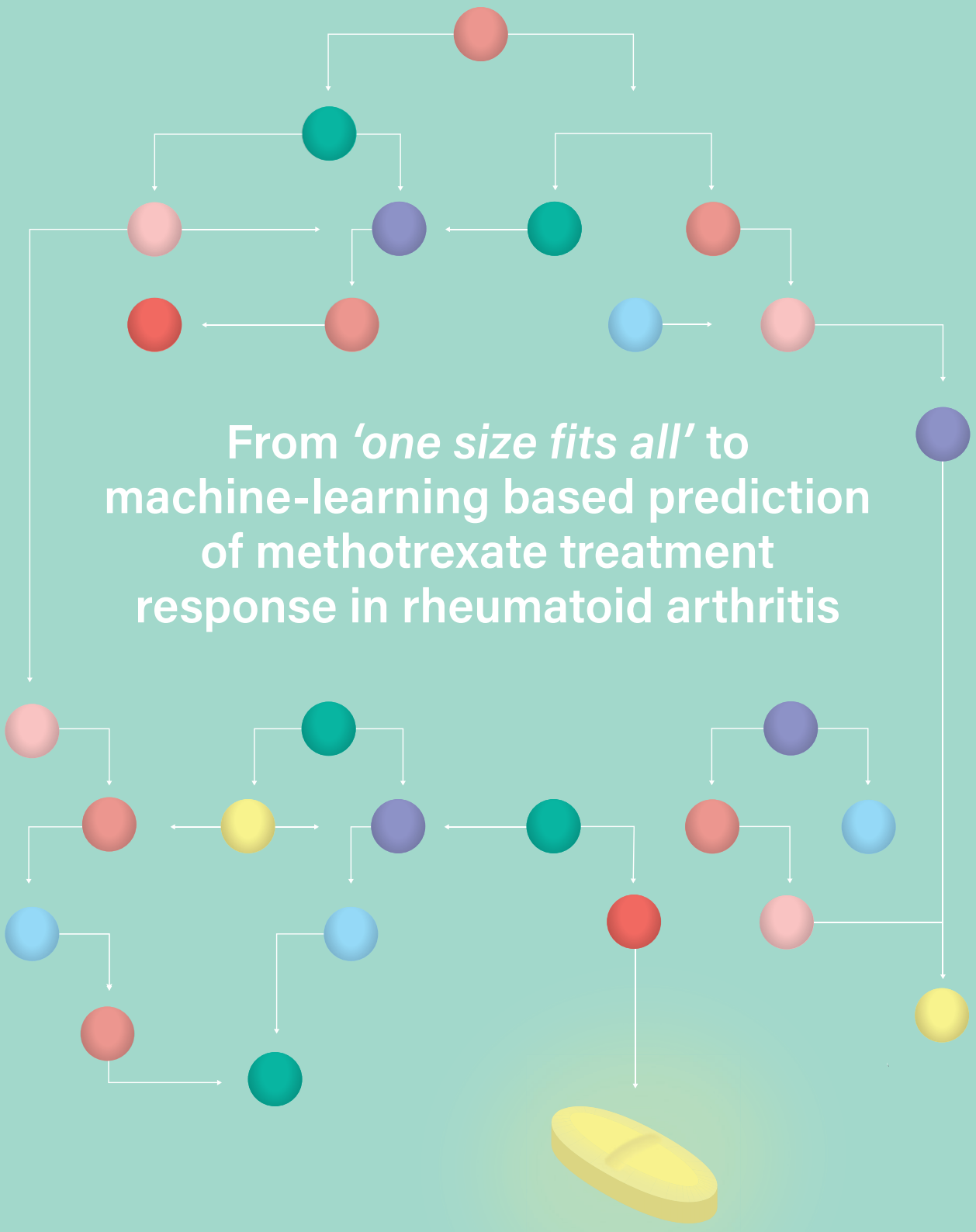

From *'one size fits all'* to machine-learning based prediction of methotrexate treatment response in rheumatoid arthritis

Helen Gosselt

From 'one size fits all' to Machine-learning based Prediction of Methotrexate Treatment Response in Rheumatoid Arthritis

Van 'one size fits all' naar op machine-learning gebaseerde predictie van methotrexaat behandeling response in reumatoïde artritis

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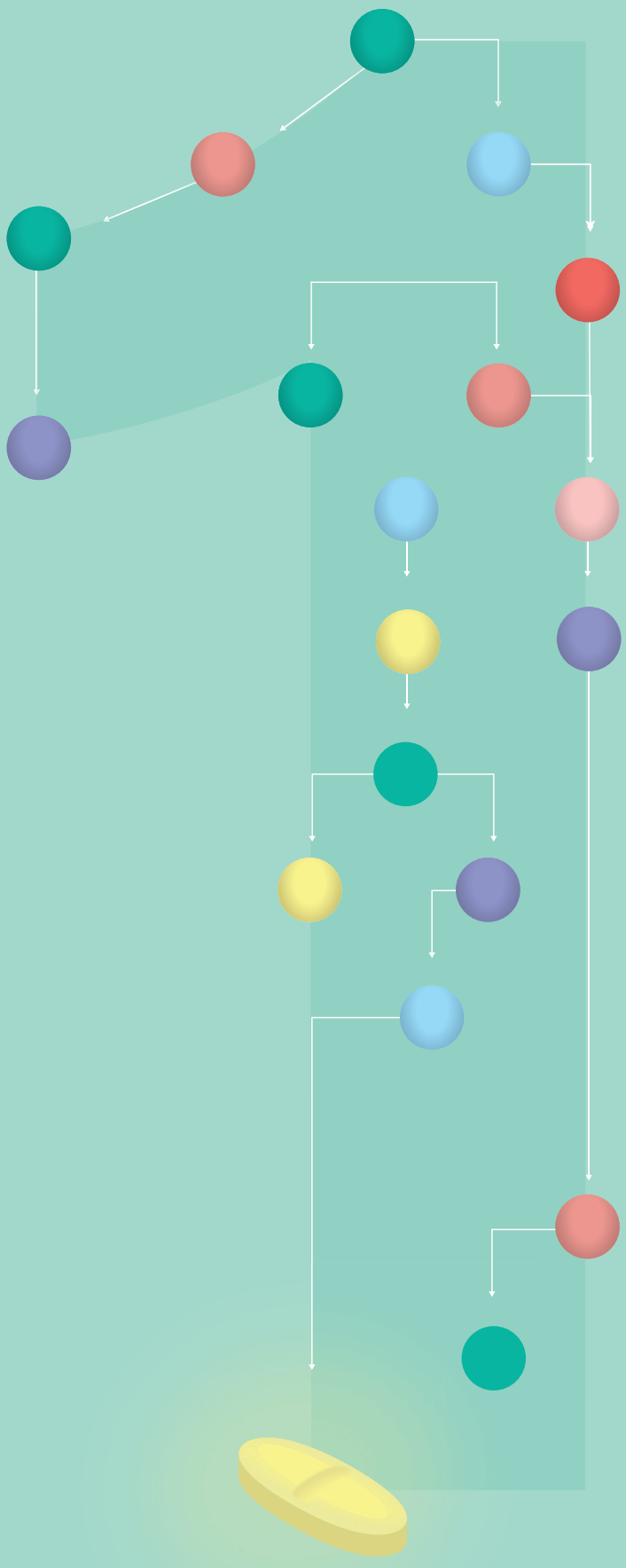

Chapter 1

General introduction

GENERAL INTRODUCTION

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic progressive autoimmune disease with a worldwide estimated prevalence of 0.24% [1], affecting twice as many women compared to man [2]. In RA, cartilage lining the joints are mistakenly recognized as foreign tissue and therefore attacked by leukocytes [3–5]. This causes inflammation and joint swelling, which can influence a patient's mobility and daily activities. In addition, the inflammation can damage other organs, causing serious comorbidities such as: cardiovascular disease, osteoporosis and gastrointestinal disease [6,7]. The first few months from diagnosis form the so-called 'window of opportunity', in which joint damage can be restricted and the disease pathogenesis can still be modified [8–12]. If patients are not treated, serious bone deformities can occur, which is why early aggressive treat-to-target interventions are required [13,14].

