

1 **DNA methylation at diagnosis is associated with response to disease-**
2 **modifying drugs in early rheumatoid arthritis**

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35

36 **Running footnote:** DNA methylation and treatment response in early RA

37 **Abstract**

38 **Aims.** A proof-of-concept study to explore whether DNA methylation at first diagnosis
39 is associated with response to disease-modifying antirheumatic drugs (DMARDs) in
40 patients with early rheumatoid arthritis (RA).

41 **Patients & Methods.** DNA methylation was quantified in T-lymphocytes from 46
42 treatment-naïve patients using HumanMethylation450 BeadChips. Treatment response
43 was determined at six months using the EULAR response criteria.

44 **Results.** Initial filtering identified 21 CpGs that were differentially methylated between
45 responders and non-responders. After conservative adjustment for multiple testing, six
46 sites remained statistically significant, of which four showed high sensitivity and/or
47 specificity ($\geq 75\%$) for response to treatment. Moreover, methylation at two sites in
48 combination was the strongest factor associated with response (80.0% sensitivity,
49 90.9% specificity, AUC 0.85).

50 **Conclusions.** DNA methylation at diagnosis is associated with DMARD treatment
51 response in early RA.

52

53 **Introduction**

54 Rheumatoid arthritis (RA) is a chronic inflammatory joint disease of autoimmune origin
55 that affects 0.5–1.0% of the adult population [1, 2]. Treatment of patients with centres
56 on the use of a variety of synthetic disease-modifying antirheumatic drugs (DMARDs).
57 Methotrexate is the first-line DMARD of choice for the treatment and management of
58 RA, prescribed as monotherapy or in combination with other DMARDs. Although these
59 agents are efficacious for the treatment of RA [3-5], clinically meaningful responses are
60 not observed in all patients and a significant proportion remain refractory to treatment.

61

62 A substantial body of literature supports an important role for epigenetic dysregulation,
63 including of DNA methylation, in the pathogenesis of RA [reviewed in 6-8]. Evidence
64 also suggests that disease modifying agents such as methotrexate may influence DNA
65 methylation [9, 10]. Moreover, methylation status as a potential biomarker associated
66 with response to therapy has been demonstrated in other conditions [11] and proposed
67 for use in RA by several investigators [12, 13]. DNA is methylated through enzymatic
68 conversion of cytosine to methylcytosine; this occurring almost invariably at cytosine-
69 phosphate-guanine sites (CpGs). In the context of promoter-associated sites,
70 methylation is associated with transcriptional repression and gene silencing [14]. In RA,
71 alterations to the DNA methylome are apparent in multiple cell types important in the
72 disease process, including peripheral blood-derived mononuclear cells, lymphocytes and
73 joint-derived fibroblasts. Recently, we were the first to define disease-associated
74 methylation changes that were distinct to individual T- and B-lymphocyte populations
75 [15]. Moreover, we reported methylation differences in these lymphocyte populations in
76 treatment-naïve patients at first RA diagnosis [16]. Whilst providing evidence for a role

77 in the development of the disease, our findings support DNA methylation profiling at
78 diagnosis as a potential source of biomarkers for response to treatment in RA.

79

80 It is clear that the ability to identify which patients will respond to treatment offers
81 considerable benefits for the management of RA. For example, it would (i) facilitate
82 rapid dose-escalation and reduce time to effective response in those likely to be poor
83 responders to traditional regimens, and (ii) avoid unwanted side-effects in those likely
84 to show an effective response to lower doses or monotherapy. These benefits are all the
85 more important given evidence that response to first treatment with disease-modifying
86 agents is strongly associated with long-term outcome in these patients [17]. The search
87 for biomarkers associated with response has encompassed demographic and clinical
88 factors as well as genetic associations and expression profiling of proinflammatory and
89 other mediators [18-20]. However, no single factor or combination of factors have thus
90 far proven to be accurate and reliable in determining which patients will respond to
91 DMARD therapy.

92

93 Our aim therefore, in this proof-of-concept study, was to determine whether genome-
94 wide DNA methylation profiles at first diagnosis are associated with response to
95 treatment with conventional DMARDs (as determined by improvement in disease
96 activity using the validated European League Against Rheumatism (EULAR) response
97 criteria) in a typical population of newly-diagnosed, treatment-naïve patients with RA.
98 As in our previous work, we examined methylation in purified T-lymphocyte
99 populations, cells that are instrumental in the disease process and chronic inflammation

100 [21], and for which relationships with disease activity have recently been described [22-
101 24].

102 **Patients and Methods**

103 **Study population**

104 A prospective cohort of 46 Caucasian patients attending the early synovitis clinic at the
105 Haywood Rheumatology Centre in Stoke-on-Trent, UK, and presenting with
106 symptomatic inflammatory arthritis suspected to be RA was recruited. All patients were
107 subsequently classified as having RA, according to the 2010 ACR/EULAR
108 classification criteria, by a consultant rheumatologist [25]. No patients had been treated
109 with DMARDs or biological agents at the time of recruitment. Clinical data collected at
110 baseline included disease activity, erythrocyte sedimentation rate (ESR), rheumatoid
111 factor (RF) and anti-citrullinated peptide antibodies (ACPA). Demographic and clinical
112 characteristics are presented in **Table 1**. At diagnosis with RA, all patients began
113 treatment with one or more DMARDs (methotrexate, hydroxychloroquine, and
114 sulphasalazine) and the majority received parenteral corticosteroids, solely for the
115 clinical management of RA and as directed by a consultant rheumatologist. Patients
116 were followed for six-months and remained on treatment throughout. The study was
117 approved by the East Midlands (Derby) Research Ethics Committee. All patients
118 provided written informed consent.

