A, ROCK1, CDC42EP3, ACTR3, RDX	3.10E+00
Apoptosis Signaling	MAP2K7, KRAS, PARP1, ROCK1, CYCS	2.92E+00
Signaling by Rho Family GTPases	MAP2K7, GNG5, MYL12B, PIP5K1A, ROCK1, CDC42EP3, ACTR3, RDX	2.91E+00
Death Receptor Signaling	MAP2K7, PARP1, TNKS2, ROCK1, CYCS	2.86E+00
Sphingosine and Sphingosine-1-phosphate Metabolism	SGPP1, ASAH1	2.67E+00
fMLP Signaling in Neutrophils	KRAS, Calm1 (includes others), GNG5, PPP3CB, ACTR3	2.55E+00
Cardiac Hypertrophy Signaling	MAP2K7, KRAS, Calm1 (includes others), GNG5, MYL12B, PPP3CB, ROCK1	2.41E+00
autophagy	NBR1, LAMP2, BECN1	2.40E+00
RhoGDI Signaling	GNG5, MYL12B, PIP5K1A, ROCK1, ACTR3, RDX	2.34E+00
Ephrin Receptor Signaling	KRAS, GNG5, RAP1B, ABI1, ROCK1, ACTR3	2.32E+00
B Cell Receptor Signaling	MAP2K7, KRAS, BCL6, Calm1 (includes others), RAP1B, PPP3CB	2.30E+00
Role of NFAT in Cardiac Hypertrophy	MAP2K7, CSNK1A1, KRAS, Calm1 (includes others), GNG5, PPP3CB	2.27E+00
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	MAP2K7, KRAS, Calm1 (includes others), PPP3CB	2.26E+00
Axonal Guidance Signaling	KRAS, GNG5, TUBB6, MYL12B, NRP1, RAP1B, PPP3CB, ROCK1, BRCC3, ACTR3	2.25E+00
Regulation of the Epithelial-Mesenchymal Transition Pathway	MAP2K7, ESRP2, KRAS, PSEN2, FRS2, ZEB2	2.21E+00
Telomere Extension by Telomerase	TNKS2, HNRNPA2B1	2.11E+00

Pathways	Gene Symbols	-log (P-value)
UVA-Induced MAPK Signaling	KRAS, PARP1, TNKS2, CYCS	2.10E+00
Granzyme B Signaling	PARP1, CYCS	2.06E+00
Regulation of Actin-based Motility by Rho	MYL12B, PIP5K1A, ROCK1, ACTR3	2.05E+00
RAN Signaling	RAN, KPNB1	2.01E+00

538 Abbreviations: DMF = dimethyl fumarate; MEF = monoethyl fumarate; MS = multiple sclerosis; RRMS = relapsing-

539 remitting multiple sclerosis.

540

541

542

543 **(NEW) Table e-3 Specific pathways in mice most impacted by a combination of DMF and MEF**

Tissue	Ingenuity Canonical Pathways	Proportion of pathway molecules represented in DEG list	Molecules	Pvalue
Blood	Aryl Hydrocarbon Receptor Signaling	1.17E-02	NQO1,TGM2	1.10E-03
Blood	Superoxide Radicals Degradation	1.25E-01	NQO1	2.19E-03
Blood	Pregnenolone Biosynthesis	7.69E-02	MICAL3	2.19E-03
Blood	Histidine Degradation VI	5.00E-02	MICAL3	3.31E-03
Blood	Ubiquinol-10 Biosynthesis (Eukaryotic)	3.33E-02	MICAL3	4.79E-03
Brain	Superoxide Radicals Degradation	1.25E-01	NQO1	6.31E-04
Brain	Nicotine Degradation III	1.37E-02	Aox3	5.37E-03
Brain	Nicotine Degradation II	1.18E-02	Aox3	6.31E-03
Brain	Hypoxia Signaling in the Cardiovascular System	1.47E-02	NQO1	6.92E-03
ILN	Aryl Hydrocarbon Receptor Signaling	1.17E-02	GSTM5,NQO1	7.76E-04
ILN	NRF2-mediated Oxidative Stress Response	1.03E-02	GSTM5,NQO1	1.29E-03
ILN	Superoxide Radicals Degradation	1.25E-01	NQO1	1.86E-03
ILN	Xenobiotic Metabolism Signaling	6.94E-03	GSTM5,NQO1	2.88E-03
ILN	Glutathione-mediated Detoxification	2.27E-02	GSTM5	8.71E-03
Jejunum	Xenobiotic Metabolism Signaling	5.56E-02	ABCC2,ABCC3,ALDH1A1,CES1,Ces1e ,GCLC,GSTA3,GSTA5,GSTK1,GSTM1,	1.58E-18