Treatment management in RA

First-line treatment consists of methotrexate (MTX) alone or in combination with short-term glucocorticoids and/or leflunomide or sulfasalazine [14]. Response to MTX can be determined after three to six months using the validated response measures: disease activity score 28 (DAS28) (linear) and European League against rheumatism (EULAR) response criteria (categorical) [15]. Step-up treatment with biologic DMARDs (bDMARDs) or targeted synthetic DMARDs (tsDMARDs) is prescribed when 1) no sufficient decrease in DAS28 is observed at 3 months, or 2) or remission (DAS28 <2.6) or low dose activity (LDA: DAS28<3.2) is not reached at 6 months [14]. If step-up treatment fails, which is determined after another 3 – 6 months, the bDMARD or tsDMARD can be changed for a biosimilar or bDMARD/tsDMARD from a different class. This trial-and-error strategy continues until the treatment target is reached [14]. Following these guidelines, it can take months before an effective therapy has been found, during which the window of opportunity to restrain joint damage becomes narrower. Prediction of insufficient responders prior to MTX therapy would allow to skip the first three to six months of waiting for a first response. Using this personalized (group based) approach, insufficient responder to MTX could step-up treatment from the start, taking advantage of the window of opportunity. Good responders on the other hand, could safely start MTX treatment and be spared costly bDMARDs. However, to enable this personalized treatment approach, baseline predictors for response to MTX are required. At the moment, there are no reliable models available to predict MTX inefficacy in the early course of treatment leading to 'trial-and-error' approach finding the right drug (and concentration).

Methotrexate in rheumatoid arthritis

Methotrexate (MTX) is a disease-modifying anti-rheumatic drug (DMARD), which was initially developed as chemotherapeutic agent and prescribed in high dosages (single doses up to 1000 mg) in leukaemia. In 1951, Aminopterin, the forerunner of MTX was first used in inflammatory diseases, and appeared to have anti-inflammatory effects in lower doses (15-25 mg/week) [16]. MTX still is the anchor drug in RA after all these years [14], despite development of new treatments in RA, such as: bDMARDs (e.g. TNF-alpha inhibitors, IL-6 inhibitors[17]) or more recently tsDMARDs (e.g. JAK-inhibitors [18–21]). This is due to its low costs, its safety and relatively high and similar response rates, as compared to MTX in combination with bDMARDs as recently compared [22]. MTX requires some time to accumulate and reach a steady-state in red blood cells [23,24], hence glucocorticoids are often prescribed concomitant during the first months as bridging therapy [25]. Still, up to 40% of RA patients does not benefit from MTX treatment and discontinue or step-up therapy due to adverse events or insufficient response.

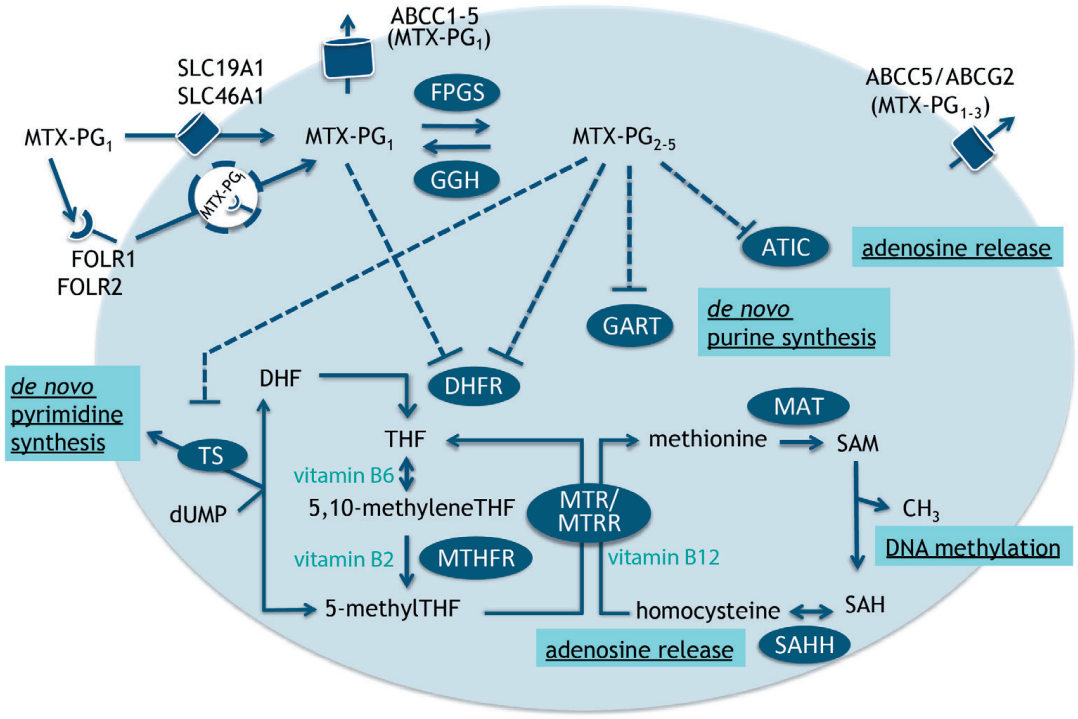

Figure 1. Simplified schematic representation of MTX metabolomic pathway. FOLR=folate receptor, SLC=solute carrier receptor family, ABCC=ATP-binding cassette transporter family, ABCG2=ATP-binding cassette super family G member2, MTX-PG=methotrexate-polyglutamate, FPGS=folylpolyglutamate synthetase, GGH=gamma-glutamyl hydrolase, DHFR=dihydrofolate reductase, MTHFR=methylenetetrahydrofolate reductase, TS=thymidylate synthase, ATIC=5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) formyltranfsferase, GART=phosphoribosylglycinamide formyltransferase, MTR=methionine synthase, MTRR=methionine synthase reductase, MAT=methionine adenosyltransferase, SAM=S-adenosylmethionine, SAH=S-adenosylhomocysteine, SAHH= SAH hydrolase.

Mechanism of action of MTX

MTX is an antifolate and shares a similar chemical structure with folate, containing one glutamate group. Circulating MTX is transported into tissue mainly through the solute carrier family 19 member 1/reduced folate carrier (SLC19A1/RFC) [26,27], for which MTX has highest affinity (Km= 1-10 μM). In the intestine, MTX is mainly transported through the solute carrier family 46 member 1/proton coupled folate transporter (SLC46A1/PCFT), which functions well in an acidic environment [28]. Furthermore, MTX is transported through folate receptors (FOLR) 1 and 2 via endocytosis and for which MTX has much lower binding affinity (Kd = 10–300 nM) (Figure 1) [29,30].

Intracellularly, the enzyme folylpolyglutamate synthetase (FPGS) attaches additional polyglutamate groups to MTX in a process called polyglutamylation, while gamma-glutamyl hydrolase (GGH) reverts this action through de-polyglutamylation. Polyglutamylation of MTX is required to remain intracellular, because longer MTX-PG chains (MTX-PG_{4-5}) are no targets for the ATP binding cassette (ABC) efflux transporter. In RA patients, polyglutamylation of up to 5 MTX-PGs has been observed [24,31–34]. Long-chain MTX-PGs have been associated with better response [24,34] and have shown to have higher affinity to its downstream targets. Yet, large longitudinal studies on cellular MTX pharmacokinetics and pharmacodynamics are lacking and large inter-patient variability in erythrocyte MTX-PGs has been observed, which complicates its use for therapeutic drug monitoring. Intracellular downstream targets of MTX include: thymidylate synthase (TS) and phosphoribosylglycinamide formyltransferase (GART), which are required for DNA synthesis. Additionally, it inhibits dihydrofolate reductase (DHFR) in the folate pathway, which is involved in donation of methyl groups for (DNA) methylation reactions [35,36]. Another target is the 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) formyltransferase (ATIC), resulting in increased AICAR and enhanced adenosine release, which has anti-inflammatory effects [37,38]. Folic acid is prescribed along (but not on the same day as) MTX to reduce adverse events, which does not reduce the efficacy of MTX [39,40]. As folic acid restores the intracellular folate pool required for DNA synthesis and is not related to the release of adenosine, the adenosine pathway is proposed to be the most likely mechanism of anti-inflammatory action of MTX [26].

PREDICTION OF MTX INEFFICACY

Clinical predictors of response to MTX

Clinical parameters would be easiest to use as predictors for response, since these are often readily available without the need for laboratory assessments. Several clinical variables have been associated with response to MTX such as symptom duration, baseline Health Assessment Questionnaire (HAQ), baseline DAS28 (components), smoking, Body Mass Index (BMI), and psychological predictors (i.e. higher Hospital Anxiety and Depression Scale anxiety score) [41–43]. However, not all of these predictors have been validated or could

not accurately predict insufficient response. Compared to clinical outcomes, biological predictors are objective measures of response and are not influenced by the feeling of a patient [44], which may therefore improve prediction of treatment response.