119

120 Disease activity was determined at recruitment (prior to initiation of DMARD therapy)
121 and after three and six months of treatment using the disease activity score with 28-joint
122 counts (DAS28) [26], though data at three months was excluded from further analysis
123 due to the known short-term effect of corticosteroid treatment on DAS28 scores.

124 DAS28 scores range from 0-10: a score >5.1 indicates high disease activity while one of
125 ≤ 3.2 denotes low disease activity. Response to treatment was determined at six months

126 according to the DAS28-based EULAR response criteria [26-28], which evaluate
127 response in patients with RA based on a composite categorization incorporating both
128 change in DAS28 from baseline (Δ DAS28) and final absolute DAS28 score.
129 Specifically, these criteria classify response as ‘good’ (Δ DAS28 >1.2 , current DAS28
130 ≤ 3.2), ‘moderate’ (Δ DAS28 >1.2 , current DAS28 >3.2 , or Δ DAS28 $>0.6-1.2$, current
131 DAS28 ≤ 5.1) and ‘no’ (Δ DAS28 ≤ 0.6 , or Δ DAS28 $>0.6-1.2$, current DAS28 >5.1) [28].
132 According to these criteria, responders were defined as patients with a ‘good’ or
133 ‘moderate’ response to treatment, and non-responders as patients with ‘no’ response to
134 treatment.

135

136 **Isolation of T-lymphocytes**

137 Fresh peripheral blood samples (35 ml, EDTA) were collected from each patient at
138 baseline, prior to the initiation of treatment. CD3⁺ T-lymphocytes were isolated from
139 mononuclear cell preparations using positive selection with magnetic microbeads
140 (MACS® Separation System; Miltenyi Biotec). We have previously shown this method
141 to yield high-purity T-lymphocyte populations (mean $\geq 99\%$) in RA patients [15].
142 Genomic DNA was extracted using an AllPrep DNA/RNA/miRNA Universal kit
143 (Qiagen) and stored at -20°C prior to use.

144

145 **Genome-wide DNA methylation profiling**

146 DNA methylation was quantified at $>480,000$ CpG sites using the
147 HumanMethylation450 BeadChip (Illumina Inc.; hereafter referred to as ‘array’).
148 Details of array design and coverage have been described elsewhere [29]. Genomic
149 DNA samples ($n = 46$) were treated with sodium bisulfite using an EZ DNA

150 Methylation Kit (Zymo Research) and subsequently were hybridized to arrays according
151 to manufacturer recommended protocols, as previously described (performed by
152 Hologic Tepnel Pharma Services, Manchester, UK) [30]. All samples passed stringent
153 internal array quality control, including sample-independent (*e.g.* staining,
154 hybridization) and sample-dependent (*e.g.* bisulfite conversion) controls. Methylation at
155 individual CpG sites is reported as a β -value ranging from 0 to 1 (unmethylated to fully
156 methylated, respectively) [29].

157

158 **Sodium bisulfite Pyrosequencing**

159 Array candidates were independently validated by bisulfite Pyrosequencing using a
160 PyroMark Q24 instrument and analysis software (Qiagen), as we have previously
161 described [15, 30]. Briefly, fresh genomic DNA aliquots were sodium bisulfite-
162 converted and amplified using whole genome amplification [30, 31]. Thereafter,
163 Touchdown PCR [32, 33] was used to prepare PCR amplicons containing CpGs of
164 interest. Assay details are provided in **Supplementary Table 1**.

165

166 **Data analysis**

167 Array data (idat files) were processed and analyzed using the Bioconductor package
168 Minfi [34]. We removed from analysis all CpGs with a detection p-value >0.01 in any
169 one or more of the 46 samples and all probes targeting sites on the X and Y
170 chromosomes (a total of 12,295 CpGs). Data were normalized by Subset-quantile
171 Within Array Normalization (SWAN), as described by Maksimovic *et al.* [35], and
172 multi-dimensional scaling plots were examined to confirm appropriate adjustment for
173 potential confounding due to batch effects (processing date, array position and slide).

174

175 To identify methylation differences associated with treatment response, patients were
176 stratified into responders and non-responders. CpGs showing altered methylation
177 between the two groups were identified using the ‘dmpFinder’ function in Minfi. This
178 function performs an F-test to compare groups and was used with logit-transformed β -
179 values (M-values), as recommended by Du *et al.* [36]. P-values <0.05 were considered
180 statistically significant and, together with a mean β -value difference ≥ 0.1 between the
181 groups, were used as an initial screening tool to identify sites displaying differential
182 methylation. Two further filtering steps were subsequently applied to identify
183 differentially methylated CpGs as those sites where: 1) at least two-thirds of non-
184 responders showed a β -value difference ≥ 0.1 relative to the responder mean; and 2) at
185 least two-thirds of responders displayed a β -value equal to or in excess of the responder
186 mean. Filtering criteria are summarized in **Figure 1**. We then applied a Bonferroni
187 adjustment at stage 5, based on comparisons conducted using the final 21 CpGs
188 identified.