			Gstm3,GSTM4,GSTM5,NQO1,UGT2B15,UGT2B7	
Jejunum	Glutathione-mediated Detoxification	1.82E-01	GSTA3,Gsta4,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5	2.00E-15
Jejunum	NRF2-mediated Oxidative Stress Response	5.64E-02	ABCC2,CBR1,GCLC,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,NQO1	5.01E-13
Jejunum	LPS/IL-1 Mediated Inhibition of RXR Function	4.49E-02	ABCC2,ABCC3,ACOX2,ALDH1A1,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5	5.01E-12
Jejunum	Aryl Hydrocarbon Receptor Signaling	5.26E-02	ALDH1A1,GSTA3,GSTA5,GSTK1,GSTM1,Gstm3,GSTM4,GSTM5,NQO1	5.01E-11
Jejunum	PXR/RXR Activation	5.43E-02	ABCC2,ABCC3,ALDH1A1,Aldh1a7,GSTM1	6.17E-07
Jejunum	Serotonin Degradation	5.13E-02	ALDH1A1,Aldh1a7,UGT2B15,UGT2B7	1.51E-05
Jejunum	Glutathione Biosynthesis	1.82E-01	GCLC,GSS	1.78E-05
Jejunum	Histamine Degradation	6.90E-02	ALDH1A1,Aldh1a7	4.47E-04
Jejunum	Î³-glutamyl Cycle	7.14E-02	GCLC,GSS	6.03E-04
Jejunum	Fatty Acid Î±-oxidation	8.70E-02	ALDH1A1,Aldh1a7	6.92E-04
Jejunum	Oxidative Ethanol Degradation III	5.00E-02	ALDH1A1,Aldh1a7	6.92E-04
Jejunum	Putrescine Degradation III	6.67E-02	ALDH1A1,Aldh1a7	7.76E-04
Jejunum	Tryptophan Degradation X (Mammalian, via Tryptamine)	6.90E-02	ALDH1A1,Aldh1a7	8.71E-04
Jejunum	Ethanol Degradation IV	6.90E-02	ALDH1A1,Aldh1a7	8.71E-04
Jejunum	Dopamine Degradation	5.26E-02	ALDH1A1,Aldh1a7	1.58E-03

Jejunum	Sorbitol Degradation I	2.00E-01	SORD	2.45E-03
Jejunum	Retinoate Biosynthesis I	5.41E-02	AKR1B10,ALDH1A1	2.82E-03
Jejunum	Thyroid Hormone Metabolism II (via Conjugation and/or Degradation)	3.77E-02	UGT2B15,UGT2B7	2.82E-03
Jejunum	Ethanol Degradation II	4.65E-02	ALDH1A1,Aldh1a7	2.95E-03
Jejunum	Retinol Biosynthesis	4.44E-02	CES1,Ces1e	3.31E-03
Jejunum	Noradrenaline and Adrenaline Degradation	3.77E-02	ALDH1A1,Aldh1a7	3.55E-03
Jejunum	Nicotine Degradation III	2.74E-02	UGT2B15,UGT2B7	7.24E-03
Jejunum	L-serine Degradation	1.67E-01	SRR	7.41E-03
Jejunum	Melatonin Degradation I	3.03E-02	UGT2B15,UGT2B7	8.13E-03
Jejunum	Superpathway of Melatonin Degradation	2.47E-02	UGT2B15,UGT2B7	9.55E-03
Jejunum	Heme Degradation	9.09E-02	BLVRB	9.77E-03
Jejunum	Nicotine Degradation II	2.35E-02	UGT2B15,UGT2B7	9.77E-03
Kidney	LXR/RXR Activation	6.47E-02	ALB,APOA1,APOC1,APOC2,APOC3,APOE,GC,SERPINA1,TTR	7.41E-08
Kidney	LPS/IL-1 Mediated Inhibition of RXR Function	4.49E-02	ALAS1,ALDH3A1,APOC1,APOC2,APOE,FABP5,GSTA3,Gstm3,GSTM4,GSTM5,GSTP1	1.58E-07
Kidney	NRF2-mediated Oxidative Stress Response	5.13E-02	AOX1,EPHX1,GSR,GSTA3,Gstm3,GSTM4,GSTM5,GSTP1,HMOX1,NQO1	2.14E-07
Kidney	Glutathione-mediated Detoxification	1.14E-01	GSTA3,Gstm3,GSTM4,GSTM5,GSTP1	8.13E-07
Kidney	Atherosclerosis Signaling	5.76E-02	ALB,APOA1,APOC1,APOC2,APOC3,APOE,PLA2G7,SERPINA1	1.07E-06