Biological predictors of response to MTX

A biomarker is defined by the World Health Organization (WHO) as: "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [45]. An ideal biomarker for MTX response is easily accessible and can identify insufficient responders from sufficient responders with high sensitivity and specificity. Single nucleotide polymorphisms (SNPs) are an example of biomarkers that have the advantage that they are stable over time, easy to measure and are present long before clinical symptoms show. The relationship between SNPs and response to MTX has therefore been extensively examined in genetic candidate studies for genes related to the metabolic pathway of MTX (e.g. in the RFC-1 [46], FPGS, ABCC1, ABCC3 [47,48]), in downstream effectors of the MTX pathway (e.g. ATIC, MTHFR, AICAR) [46, 49–53], or in genome-wide association studies (GWAS) [50,54]. Previously, we have also performed a candidate study, where ABCC3 and ABCB1 were significantly associated with treatment response to MTX [47]. Unfortunately, results so far have mainly been inconsistent as extensively reviewed [55,56], or could not be replicated due to homogeneous cohorts and different outcome measures, except for SLC19A1 rs1051266 [57].

Besides SNPs, epigenetic markers can alter gene expression without changing the DNA sequence. A monozygotic twin pair discordant for RA showed that external (lifestyle) and internal (pathogenic) factors affected the aetiology of RA through differences in DNA methylation [58]. This is in line with other studies that showed a relationship between epigenetic changes in relation to RA aetiology and development [59–63]. Hypothetically, differences in epigenetic regulation could therefore also be predictive for treatment response. So far, few studies have observed a relationship between DNA methylation and response to MTX treatment [36,64–68]. Also, non-coding microRNAs [69,70] and a splice variant (partial retention in intron 8 of FPGS: 8PR), leading to a dysfunctional FPGS enzyme, were associated with non-response to MTX [71]. Overall, much is still unknown about the role of epigenetic biomarkers and prediction of MTX response [72,73], hence further investigation is required.

Once the disease has been diagnosed and symptoms have shown, other biomarkers for response may be more predictive for treatment response, which comprise cytokines, autoantibodies or metabolites that all play a role in RA pathogenesis and other inflammatory diseases [74–76]. Metabolomics comprises all chemical processes and therefore directly reflects the current pathogenic phenotype of a cell. Up to now, only a handful of metabolic studies in relation to response to MTX in RA patients have been performed [75,77–80]. Most studies were metabolic profiling studies and targeted metabolites in the MTX pathway [77,81]. Besides low erythrocyte-folate, that was suggested to reflect insufficient

uptake/metabolism of MTX, other 1-carbon metabolites such as homocysteine, vitamin B12 or B6 were assessed without success [81]. To date, still not all metabolic targets of MTX have been fully understood; hence new biomarkers could have been missed using a targeted approach. Moreover, metabolites are components of large interconnected pathways and information on underlying pathways may give more insight in relation to response. That, and the fact that these cover all possible targets is why untargeted "-omic" studies (e.g. transcriptomics, proteomics, metabolomics) may be more suitable for the search of new potential biomarkers of insufficient response to MTX. Of all -omic fields, metabolomics is closest to the biological phenotype of a cell and directly reflects current chemical cellular processes. Therefore, metabolomics might provide the highest chance of finding new predictors for insufficient response.

Prediction models: the era of machine learning

Given that RA is a multidisciplinary disease, single predictors are unlikely to be strong enough to predict insufficient treatment response. That is why we and others already proposed prediction models combining multifactorial predictors (e.g. genetics, life style predictors, clinical and laboratory variables) to identify insufficient responders [42,82–89]. These models were all based on multivariable logistic regression.

In view of the development of untargeted/-omic approaches, more complex and much larger datasets are obtained. This pile of data has lead to the introduction of machine learning algorithms into the healthcare domain [90–93]. Machine learning algorithms facilitate examination of complex (non-) linear associations in large datasets. As a matter of fact, these algorithms are data-driven instead of hypothesis driven and are designed to make predictions on unseen data. Besides, internal cross-validation of the models leads to increased generalization of the models and improve clinical applicability. Considering this, machine-learning algorithms may outperform classical statistical approaches in prediction of non-response to MTX therapy in RA. The use of machine-learning algorithms already showed promising results in the prediction of treatment response (MTX and Etanercept) in juvenile idiopathic arthritis (JIA) patients [94,95] and to bDMARDs in RA patients [96,97]. However, these studies did not compare conventional logistic regression to machine-learning algorithms in parallel, hence it is unclear whether machine-learning algorithms result in superior prediction of insufficient response or whether similar predictive results could be achieved using logistic regression.

AIMS AND OUTLINE OF THIS THESIS

The ultimate aim is to enable prescription of personalized treatment in RA patients according to a personal risk score instead of "one-drug-fits-all" approach (Figure 2). To reach this goal, the primary aim of this thesis is to develop a prediction model to identify insufficient responders to MTX prior to treatment start. Secondary aims were to examine potential new 1) epigenetic predictors of insufficient response: global DNA (hydroxy) methylation and genome wide DNA methylation and 2) metabolomic predictors using an untargeted approach. Finally, we will externally validate a previously developed prediction model [82] and assess whether machine learning algorithms can improve the predictive power of the model.

The thesis outline, covering above mentioned aims will be as follows: **Chapter 2** we examine whether DNA methylation and hydroxymethylation are stable upon storage to investigate if stored material can be used in epigenetic studies. In **Chapter 3,** we investigate if global DNA methylation is associated to response to MTX in RA patients. This research is extended in **Chapter 4,** where genome-wide DNA methylation and response to MTX in treatment naïve RA patients is assessed. In **Chapter 5,** we focus on potential metabolic predictors and therefore perform an untargeted metabolomics study prior to treatment in relation to response to MTX. A previously developed prediction model for insufficient response to MTX will be validated in an external cohort in **Chapter 6,** where we will also enhance the model for clinical implementation. Finally, in **Chapter 7,** we study whether machine-learning algorithms perform better in the prediction of insufficient response compared to conventional multivariable logistic regression analysis.

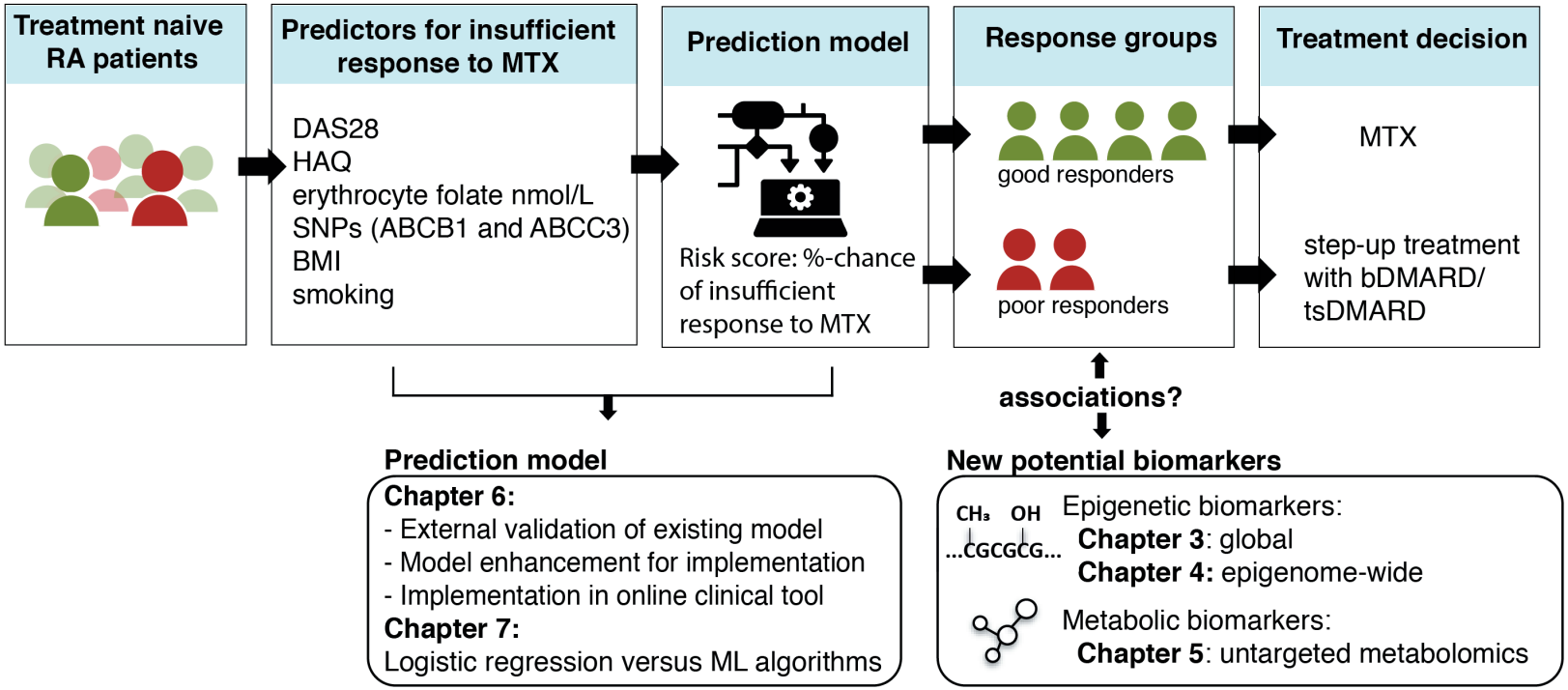

Figure 2. Graphical representation of aims and chapters discussed in this thesis. Abbreviations: DAS28= disease activity score 28, BMI= body mass index, MTX= methotrexate, ML= machine learning, b/tsDMARD= biologic/targeted synthetic disease modifying anti-rheumatic drug. Predictors for insufficient response were previously determined a prediction model was published [81].