189

190 The McNemar test was used to examine the incidence of patients with moderate/high
191 disease activity between baseline and six-months. The association of baseline
192 methylation status with treatment response was determined by calculating sensitivity,
193 specificity, positive predictive value (PPV) and negative predictive value (NPV), and by
194 examining receiver operating characteristic (ROC) area under the curve (AUC) plots.
195 ROC curves were constructed based on logistic regression analysis with response to
196 treatment categorised as no response versus moderate/good response as described

197 above. Analyses were performed using Stata 12.0 (Intercooled; Stata Corporation, TX,
198 USA) and considering p-values <0.05 as statistically significant.

199 **Results**

200 **Characteristics of the patients**

201 **Table 1** summarizes the demographic and clinical characteristics for the RA patients at
202 recruitment. Most patients (43/46, 93.5%) started treatment with MTX, either as
203 monotherapy or in combination with other DMARDs. The majority of patients (33/46,
204 71.7%) remained on their indicated starting DMARD regimen throughout the course of
205 the study. Of the remaining patients, all but two introduced or discontinued a single
206 DMARD on one occasion during the six-month follow-up period.

207

208 **Disease activity and treatment response**

209 Moderate or high disease activity (DAS28 >3.2) was present in 43/46 (93.5%) patients
210 at recruitment (three patients had low disease activity, with DAS28 scores of 2.27, 2.66
211 and 3.18). After six-months of treatment, 28/46 (60.9%) patients had moderate/high
212 disease activity ($p < 0.001$ vs. baseline, McNemar test), with approximately two-thirds
213 (63.0%) achieving an improvement in $DAS28 \geq 1.2$. Classifying response by the
214 EULAR response criteria, the number of patients achieving a good, moderate and no
215 response to treatment at six-months was 16 (34.8%), 19 (41.3%), and 11 (23.9%),
216 respectively. On this basis, 76.1% (35/46) of patients were classified as responders and
217 the remainder as non-responders. Details of baseline characteristics and six-month
218 treatment regimens for the two groups are presented in **Supplementary Table 2**.

219

220 **Relationship between DNA methylation and treatment response**

221 Use of the robust filtering steps described in the Methods section and shown in **Figure 1**
222 identified 269 CpGs with a statistically significant difference in mean methylation β -

223 value ≥ 0.1 between responders and non-responders. Moreover, for a subset of 21 sites,
224 methylation differences were present in at least two-thirds of the individual patients
225 within each group (full annotation for these 21 sites is provided in **Supplementary**
226 **Table 3**). The majority of these sites were hypermethylated in responders (16/21,
227 76.2%), were linked with a gene (15/21, 71.4%) and were associated with a CpG island
228 and/or the surrounding shores/shelves (13/21, 61.9%).

229

230 To refine these sites further, we applied a conservative Bonferroni adjustment for
231 multiple testing, based on the 21 comparisons undertaken. This revealed six CpGs for
232 which the methylation differences between responders and non-responders remained
233 statistically significant ($p_{\text{adj}} < 0.05$; **Supplementary Table 3**). For each of these six
234 CpGs, we plotted methylation against treatment response to determine a percentage
235 methylation cut-off that in each case provided the greatest discrimination between
236 patients that responded to treatment and those that did not. Examples of two
237 differentially methylated CpGs are presented in **Figure 2**. We also calculated the
238 corresponding sensitivity and specificity for each site to assess the association of
239 methylation status with response. Using this approach, and as shown in **Table 2**, four
240 sites were identified with a sensitivity and/or specificity $\geq 75\%$ for discrimination
241 between responders and non-responders. Most notably, hypermethylation of CpG-2 and
242 hypomethylation of CpG-3 (shown in **Figure 2** and validated by Pyrosequencing in
243 **Supplementary Figure 1**) each demonstrated a sensitivity and PPV of approximately
244 90%, although the corresponding specificity and NPV were lower (63.6% and 70.0%,
245 and 63.6% and 63.6%, for CpG-2 and CpG-3, respectively). Using ROC curve analysis

246 to further evaluate the association with response, CpG-2 and CpG-3 also demonstrated
247 the highest AUC values (0.78 and 0.76, respectively).

248

249 **Combinations of CpGs associated with treatment response**

250 Focusing on the four sites identified above, we next examined the ability to discriminate
251 between responders and non-responders for each of the six possible pairs of sites. The
252 combination of hypermethylation of CpG-2 and hypomethylation of CpG-3
253 demonstrated the best overall performance with a sensitivity of 80.0% and specificity of
254 90.9% (**Table 2**). As shown in **Figure 3**, 28 of 29 patients with this combination were
255 responders (14 good and 14 moderate response; right chart, **Figure 3**). In contrast, all
256 four patients failing to satisfy either cut-off were non-responders (left chart, **Figure 3**).
257 The strength of the association of the CpG-2 + CpG-3 combination with response was
258 also reflected in a ROC AUC of 0.85 (95% confidence interval [CI] 0.71, 0.94).

259 **Discussion**

260 This is the first study to examine the link between DNA methylation and first-line
261 treatment response in RA. Using a prospective cohort of patients recruited at first
262 diagnosis and prior to the initiation of treatment, our data indicate that baseline DNA
263 methylation levels for a discrete subset of sites are significantly associated with
264 response to treatment with disease-modifying agents. The methylation status at two
265 specific sites assessed in combination, and which independently were associated with
266 response, proved to be the strongest factor associated with treatment response.