Kidney	Xenobiotic Metabolism Signaling	3.82E-02	ALDH3A1,Ces2b/Ces2c,GSTA3,Gstm3,GSTM4,GSTM5,GSTP1,HMOX1,NQO1,UGT2B10,UGT2B15	1.20E-06
Kidney	Nicotine Degradation III	6.85E-02	AOX1,CYP2D6,CYP2J2,UGT2B10,UGT2B15	1.91E-05
Kidney	IL-12 Signaling and Production in Macrophages	4.46E-02	ALB,APOA1,APOC1,APOC2,APOC3,APOE,SERPINA1	2.29E-05
Kidney	Clathrin-mediated Endocytosis Signaling	4.04E-02	ALB,APOA1,APOC1,APOC2,APOC3,APOE,ITGB6,SERPINA1	2.29E-05
Kidney	Aryl Hydrocarbon Receptor Signaling	4.09E-02	ALDH3A1,GSTA3,Gstm3,GSTM4,GSTM5,GSTP1,NQO1	3.02E-05
Kidney	Pentose Phosphate Pathway	1.30E-01	G6PD,PGD,TKT	3.89E-05
Kidney	Nicotine Degradation II	5.88E-02	AOX1,CYP2D6,CYP2J2,UGT2B10,UGT2B15	4.17E-05
Kidney	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	3.30E-02	ALB,APOA1,APOC1,APOC2,APOC3,APOE,SERPINA1	1.41E-04
Kidney	Heme Degradation	1.82E-01	BLVRB,HMOX1	2.34E-04
Kidney	Pentose Phosphate Pathway (Oxidative Branch)	1.82E-01	G6PD,PGD	3.89E-04
Kidney	Melatonin Degradation I	6.06E-02	CYP2D6,CYP2J2,UGT2B10,UGT2B15	3.98E-04
Kidney	Superpathway of Melatonin Degradation	4.94E-02	CYP2D6,CYP2J2,UGT2B10,UGT2B15	5.62E-04
Kidney	Coagulation System	7.89E-02	PLAU,PLAUR,SERPINA1	1.38E-03
Kidney	FXR/RXR Activation	3.64E-02	APOA1,APOC2,APOC3,APOE	2.14E-03
Kidney	Acute Phase Response Signaling	2.76E-02	ALB,APOA1,HMOX1,SERPINA1,TTR	4.37E-03
Kidney	Serotonin Degradation	3.85E-02	ALDH3A1,UGT2B10,UGT2B15	6.76E-03