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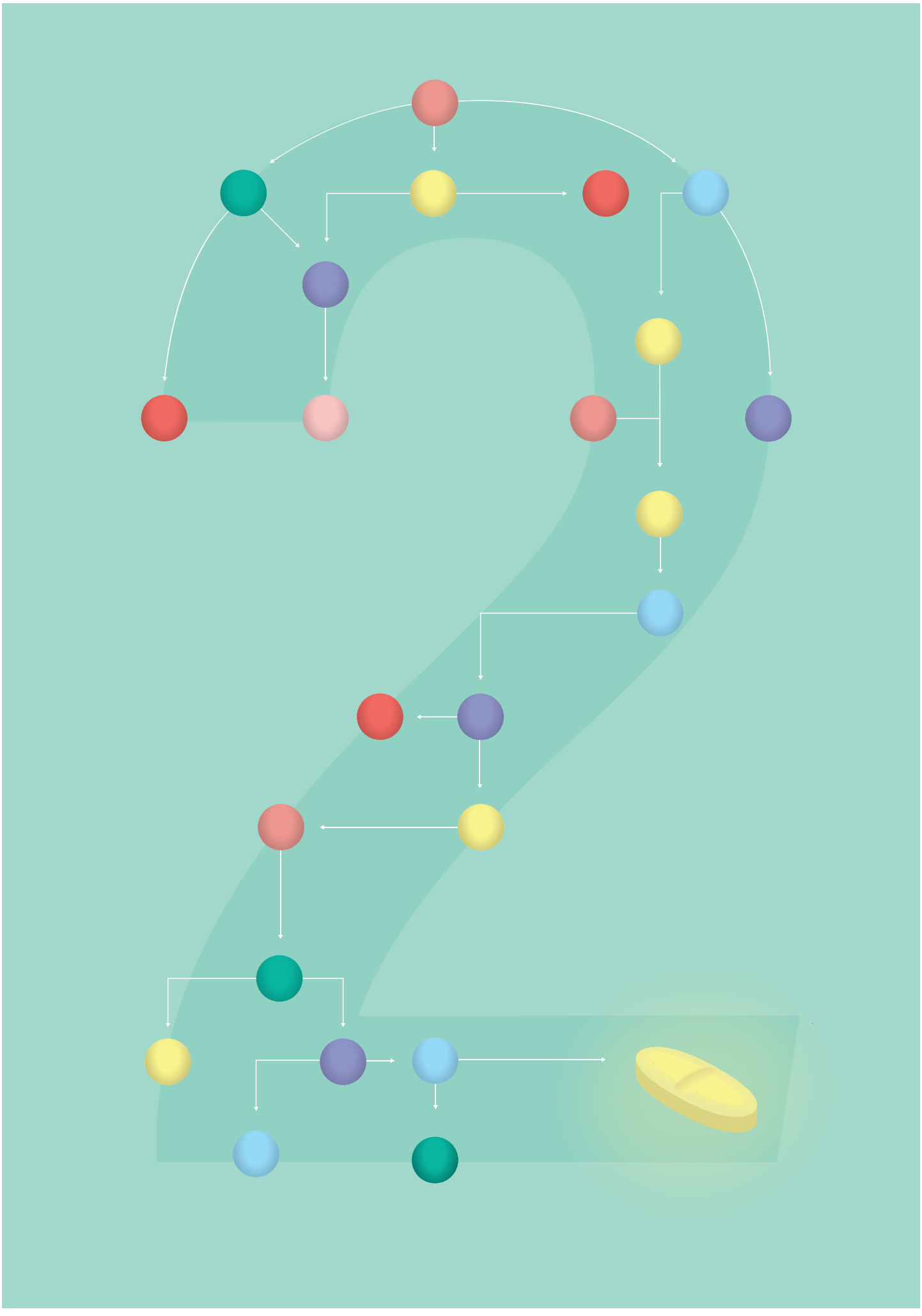

Chapter 2

Global DNA (hydroxy)methylation is stable over time under several storage conditions and temperatures

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ABSTRACT

Background: Epigenetic markers are often quantified and related to disease in stored samples. While, effects of storage on stability of these markers have not been thoroughly examined. In this longitudinal study, we investigated the influence of storage time, material, temperature, and freeze-thaw cycles on stability of global DNA (hydroxy)methylation.

Methods: EDTA blood was collected from 90 individuals. Blood (n = 30, group 1) and extracted DNA (n = 30, group 2) were stored at 4°C, -20°C and -80°C for 0, 1 (endpoint blood 4°C), 6, 12 or 18 months. Additionally, freeze-thaw cycles of blood and DNA samples (n = 30, group 3) were performed over three days. Global DNA methylation and hydroxymethylation (mean ± SD) were quantified using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) with between-run precision of 2.8% (methylation) and 6.3% (hydroxymethylation). Effects on stability were assessed using linear mixed models.

Results: global DNA methylation was stable over 18 months in blood at -20°C and -80°C and DNA at 4°C and -80°C. However, at 18 months DNA methylation from DNA stored at -20°C relatively decreased -6.1% compared to baseline. Global DNA hydroxymethylation was more stable in DNA samples compared to blood, independent of temperature ($p = 0.0131$). Stability of global DNA methylation and hydroxymethylation was not affected up to three freeze - thaw cycles.

Conclusion: Global DNA methylation and hydroxymethylation stored as blood and DNA can be used for epigenetic studies. The relevance of small differences occuring during storage depend on the expected effect size and research question.

INTRODUCTION

With the introduction of the term 'epigenetics' and the development of more accurate techniques to quantify epigenetic changes, this field has gained more interest over the last thirty years [1]. Epigenetics comprises the reversible process of DNA and histone modifications that alter gene expression without changing the DNA sequence. DNA methylation is the most studied epigenetic modification, which involves the addition of a CH_3 group to the DNA, which is in humans predominantly found on cytosines (5-methylcytosine; 5mC) [2]. DNA methylation can be actively de-methylated through the oxidation by Ten-eleven-Translocation (TET) enzymes to 5-hydroxymethylcytosine (5hmC) which can also act as an epigenetic modifier itself [3,4]. DNA methylation and hydroxymethylation have previously been associated with several diseases like cancer, heart disease, autoimmune diseases, and neurological disorders [5–10], and are therefore potential biomarkers for disease onset, progression, or response to medication [11]. DNA is required for epigenetic studies, which is mainly extracted from blood samples that have been collected from patients over several years and has therefore been stored for different time periods prior to the start of a study. Despite the increasing number of studies that relate differences in DNA (hydroxy)methylation to disease, only a handful of studies have examined the effect of storage material and temperature on the stability of global DNA methylation over longer storage duration. These studies, focused either at DNA methylation of targeted regions for specific research questions or only included a few subjects [12–18]. Stability of DNA hydroxymethylation was examined in even fewer studies. An increase in DNA hydroxymethylation was observed at two different sites in brain tissue of Alzheimer patients compared to age-matched controls [19], which was not influenced by tissue storage time. Unfortunately, storage duration and temperature were not specified in this study.

In this longitudinal study, we examined the stability of global DNA methylation and DNA hydroxymethylation in EDTA blood and extracted DNA samples at three different temperatures (4°C, −20°C, −80°C) for the duration of 0, 1, 6, 12 and 18 months. Additionally, we investigated the effect of freeze-thaw cycles on the stability of global DNA (hydroxy) methylation.