267

268 Since early, effective intervention in RA reduces disease activity and inflammation, and
269 improves long-term outcome [37-40], identification of baseline factors associated with
270 treatment response has been a priority. However, examination of a broad range of
271 clinical, molecular and genetic factors has not produced definitive biomarkers [18, 19].
272 Our findings now provide the first evidence that epigenetic profiling, in this case of
273 DNA methylation, may have significant value in identifying which patients with RA
274 may respond to first-line DMARD treatment. Furthermore, DNA methylation is an
275 attractive biomarker since it is typically stable over time, is minimally affected by short-
276 term stimuli and is readily measured [12]. The potential utility of methylation profiling
277 is further supported by a very recently reported association between differential DNA
278 methylation and response to second-line anti-TNF therapy in RA [41].

279

280 We were unable to formally examine the independence of the CpG-2 + CpG-3
281 association with treatment response in this proof-of-concept study. However, a
282 preliminary assessment using our data suggested that it was independent of baseline

283 clinical variables including disease activity, autoantibodies and systemic inflammatory
284 markers, which individually did not appear to be associated with response. This would
285 be in agreement with the main body of literature, which indicates that ESR, RF and
286 ACPA are not independently associated with response to methotrexate and/or other
287 DMARDs [reviewed in 18]. Although not reported by all studies [42], evidence does
288 indicate that male sex is associated with a better response to methotrexate [43-45]. Our
289 data suggest a possible trend towards better response in males ($p < 0.1$), which may
290 reflect treatment with methotrexate for over 90% of the patients studied.

291

292 The CpG-2 + CpG-3 combination, which we identified as the strongest independent
293 factor associated with treatment response, comprises sites in *ADAMTSL2* (CpG-2), a
294 disintegrin and metalloproteinase with thrombospondin motif-like protein, and in
295 *BTN3A2* (CpG-3), a butyrophilin family member. Although the function of
296 *ADAMTSL2* has not been fully determined, evidence supports a role in the regulation
297 of transforming growth factor- β (TGF- β) [46]. TGF- β is a pleiotropic cytokine with
298 important immunoregulatory functions [47, 48], which is implicated in RA synovial
299 pathology [49]. Butyrophilins are transmembrane proteins that share structural
300 similarities with B7 co-stimulatory molecules and are emerging as novel regulators of
301 T-lymphocyte function and immune responses [50, 51].

302

303 We focused on DNA methylation factors associated with response in the context of
304 DMARD treatment strategies that reflected standard clinical practice. Both responder
305 and non-responder groups included patients receiving methotrexate monotherapy and
306 patients receiving combination therapy, the proportions of which were not significantly

307 different either at baseline or at six-months follow-up (**Supplementary Table 2**).

308 Importantly, methylation at two CpGs in combination was strongly associated with

309 treatment response despite the limited variation in treatment regimens, supporting its

310 potential utility as a marker of response at diagnosis in a real-world clinical setting.

311 Furthermore, we purposefully used the EULAR criteria as the response measure in this

312 study as these are universally accepted and encompass both improvement in disease

313 activity over time and end-point disease activity. Reassuringly, the proportion of

314 responders in this study is consistent with previous reports using these criteria [44, 52].

315 By quantifying methylation at baseline, we are also able to exclude potential

316 confounding associated with DMARDs, including methotrexate, an impact of which on

317 methylation has been suggested by several groups [9,10,53,54].

318

319 Although our proof-of-concept study is the first of its kind in RA, a limitation of our

320 work was the relatively small number of patients that we were able to recruit. In an

321 attempt to address this, we used a number of sequential filtering steps to identify sites

322 differentially methylated between responders and non-responders to treatment.

323 Furthermore, for the two CpGs comprising the strongest biomarker associated with

324 response, we validated the array data by also quantifying methylation using an

325 independent method (Pyrosequencing). This significantly reduces the risk of type I

326 errors associated with genome-wide approaches. However, we recognise that an

327 important next step will be to confirm our findings and determine the true predictive

328 value of this biomarker in larger, independent patient cohorts.

329 **Conclusions**

330 In conclusion, we report the identification of a novel DNA methylation combination
331 that is associated with response to treatment with conventional disease-modifying drugs
332 in newly diagnosed patients with RA. Whilst our findings will require verification in
333 larger, independent early RA cohorts, they provide the first evidence to support
334 epigenetic profiling as a novel approach to identifying biomarkers associated with
335 response to DMARD therapy. Ultimately, this has the potential to inform clinical
336 management and patient care, towards the goal of a stratified, personalized medicine
337 approach to treatment.

338 **Executive Summary**

339 **Background**

- 340 • Newly diagnosed patients with rheumatoid arthritis (RA) demonstrate variability of
341 response to treatment with disease-modifying antirheumatic drugs (DMARDs).
- 342 • To date, no definitive biomarkers associated with response have been identified.
- 343 • This proof-of-concept study explored whether DNA methylation at first diagnosis is
344 associated with response to treatment with DMARDs in patients with treatment-
345 naïve early RA.

346 **Patients & Methods**

- 347 • HumanMethylation450 BeadChips were used to quantify genome-wide DNA
348 methylation at diagnosis in T-lymphocytes from 46 treatment-naïve patients with
349 early RA.
- 350 • Response to DMARD treatment was determined at six months using the DAS28-
351 based EULAR response criteria. Sensitivity, specificity and receiver operating
352 characteristic AUC data were used to assess associations of baseline methylation
353 with treatment response.

354 **Results**

- 355 • At six-months, the numbers of patients achieving a good/moderate/no response to
356 treatment were 16/19/11 (35/41/24%), respectively.
- 357 • Array analysis identified 21 CpGs displaying methylation differences between
358 responders and non-responders, of which four statistically significant sites (p_{adj}
359 <0.05 , Bonferroni) showed high sensitivity and/or specificity $\geq 75\%$ for treatment
360 response.