MLN	Airway Pathology in Chronic Obstructive Pulmonary Disease	1.82E-01	MMP2,MMP9	1.00E-04
MLN	NRF2-mediated Oxidative Stress Response	2.05E-02	GSTA3,GSTM5,HMOX1,NQO1	3.89E-04
MLN	Glutathione-mediated Detoxification	4.55E-02	GSTA3,GSTM5	1.32E-03
MLN	Xenobiotic Metabolism Signaling	1.39E-02	GSTA3,GSTM5,HMOX1,NQO1	1.78E-03
MLN	Hepatic Fibrosis / Hepatic Stellate Cell Activation	1.94E-02	AGTR1,MMP2,MMP9	2.40E-03
MLN	Aryl Hydrocarbon Receptor Signaling	1.75E-02	GSTA3,GSTM5,NQO1	2.45E-03
MLN	Inhibition of Matrix Metalloproteases	5.00E-02	MMP2,MMP9	2.57E-03
MLN	IL-8 Signaling	1.33E-02	HMOX1,MMP2,MMP9	5.62E-03
MLN	Glioma Invasiveness Signaling	3.03E-02	MMP2,MMP9	5.62E-03
MLN	Eicosanoid Signaling	2.33E-02	LTC4S,PTGDS	6.61E-03
MLN	Heme Degradation	9.09E-02	HMOX1	7.76E-03
MLN	LPS/IL-1 Mediated Inhibition of RXR Function	1.22E-02	GSTA3,GSTM5,HMGCS2	8.71E-03
Spleen	NRF2-mediated Oxidative Stress Response	1.54E-02	AOX1,GSTA3,GSTM5	8.13E-06
Spleen	Glutathione-mediated Detoxification	4.55E-02	GSTA3,GSTM5	2.04E-05
Spleen	Aryl Hydrocarbon Receptor Signaling	1.17E-02	GSTA3,GSTM5	5.25E-04
Spleen	LPS/IL-1 Mediated Inhibition of RXR Function	8.16E-03	GSTA3,GSTM5	1.29E-03
Spleen	Xenobiotic Metabolism Signaling	6.94E-03	GSTA3,GSTM5	1.95E-03
Spleen	Guanosine Nucleotides Degradation III	4.35E-02	AOX1	3.39E-03

Spleen	Urate Biosynthesis/Inosine 5'-phosphate Degradation	4.35E-02	AOX1	3.63E-03
Spleen	Adenosine Nucleotides Degradation II	3.57E-02	AOX1	4.47E-03
Spleen	Purine Nucleotides Degradation II (Aerobic)	2.70E-02	AOX1	5.25E-03

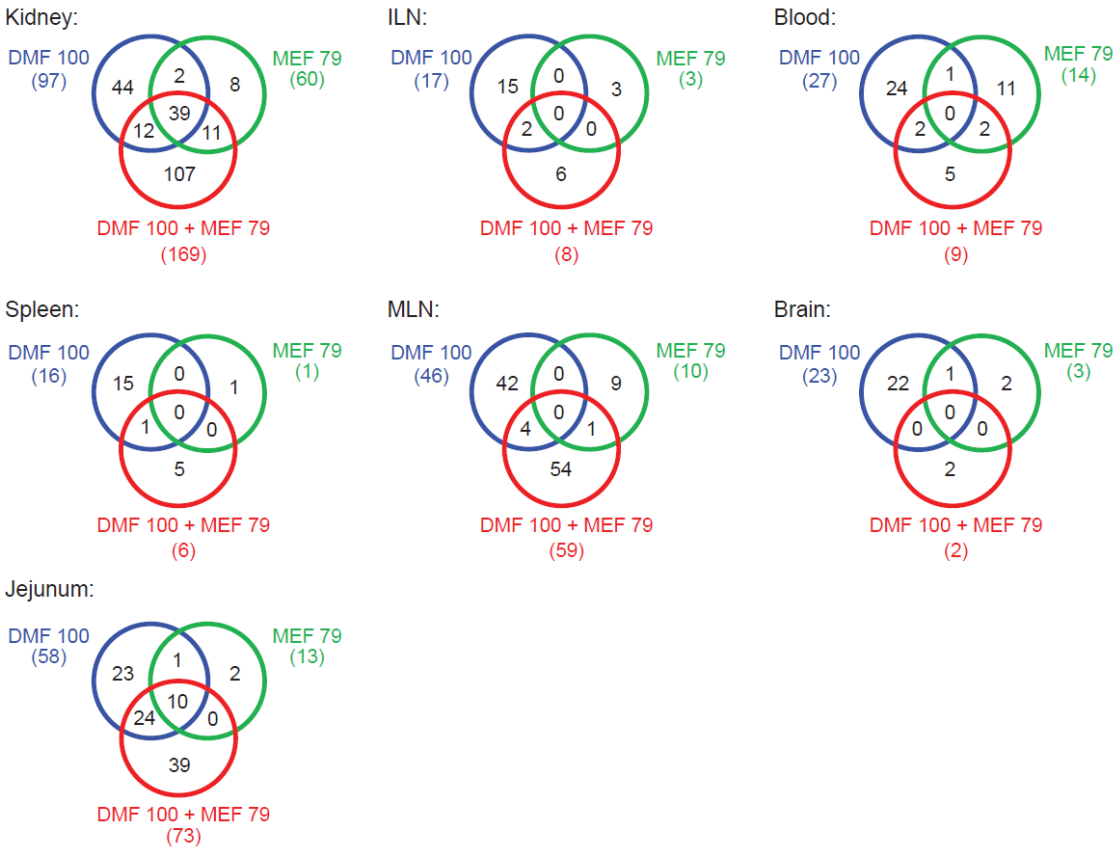
544 Abbreviations: DEG = differentially expressed gene; ILN = inguinal lymph node; MLN = mesenteric lymph node.