METHODS

Subjects and study design

EDTA whole blood was collected from leftover blood samples of the department of clinical chemistry at the Erasmus MC University medical centre of 90 individuals and divided into three groups of 30 individuals. Blood from patients from the oncology department was excluded due to the chance of decreased white blood cell concentrations, required for DNA extraction. None of the subjects included in the study objected for the use of their material for scientific research (opt in-opt out procedure) and all samples were anonymized

after collection and the study follows the principles of the Declaration of Helsinki of 1975. 4 mL EDTA blood of the first 30 subjects (group 1) was stored in aliquots of 375 µL. From the second group, 4 mL EDTA blood was used to extract DNA, where after DNA samples were stored in aliquots of 25 µL. Blood and DNA aliquots were stored at three different temperatures (4°C, −20°C, −80°C) for 1, 6, 12 or 18 months prior to (hydroxy)methylation quantification (***Figure 1***). Blood samples stored at 4°C were stored for 1 month, as blood storage at this temperature is usually short term. From all subjects, one sample was processed and quantified immediately, which was used as baseline measurement. From the third group, 1.6 mL EDTA blood was collected of which half was used to store blood samples and half was used to extract and store DNA. Blood and DNA samples of this group were used to assess the effect of freeze-thaw cycles over three consecutive days after blood collection (0, 1, 2, 3 times) on the stability of DNA (hydroxy)methylation. DNA and blood samples were frozen at −80°C and thawed at room temperature on a laboratory tube roller for 2 h (=1 cycle) before they were refrozen or further processed for DNA (hydroxy)methylation quantification. DNA (hydroxy)methylation was quantified within the same day in all samples.

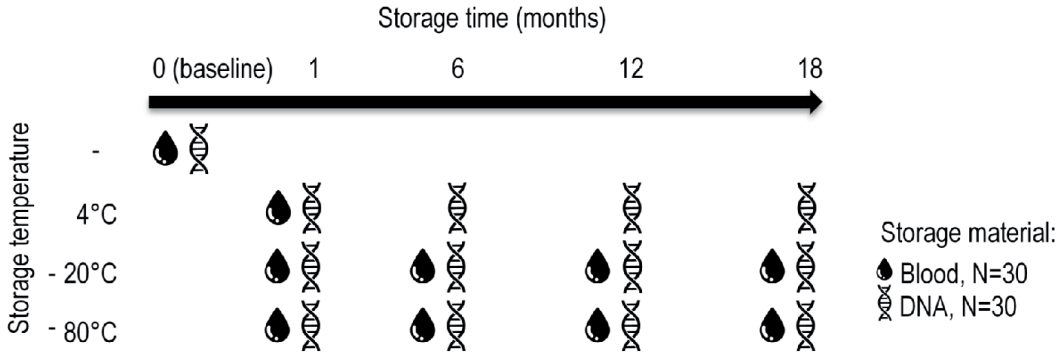

Figure 1. Experimental set up of material stored at different temperatures. EDTA blood of 30 individuals and extracted DNA samples of another 30 individuals were aliquoted and stored at three different temperatures for different time durations as indicated. Blood samples stored at 4°C were stored for one month only. Baseline samples were immediately processed and quantified which is why no storage temperature was given (-).

DNA extraction

DNA was extracted from EDTA whole blood using the MagNA Pure Compact Nucleic Acid Isolation Kit I, following manufacturer's protocol (cat.no: 03730964001, Roche Molecular Biochemicals®) [10]. DNA concentrations were assessed using a NanoDrop ND-1000 Spectrophotometer with DNA-50 default settings (NanoDrop Technologies, Wilmington, DE, USA).

DNA (hydroxy)methylation quantification

Quantification of DNA (hydroxy)methylation was performed using a liquid chromatography – electrospray ionization – tandem mass spectrometry (LC-ESI-MS/MS) method, as previously described [10]. To reduce technical variances, a batch sample of internal standard and a batch calibration curve (stored at −80°C) were used for (hydroxy) methylation quantification at each time point. For hydroxymethylation, samples with analyte areas <50 000 were excluded, as these were too low to correctly quantify DNA hydroxymethylation. For DNA methylation, all areas were above 50 000. As an additional quality check, we assessed the ratios between areas of the quantifier and qualifier of 2'-deoxyguanosine, as these should be similar each day. DNA methylation samples in which this ratio deviated >10% from the mean of that day, were excluded. A random DNA sample was stored in batches of 27 ng/µL at −80°C which was used as batch control: these samples were digested and quantified in each run together with the rest of the samples and acted as quality control. The method was previously validated. To determine precision 5-hmdC/2-dG and 5-mdC/2-dG were quantified in fourfold over 5 days in DNA samples with low (27 ng/µL) and high (54 ng/µL) concentrations. Results of the precision experiment are shown in Table 1. Samples were all diluted to 30 ng/µL, hence for this study, we compared samples to the precision of the method for the low DNA concentration (27 ng/µL). The LLOQ for DNA hydroxy(methylation) was 10 ng/mL, which was determined according to Clinical Laboratory and Standards Institute (CLSI) protocol EP-17a. To determine the dynamic range of the method, a random DNA sample of 27 ng/ul was spiked with 6 increasing concentrations, each measured in quadruple. Linearity was assessed using a lack-of-fit test using the Excel plug-in Analyse-it, where values <3.29 were considered linear. The range and corresponding lack-of-fit measure were as follows: 5-hmdC between 5.9 and 36 nM (lack-of-fit = 0.16), 5-mdC between 552 and 3553 nM (lack-of-fit = 2.47) and 2-dG between 13159 and 23159 nM (lack-of-fit = 2.22).

Table 1. Precision results of method validation.

Component	DN concentration (ng/µL)	Within-run CV%	Between-run CV%
5-hmdC/2-dG	27	6.3	6.3
	54	7.6	7.7
5-mdC/2-dG	27	1.7	2.8
	54	1.5	2.5

CV=coefficient of variation.

Statistics

Percentages of global DNA (hydroxy)methylation were presented as mean ± standard deviation (SD). Linear mixed models were used to assess changes in DNA (hydroxy) methylation over time. Main effects included in the models were: temperature, material, and time. To assess whether DNA (hydroxy)methylation changed differently over time for

DNA and blood samples stored at different temperatures, two and three-way interaction terms between temperature, material, and time were examined. The models with two-way interaction terms and a model with a three-way interaction term between time, material, and temperature were compared using a chi-square log likelihood ratio test, where a p-value <0.05 was considered a significant difference. Additionally, overall model qualities were assessed using the Akaike information criterion (AIC), where the lower the AIC the better the model fits the data. Here, a difference of at least two AIC units was considered a significantly better fit [29]. Upon significance of (higher-term) interactions, all lower-term interactions were kept in the model to keep the models hierarchically well formulated (HWF) [30,31]. Additionally, to assess whether DNA (hydroxy) methylation was stable over time, we completely removed time from the model and compared the model with and without time component. Upon a change of ≥2 units in AIC upon removal of the time component, time was considered significantly important for the model, meaning that there are changes over time. Random effects included in the model were: (A) random intercept for time (accounting for inter-subject variability in (hydroxy)methylation levels at baseline, which may influence stability over time) and (B) random slopes for subjects (hydroxymethylation) or for subjects grouped within storage temperature (methylation), adjusting for the fact that samples from the same subject were higher correlated over time than from different subjects. Final model for the analysis of DNA methylation over time per condition was: lme(fixed = methylation ~ material * temperature * time, random = ~time|id/temperature) and for DNA hydroxymethylation: lme(fixed = hydroxymethylation ~ temperature + material * time, random = ~time|id). For the analysis of freeze-thaw cycles on the stability of DNA (hydroxy)methylation we used a linear mixed model including fixed effects for: material and number of cycles (cycles) and the following random effects: random intercept and random slope for subjects (id). Final models for both DNA methylation and hydroxymethylation upon freezing and thawing were as follows: lme(data = DF, fixed = (hydroxy)methylation ~ material * cycles, random = ~ 1 | id). All analyses were performed in R studio (R version 3.6.1 (2019–07-05)) using the linear mixed effect model (lme) function of the 'nlme' package and were fitted using the maximum likelihood 'ML' method [32].