361 • Methylation at two individual sites in combination (cg0301849 and cg14345882)
362 was the strongest factor associated with response, with 80.0% sensitivity and 90.9%
363 specificity (AUC 0.85). 28 of 29 patients with this combination were responders.

364 **Conclusions**

365 • DNA methylation of a novel CpG combination is associated with treatment response
366 at first diagnosis in early RA patients prior to commencing treatment with
367 DMARDs.

368 • These findings provide the first evidence to support epigenetic profiling as a novel
369 approach to identifying biomarkers associated with DMARD treatment response in
370 RA. This may ultimately have the potential to inform clinical management and
371 patient care.

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- 544

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554 **Figure legends**

555 **Figure 1. Filtering criteria for identification of CpGs differentially methylated at**
556 **baseline (pre-treatment) between treatment responders and non-responders in**
557 **patients with early RA.** The starting number of CpGs indicated (482,421) is the total
558 number of CpGs on the methylation array platform. Following initial processing (step
559 1), data were normalized using SWAN [35], implemented in the Bioconductor package
560 Minfi [34]. Numbers in the figure indicate the number of CpGs remaining at each
561 successive step.

562 *Abbreviations:* RA, rheumatoid arthritis; SWAN, subset-quantile within array
563 normalization.

564

565 **Figure 2. Pre-treatment methylation status discriminates responders and non-**
566 **responders in patients with early RA.** In (A) CpG-2 (cg03018489) and (B) CpG-3
567 (cg14345882), non-responders (n = 11) and responders (n = 35) are depicted by open
568 circles and filled triangles, respectively, and where responders are divided into those
569 showing a moderate (centre, n = 19) and good (right, n = 16) response to treatment.
570 Good, moderate and no response categories are defined in the EULAR response criteria
571 [23-25]. The horizontal dashed line indicates the methylation cut-off for distinguishing
572 between responders and non-responders, and the short horizontal bar in each group
573 indicates the mean value.

574 *Abbreviations:* RA, rheumatoid arthritis; EULAR, European League Against
575 Rheumatism).

576

577 **Figure 3. Pre-treatment methylation status at two CpG sites in combination is**
578 **associated with response to treatment in patients with early RA patients.** For CpG-
579 2 (cg03018489) and CpG-3 (cg14345882) methylation status was defined as
580 hypermethylated (above) or hypomethylated (below) relative to a cut-off of 60% and
581 20%, respectively. Shown on the x-axis are the four possible methylation combinations,
582 with methylation status of CpG-2 given first and of CpG-3 given second, as indicated
583 (the two combinations in which only one CpG satisfied the cut-off value are grouped
584 together (centre chart)). Each chart depicts the proportion of patients achieving a good
585 (white), moderate (striped) and no response (dark grey) to treatment, stratified by
586 methylation status for the CpG-2/CpG-3 combination.

587 *Abbreviations:* RA, rheumatoid arthritis; Hypo, hypomethylated; Hyper,
588 hypermethylated.

589

590 **Supplementary Figure 1. Technical validation by bisulfite pyrosequencing of**
591 **baseline methylation status for two CpGs differentially methylated between**
592 **responders and non-responders in patients with early RA.** In both (A) CpG-2
593 (cg03018489) and (C) CpG-3 (cg14345882), responders (n = 35) and non-responders (n
594 = 11) are depicted by triangles and circles respectively. The short red horizontal bar
595 shown in each group indicates the mean value. For each CpG, methylation values are
596 shown for the array (filled symbols; left) and Pyrosequencing (open symbols; right).
597 Bland-Altman plots in (B) CpG-2 (cg03018489) and (D) CpG-3 (cg14345882) show the
598 agreement between % methylation levels as determined by 450K array and
599 pyrosequencing analysis. Each point represents an individual patient. Shown by
600 horizontal lines are the mean difference between the methods (bias) and the upper and

601 lower boundaries of the 95% limits of agreement (± 1.96 SD). The intraclass correlation
602 coefficient between the methods is 0.963 for CpG-2, and 0.690 for CpG-3.

603 *Abbreviations:* RA, rheumatoid arthritis; 450K, HumanMethylation450 BeadChip

604

605 **Supplementary Table 1. Assay details for candidate CpGs/genes interrogated by**
606 **bisulfite Pyrosequencing.***

607 *Further information that is not included here is available upon request.

608 †The prefix 'b-' denotes biotin labeling at the 5' end.

609 ‡The sequence indicated is post-bisulfite conversion. Letters 'Y' and 'R' denote the
610 cytosine of the CpG site interrogated by the assay ('Y' and 'R' refer to sequencing in the
611 forward and reverse orientation, respectively).

612 *Abbreviations:* bp, base pairs.

613

614 **Supplementary Table 2. Baseline demographic and clinical characteristics in early**
615 **RA patients who responded and did not respond to DMARD treatment at 6-**
616 **months follow-up.**

617 * Mann-Whitney U test (continuous variables) or Fisher's exact test (categorical), as
618 appropriate.

619 † data unavailable for two patients.

620 ‡ data unavailable for one patient.

621 § 26/45 (57.8%) patients were positive for ACPA/ RF (data unavailable for one patient).

622 ¥ The total number of patients starting treatment with a given DMARD, whether
623 received as monotherapy or in combination with other DMARDs.

624 * One further patient received monotherapy with hydroxychloroquine.

625 # One patient was not receiving DMARD treatment.