545 Pathways with significant changes ($p<0.01$) after treatment of mice with the combination of DMF and MEF.

546 Pathways with significant changes ($p<0.01$) after treatment of mice with the combination of DMF and MEF.

547 **Figure e-1** Steady-state tissue-specific DEGs in response to chronic DMF, MEF,

548 and DMF/MEF administration in mice



549

550 Tissue was harvested after 10 days of daily treatment with DMF, MEF, or

551 DMF/MEF. DEGs were identified by comparing the groups DMF-vs-vehicle,

552 MEF-vs-vehicle, and DMF/MEF-vs-vehicle in each tissue. The number in

553 parentheses designates the total number of DEGs for that treatment. DEG =

554 differentially expressed gene; DMF = dimethyl fumarate; ILN = inguinal lymph

555 node; MLN = mesenteric lymph node; MEF = monoethyl fumarate.

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558 **Appendix 1** Author Contributions

Name	Location	Contribution
Brian T. Wipke, PhD	Biogen, Inc., Cambridge, MA	Designed and conceptualized study, interpreted the data, drafted the manuscript for intellectual content, revised the manuscript for intellectual content
Robert Hoepner, MD PhD	Inselspital, Bern University Hospital, University of Bern, Switzerland	Generated, analyzed, and interpreted data; revised manuscript for intellectual content
Katrin Strassburger-Krogias, MD	St. Josef Hospital, Ruhr University Bochum, Germany	Role in acquisition of data, interpreted the data, revised the manuscript for intellectual content
Ankur Thomas, MS	Biogen, Inc., Cambridge, MA	Designed and conceptualized study; major role in acquisition of data; generated, analyzed and interpreted data; revised manuscript for intellectual content
Davide Gianni, PhD	Biogen, Inc., Cambridge, MA	Designed and conceptualized study; major role in acquisition of data; generated, analyzed and interpreted data; revised manuscript for intellectual content
Suzanne Szak, PhD	Biogen, Inc., Cambridge, MA	Analyzed the data; interpreted the data; major role in revising the manuscript for intellectual content
Melanie S. Brennan, PhD	Biogen, Inc., Cambridge, MA	Generated, analyzed and interpreted data, revised manuscript for intellectual content
Maximilian Pistor, MD	Inselspital, Bern University Hospital, University of Bern, Switzerland	Analyzed the data; interpreted the data
Ralf Gold, MD, PhD	St. Josef Hospital, Ruhr University Bochum, Germany	Major role in study design and drafting of the manuscript; revised manuscript for intellectual content
Andrew Chan, MD	Inselspital, Bern University Hospital, University of Bern, Switzerland	Designed and conceptualized study; drafted the manuscript for intellectual content; major role in the acquisition of data; interpreted the data; revised the manuscript for intellectual content
Robert H. Scannevin, PhD	Biogen, Inc., Cambridge, MA	Design and conceptualized study, analyzed the data, drafted the manuscript for intellectual content, major role in the acquisition of data, interpreted the data, revised the manuscript for intellectual content

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