RESULTS

Stability over time and under different storage conditions

Global DNA methylation

The effects of storage time, temperature, and material were assessed in the same model showing that global DNA methylation significantly changed over time, as the model including the time variable (Akaike information criterion; AIC = −819.06) significantly better fit the data ($p < 0.0001$) than the model without time variable (AIC = −685.23). To assess whether this significant change over time was affected by the type of material or

storage temperature a three-way interaction between time, material, and temperature was performed, which was significant ($p < 0.0001$; Supplementary Table S1), indicating that DNA methylation evolved significantly different over time between blood and DNA samples and between samples stored at different temperatures. Global DNA methylation was not significantly different in blood and DNA samples stored at 4°C (when compared over 1 month), nor between blood and DNA samples stored at −80°C (compared over 18 months) (***Figure 2***; Supplementary Table S2). Global DNA methylation in blood and DNA samples was stable at −20°C up to 12 months (***Figure 2***). However, after 12 months mean global DNA methylation in DNA samples stored at −20°C decreased and slightly deviated from the slope of EDTA blood samples (***Figure 2***; Supplementary Table S2). After 18 months, mean global DNA methylation in these DNA samples was 4.15 ± 0.23, which is a relative decrease of 6.1% compared to baseline (4.42 ± 0.11). In all other conditions, relative differences fluctuated between 0.5% and 4.1% compared to baseline (Supplementary Table S2), which is more comparable to the precision of the method (coefficient of variation; CV% = 2.3%).

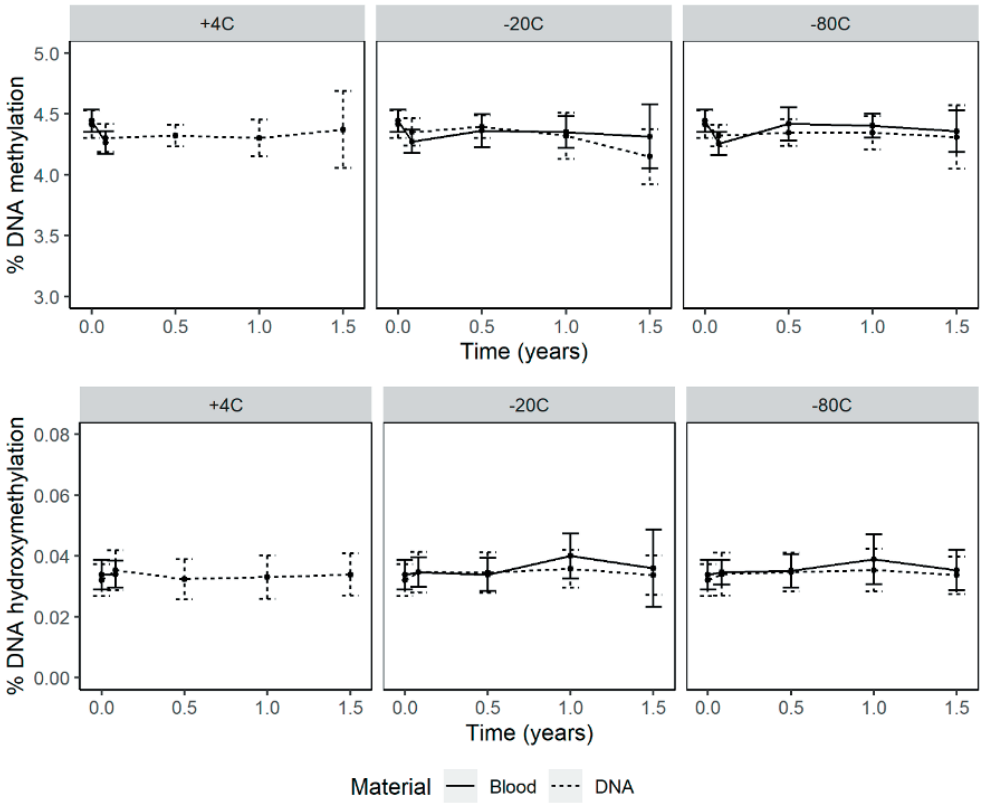

Figure 2. DNA (hydroxy)methylation over time for blood and DNA samples stored at different temperatures. Mean ± SD DNA methylation (upper panel) and hydroxymethylation (lower panel) quantified in samples stored as blood (solid line) or DNA (dashed line). The slope is calculated for 1.5 year from the linear mixed model summary. *Blood samples stored at 4°C were stored for one month only, so the slope was calculated for 1 month.

Global DNA hydroxymethylation

The influence of time, material, and temperature on the stability of global DNA hydroxymethylation was assessed in a linear mixed model. Global DNA hydroxymethylation changed significantly over time as the AIC of the model including time variable (AIC = −5944.98) fit the data significantly better ($p < 0.0001$) compared to the model without time variable (AIC = −5920.03). Changes over time were significantly different for blood and DNA samples, which is described by the significant two-way interaction between time and material ($p = 0.0131$; Supplementary Table S1), but independent of storage temperature, as a three-way interaction between time, material, and storage was not significant and therefore excluded from the model (Supplementary Table S1). Up to 6 months of storage, there was no difference in global DNA hydroxymethylation between blood and DNA samples (***Figure 2***). However, global DNA hydroxymethylation slightly increased in blood samples after 6 months but did not change in DNA samples (***Figure 2***). Over 12 months, mean global DNA hydroxymethylation in blood samples stored at −20°C and −80°C increased to 0.040 ± 0.007 and 0.039 ± 0.008, respectively, which are relative changes of 17.6% and 14.7% compared to baseline (0.034 ± 0.005). Global DNA hydroxymethylation stored at −20°C also increased relatively with 12.5% at 12 months compared to baseline. Under all other conditions, differences over time were smaller and fluctuated between 0.0% and 9.4% relative to baseline. After 12 months, global DNA hydroxymethylation in blood and DNA samples stored at these temperatures decreased again to a relative difference of ≤6.3% compared to baseline, which is within the precision of the method (CV = 7.2%). At 18 months, fewer global DNA hydroxymethylation samples could be quantified from blood due to lower DNA yield upon extraction, especially from blood samples stored at −20°C (Supplementary Table S2).

Stability after repetitive freezing and thawing

DNA methylation assessed in stored blood or extracted DNA upon freezing and thawing fluctuated between 4.23 ± 0.20 and 4.29 ± 0.17 over three cycles (***Figure 3*** and supplementary Table S3), which was no significant change ($p = 0.7904$). Changes compared to baseline were all <1%, which is within the within-run precision of the method (CV = 1.7%).

For global DNA hydroxymethylation in stored blood and extracted DNA samples, mean global DNA hydroxymethylation fluctuated between 0.034 ± 0.005 and 0.036 ± 0.008 upon freezing and thawing. Relative differences compared to baseline were all <6%. Although, this change was significant ($p = 0.0169$; Supplementary Table S3), these differences were within the within-run precision of our method (CV = 6.3%).

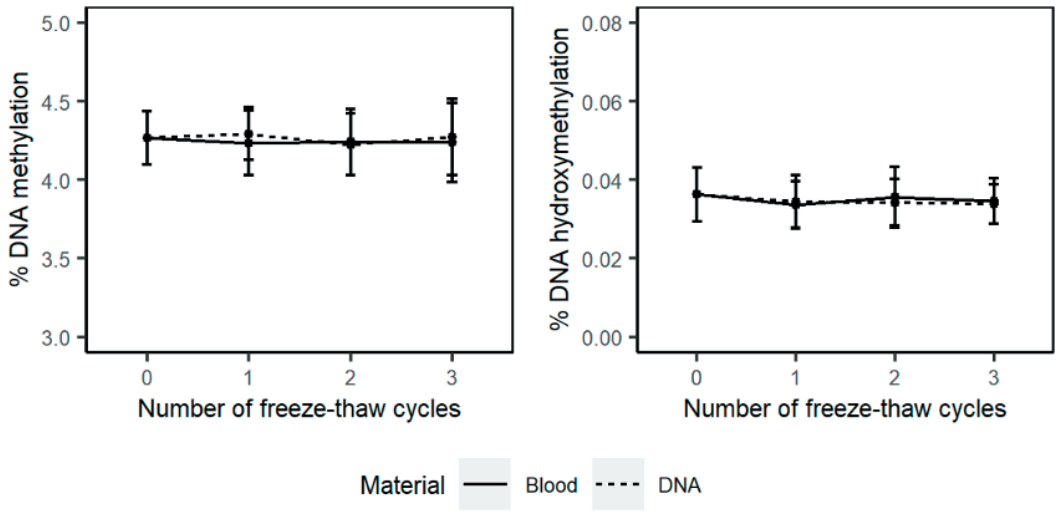

Figure 3. Effect of freezing and thawing on the stability DNA (hydroxy)methylation. Mean percentage DNA methylation (left) and hydroxymethylation (right) for 0 (fresh material), 1, 2 or 3 freeze-thaw cycles. Error bars represent the standard deviation.