626 *Abbreviations:* RA, rheumatoid arthritis; DMARDs, disease-modifying anti-rheumatic
627 drugs; RF, rheumatoid factor; ACPA, anti-citrullinated peptide antibodies; DAS28,
628 disease activity score with 28-joint count; ESR, erythrocyte sedimentation rate.

629

630 **Supplementary Table 3. Complete list and annotation for the 21 CpGs identified as**
631 **differentially methylated at baseline (pre-treatment) between responders and non-**
632 **responders in patients with early RA patients.***

633 *Bold blue font indicates CpGs with statistically significant ($p < 0.05$, Bonferroni-
634 adjusted) differences in methylation between responders and non-responders. The

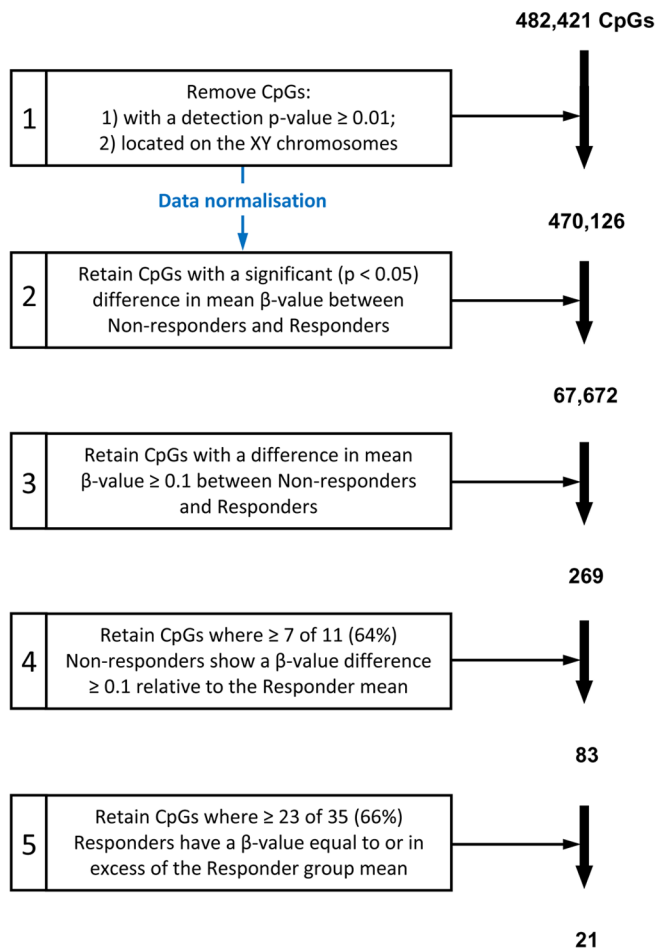
635 dashed horizontal line between rows 18 and 19 separates CpGs that were

636 hypermethylated (above) and hypomethylated (below) in responders relative to non-
637 responders.

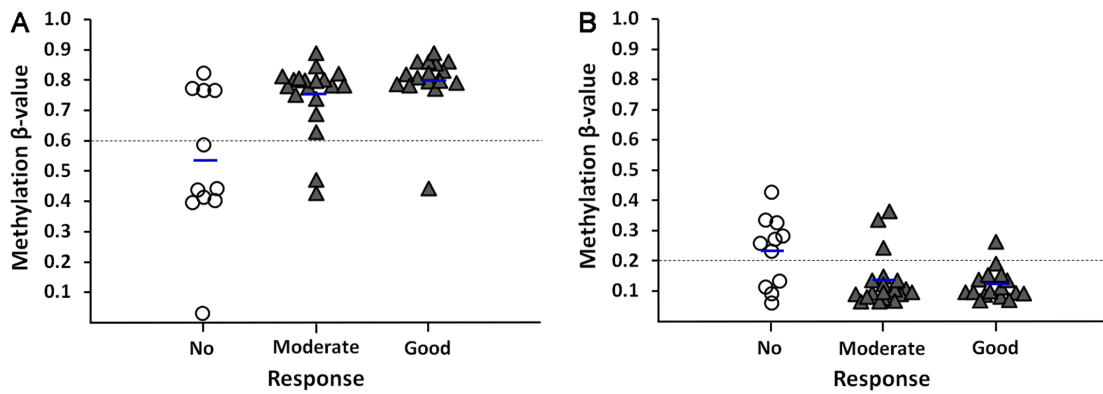
638 †The 'dmpFinder' function in Minfi [34] was used to calculate F-test p-values.

639 *Abbreviations:* RA, rheumatoid arthritis.

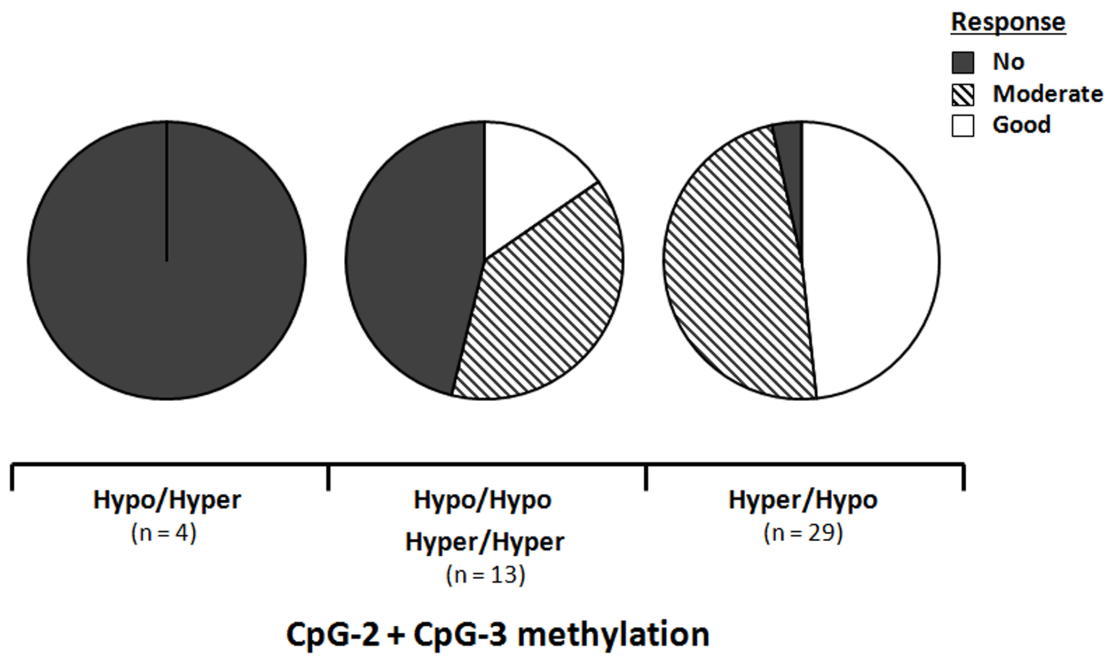
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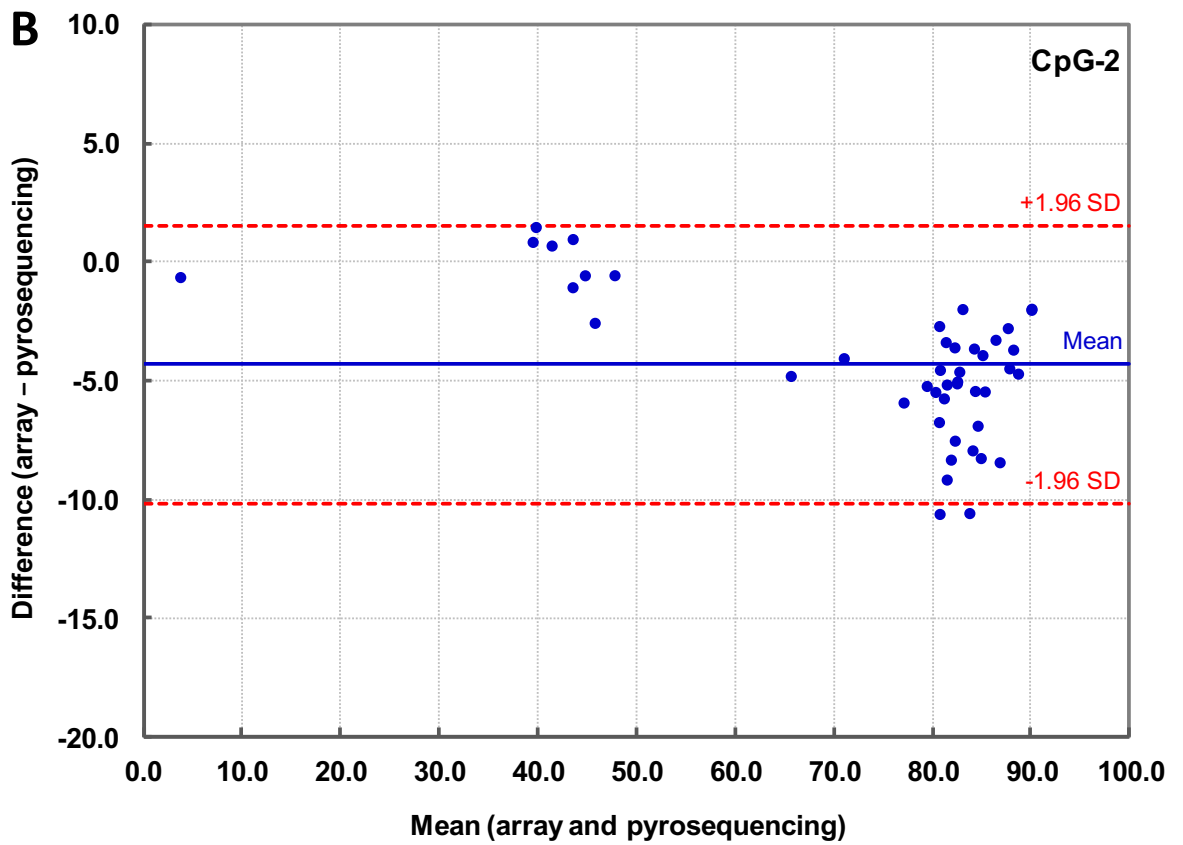
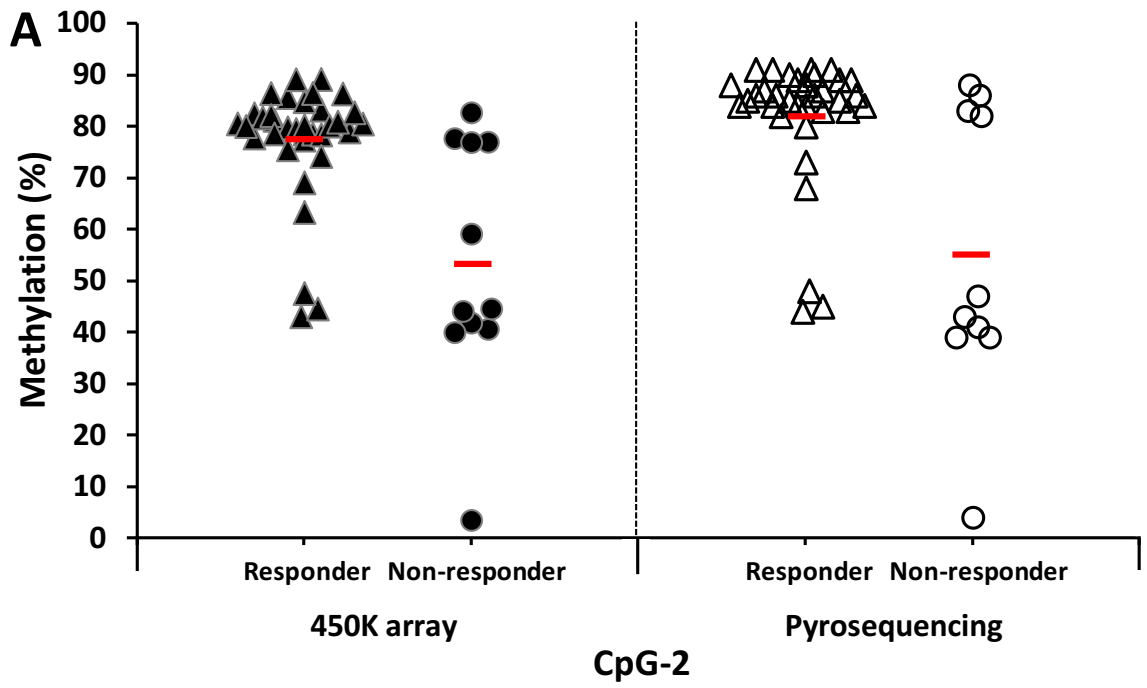