DISCUSSION

For the use of stored samples in epigenetic studies it is important to know whether the stability of epigenetic markers is affected by storage conditions like time, material and temperature. In this study, we therefore examined the effect of these storage conditions on the stability of DNA methylation and hydroxymethylation. We showed that overall global DNA methylation and global DNA hydroxymethylation are stable when stored as blood or extracted DNA over a period of 18 months. However, storage of DNA at −20°C for longer than 12 months led to a significant but small decrease of 6.1% in global DNA methylation relative to baseline. Global DNA hydroxymethylation was more stable in stored DNA compared to blood after 6 months, which was independent of storage temperature. Furthermore, freeze-thaw cycles did not influence the stability of global DNA methylation and hydroxymethylation. Considering all this, we would recommend to store samples as extracted DNA at −80°C.

We have chosen storage temperatures and time according to daily practice. Therefore, EDTA blood at 4°C was stored for 1 month as in most laboratories longer storage takes place at −20°C or −80°C. Although global DNA methylation in blood samples stored at 4°C for 1 month slightly decreased, this decrease was similar to that in blood stored at −20°C and −80°C and to DNA samples stored at 4°C during the first month, where after mean methylation in all these conditions increased again towards the mean of baseline measurements. These changes lay within the precision of the method, indicating that these are probably due to between-run variances.

Mean DNA methylation levels were comparable to previously reported levels quantified by liquid chromatography – electrospray ionization – tandem mass spectrometry

(LC-ESI-MS/MS) with mean DNA methylation between ~2 – 5% and hydroxymethylation of ~0.03% [20–22]. These DNA methylation levels are also comparable to quantification using enzyme-linked immunosorbent assay (ELISA) – based methods with mean levels in the same range [23]. However, LC-MS has been shown to be more specific compared to ELISA-based methods [24]. Other methods that are used as a proxy for global DNA methylation usually measure highly repetitive and densely methylated regions, which is the case for long interspersed nuclear elements (LINE-1) and short interspersed nuclear elements (SINE), together constituting of ~30% of the genome. Because these regions are highly methylated, quantification of DNA methylation results in higher percentages (>50%). For the quantification of actual global DNA methylation and hydroxymethylation percentages LC-MS/MS is the gold standard, while for other research questions other methods could be considered [24,25].

Few other groups investigated the stability of global DNA methylation. One study observed a strong decrease in global DNA methylation upon three days of whole blood storage at room temperature, 4°C and −80°C, assessed using a dot-blot assay [16]. They observed a strong decrease in DNA methylation, however, this was a small study, including 10 subjects only and the decrease was accompanied by a decrease in DNA concentration due to white blood cell lysis. In another study, the authors examined mean methylation of multiple CpG sites grouped within specific genomic regions (transcription start site, promoter, gene body, CpG islands, and shores) assessed by bisulphite conversion followed by pyrosequencing. In line with our results, they observed similar methylation patterns over time for DNA extracted from stored blood (4°C, -80°C) for 20 years and fresh samples. Like in our study, small differences over time were observed; however, their study design was cross-sectional and ours longitudinal. They observed that the variation between fresh samples and stored samples was similar, indicating that there was no real effect of storage [15]. In our study, we observed small differences over time that were comparable to the precision of the method, with the exception of stored DNA at −20°C after 12 months and blood after 12 months, here we saw a relative decrease in global DNA methylation and a relative increase in global DNA hydroxymethylation respectively that exceeded the precision of the method.

In contrast to our study, most studies focused on the stability of DNA methylation at candidate genes. These results depended on the targeted site, the technique used and the material stored. Schröder et al. for instance observed a strong increase in mean DNA methylation in the intron 1 of HIF3A gene of blood stored at room temperature, 2–8°C, −20°C and −70°C, after 10 months of storage, assessed using bisulphite sequencing [17]. While, others did not find significant differences in the hypermethylated promoter region of testis-specific histone 2B (TSH2B) and the hypomethylated transcription start site of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) quantified using methylated DNA immunoprecipitation coupled with next-generation sequencing (MeDIP-seq) in dried blood spots (DBS) stored at room temperature for 16 years compared to freshly extracted

material [26]. Thus, global DNA methylation is mainly stable upon storage, however, changes may occur at gene-specific sites.

Finally, stability of global DNA methylation and hydroxymethylation were not affected by freezing and thawing of blood or DNA samples for at least three freeze-thaw cycles at −80°C, which is in accordance with Li and colleagues who observed no effect of freeze-thaw cycles on DNA methylation [15]. Additionally, to our knowledge we were the first to assess the stability of global DNA hydroxymethylation, thus more studies are required to confirm our findings.

Strengths of this study are that this is a longitudinal study, excluding potential differences due to inter-subject variability over time. Relative to other studies, our study groups were large, as most studies assessing DNA methylation stability only included 6–10 subjects per condition. Additionally, for all subjects, fresh material was collected and stored at the same time, so there was no time-to storage variability between the samples and samples from similar materials were quantified at the same time to reduce between-run variability. Additionally, global DNA methylation and hydroxymethylation were quantified using a global LC-MS/MS method, which is the gold standard method for analysis of global DNA methylation and enables to assess mean stability of methylated and hydroxymethylated cytosines all over the genome [27]. An advantage of our method over other global DNA methylation methods is that no bisulphite conversion nor whole genome amplification is required to distinguish methylated from unmethylated cytosines, which minimizes the risk to introduce biases. Also, in our study DNA was digested into single nucleotides representing cytosines from the whole genome instead of a surrogate measure for global DNA methylation, like repetitive elements. Additionally, DNA hydroxymethylation was quantified simultaneously, which allows us to compare DNA methylation and hydroxymethylation within the same samples. A limitation of the method is that we cannot draw conclusions from stability at targeted CpG sites, while methylation at some sites have been shown to be more stable than others.

Other limitations of our study are that we did not account for cell type ratios. From literature, it is known that different cell types have different (hydroxy)methylomes [28]. Previously, it was suggested that some cell types are more stable than others upon storage, which may lead to different cell type ratios upon cell lysis and consequently in different DNA methylation percentages [17]. Although, in our study, this seems unlikely as we then would expect to find changes in blood samples over time and not in stored DNA samples, which was not the case.

CONCLUSION

In conclusion, overall global DNA (hydroxy)methylation was stable. However, small significant differences over time were observed in global DNA methylation and global DNA hydroxymethylation upon storage. Therefore, when samples are stored for extended periods of time or when samples are stored for different time durations, small differences in relation to disease should be interpreted with caution. Subsequently, for the assessment of global DNA (hydroxy)methylation, samples can best be stored at −80°C as extracted DNA.

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SUPPLEMENTAL MATERIAL

Supplementary Table S1. Final linear mixed model variables.

	Methylation				Hydroxymethylation			
	numDF	**denDF**	**F-value**	**p-value**	**numDF**	**denDF**	**F-value**	**p-value**
(Intercept)	1	612	243198.57	<.0001	1	706	3882.46	<.0001
Material	1	58	0.12	0.7319	1	58	0.60	0.4414
Time	1	612	9.56	0.0021	1	706	12.60	0.0004
Temperature	2	116	1.48	0.2319	2	706	3.45	0.0324
Time:temperature	2	612	4.94	0.0074			-	
Time:material	1	612	5.62	0.0180	1	706	6.18	0.0131
Material:temperature	2	116	0.70	0.4994			-	
Material:time:temperature	2	612	17.83	<.0001			-	

F test for all fixed effects in the model for the course of DNA methylation (left) and hydroxymethylation (right). Colons indicate two – or three way interactions between predictors. A dash indicates that the predictor was not included in the model as it was not significant. All lower-order interaction terms were kept in the model as long as higher order interaction terms were significant. numDF= degrees of freedom numerator , denDF= degrees of freedom denominator.

Supplementary Table S3. Linear mixed model for freeze-thaw cycles.

	Methylation				Hydroxymethylation			
	numDF	**denDF**	**F-value**	**p-value**	**numDF**	**denDF**	**F-value**	**p-value**
(Intercept)	1	194	30570.62	<.0001	1	151	882.93	<.0001
Freeze-thaw cycles	3	194	0.348	0.7904	3	151	3.510	0.0169
Material	1	194	0.771	0.3810	1	151	0.642	0.4242

F test for all fixed effects in the model for the course of DNA methylation (left) and hydroxymethylation (right) upon freeze-thaw cycles. numDF= degrees of freedom numerator , denDF= degrees of freedom denominator.