Table 1. Demographic and clinical characteristics at baseline for the cohort of 46 treatment-naïve patients with early RA.

Number	46
Male/female, No. (%)	16/30 (34.8/65.2)
Age, mean \pm SD (years)	57.7 \pm 13.9
RF positive, No. (%) ^{†§}	23 (52.3)
ACPA positive, No. (%) ^{‡§}	22 (48.9)
DAS28, mean \pm SD	5.29 \pm 1.4
ESR, mean \pm SD	30.1 \pm 23.7
Corticosteroids, No. (%)	45 (97.8)
Starting DMARD, No. (%) [¥]	
<i>Methotrexate (MTX)</i>	43 (93.5)
<i>Hydroxychloroquine (HCQ)</i>	29 (63.0)
<i>Sulphasalazine (SSZ)</i>	23 (50.0)
Starting treatment regimens, No. (%)	
<i>Monotherapy (MTX)*</i>	15 (32.6)
<i>Triple therapy (MTX+HCQ+SSZ)</i>	20 (43.5)
<i>Dual therapy (two of MTX, HCQ and SSZ)</i>	10 (21.7)

[†] of 44 patients (data unavailable for two patients).

[‡] of 45 patients (data unavailable for one patient).

[§] 26/45 (57.8%) patients were positive for ACPA/ RF (data unavailable for one patient).

[¥] The total number of patients starting treatment with a given DMARD, whether received as monotherapy or in combination with other DMARDs.

* One further patient started monotherapy with hydroxychloroquine.

Table 2. Association of baseline methylation status with treatment response in patients with early RA.*

pG ID	Methylation	Sensitivity	Specificity	PPV	NPV	ROC AUC
	in responders: Hyper/Hypo	(%)	(%)	(%)	(%)	(95% CI)
Individual sites						
pG-1 (<i>cg07225509</i>)	Hyper	77.1	72.7	90.0	50.0	0.75 (0.59, 0.86)
pG-2 (<i>cg03018489</i>)	Hyper	91.4	63.6	88.9	70.0	0.78 (0.64, 0.89)
pG-3 (<i>cg14345882</i>)	Hypo	88.6	63.6	88.6	63.6	0.76 (0.61, 0.87)
pG-4 (<i>cg23974730</i>)	Hypo	82.9	63.6	87.9	53.9	0.73 (0.59, 0.86)
Combinations						
pG-1 + CpG-2	Hyper/Hyper	71.4	90.9	96.2	50.0	0.81 (0.66, 0.91)
pG-1 + CpG-3	Hyper/Hypo	65.7	81.8	92.0	42.9	0.74 (0.59, 0.86)
pG-1 + CpG-4	Hyper/Hypo	60.0	90.9	95.5	41.7	0.75 (0.61, 0.87)
pG-2 + CpG-3	Hyper/Hypo	80.0	90.9	96.6	58.8	0.85 (0.71, 0.94)
pG-2 + CpG-4	Hyper/Hypo	77.1	72.7	90.0	50.0	0.75 (0.59, 0.86)
pG-3 + CpG-4	Hypo/Hypo	74.3	90.9	96.3	52.6	0.83 (0.69, 0.92)

*Of the six CpGs identified as significantly differentially methylated between responders and non-responders (see main text), shown are the four CpGs with a sensitivity and/or specificity $\geq 75\%$ and that showed most promise for discriminating between responders and non-responders. Also shown are the six possible CpG pairs derived from these four sites. All individual sites and combinations shown were significantly associated with treatment response ($p < 0.05$, Fisher's exact test). The CpG-2 + CpG-3 combination displayed the best overall performance ($p < 0.001$; bold font).