Supplementary Table S2. Mean DNA (hydroxy)methylation over time under different storage conditions.

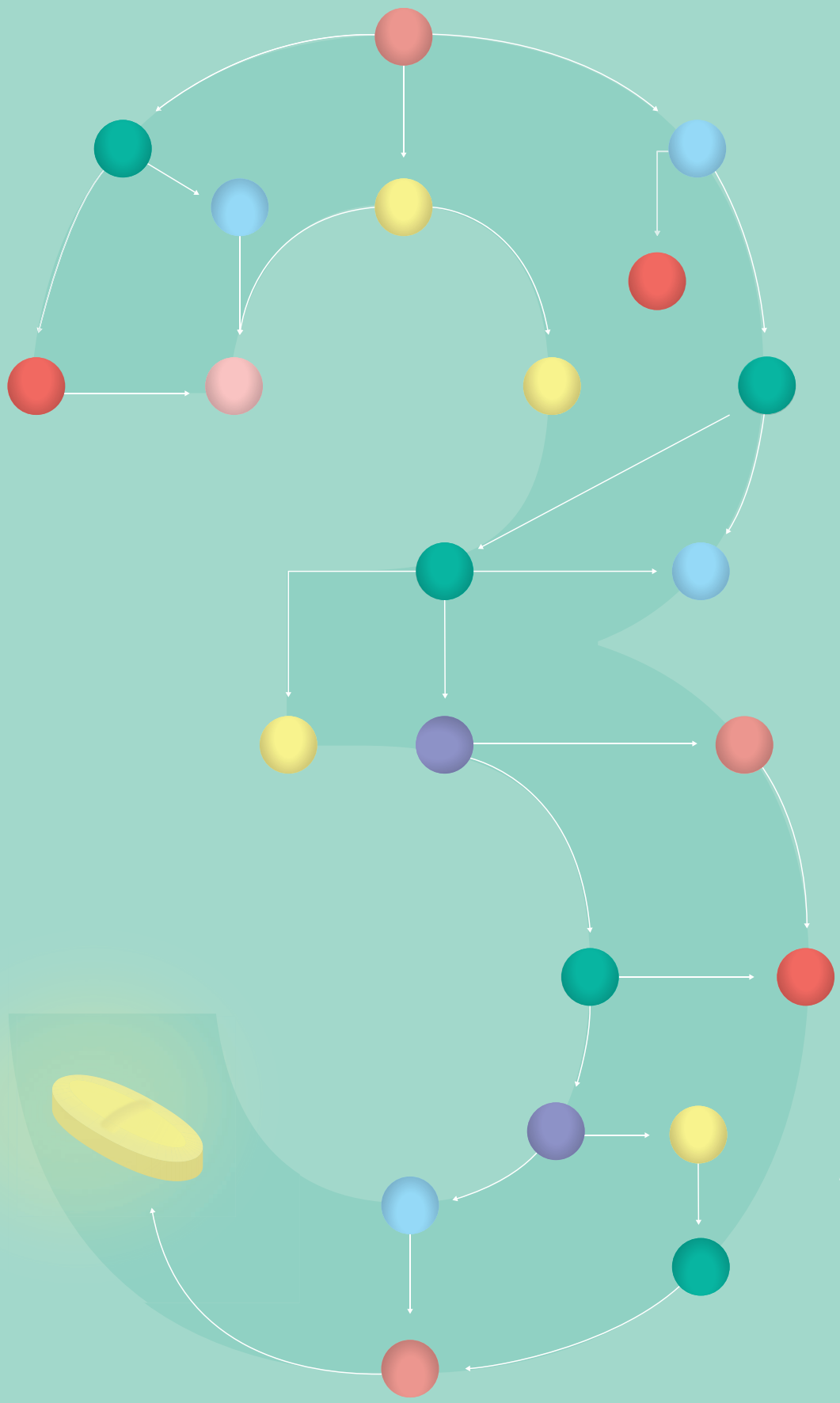
		Baseline		Temp. (°C)	1 month			6 months			12 months			18 months		
		N	Mean ± SD		N	Mean ± SD	Δbaseline (%)	N	Mean ± SD	Δbaseline (%)	N	Mean ± SD	Δbaseline (%)	N	Mean ± SD	Δbaseline (%)
Methylation	Blood	30	4.44 ± 0.09	4	30	4.26 ± 0.09	-0.18 (4.1)		-	-		-	-		-	-
				-20	30	4.27 ± 0.10	-0.17 (3.8)	30	4.36 ± 0.13	- 0.08 (1.8)	30	4.35 ± 0.13	-0.09 (2.0)	27	4.31 ± 0.26	-0.13 (2.9)
				-80	30	4.26 ± 0.09	-0.18 (4.1)	30	4.42 ± 0.14	- 0.02 (0.5)	30	4.40 ± 0.10	-0.04 (0.9)	29	4.36 ± 0.17	-0.08 (1.8)
				QC	5	4.25 ± 0.08	-0.21 (4.7)	4	4.31 ± 0.06	-0.15 (3.4)	4	4.37 ± 0.06	-0.09 (2.0)	2	4.49 ± 0.03	+0.03 (0.7)
	DNA	30	4.42 ± 0.11	4	30	4.30 ± 0.11	-0.12 (2.7)	30	4.32 ± 0.09	- 0.10 (2.3)	29	4.30 ± 0.15	-0.12 (2.7)	29	4.37 ± 0.32	-0.05 (1.1)
				-20	29	4.35 ± 0.11	-0.07 (1.6)	30	4.39 ± 0.09	- 0.03 (0.7)	29	4.32 ± 0.19	-0.10 (2.3)	30	4.15 ± 0.23	-0.27 (6.1)
				-80	30	4.32 ± 0.09	-0.10 (2.3)	29	4.35 ± 0.11	- 0.07 (1.6)	28	4.34 ± 0.14	-0.08 (1.8)	29	4.31 ± 0.26	-0.11 (2.5)
	QC	4	4.46 ± 0.002	QC	5	4.32 ± 0.19	-0.14 (3.1)	6	4.33 ± 0.10	-0.13 (2.9)	6	4.34 ± 0.07	-0.12 (2.7)	4	4.34 ± 0.24	-0.12 (2.6)
Hydroxymethylation	Blood	30	0.034 ± 0.005	4	30	0.034 ± 0.004	0.000 (0.0)		-	-		-	-		-	-
				-20	30	0.035 ± 0.005	+0.001 (2.9)	30	0.034 ± 0.005	0.000 (0.0)	27	0.040 ± 0.007	+0.006 (17.6)	13	0.036 ± 0.013	+0.002 (5.9)
				-80	30	0.035 ± 0.004	+0.001 (2.9)	30	0.035 ± 0.005	+0.001 (2.9)	29	0.039 ± 0.008	+0.005 (14.7)	25	0.035 ± 0.007	+0.001 (2.9)
				4	30	0.035 ± 0.007	+0.003 (9.4)	30	0.032 ± 0.007	0.000 (0.0)	28	0.033 ± 0.007	+0.001 (3.1)	28	0.034 ± 0.007	+0.002 (6.3)
				QC	5	0.035 ± 0.004	-0.001 (2.8)	4	0.037 ± 0.004	+0.001 (2.8)	4	0.037 ± 0.002	+0.001 (2.8)	2	0.040 ± 0.005	+0.004 (11.1)
	DNA	30	0.032 ± 0.005	-20	29	0.035 ± 0.007	+0.003 (9.4)	30	0.034 ± 0.007	+0.002 (6.3)	28	0.036 ± 0.006	+0.004 (12.5)	29	0.034 ± 0.007	+0.002 (6.3)
				-80	30	0.034 ± 0.007	+0.002 (6.3)	29	0.035 ± 0.006	+0.003 (9.4)	27	0.035 ± 0.007	+0.003 (9.4)	28	0.034 ± 0.006	+0.002 (6.3)
	QC	4	0.036 ± 0.075	QC	5	0.039 ± 0.003	+0.003 (8.3)	6	0.038 ± 0.003	+0.002 (5.6)	6	0.042 ± 0.006	+0.006 (16.7)	4	0.039 ± 0.005	+0.003 (8.3)

SD= standard deviation. Temp.= storage temperature. Blood samples stored at 4°C were stored for only one month, thus (-) represents no sample quantified. Δbaseline = the absolute change from baseline, (%)=relative change from baseline, DNA methylation and hydroxymethylation were quantified simultaneously, thus DNA hydroxymethylation was assessed in the same blood – and DNA samples as in which DNA methylation was quantified. QC= quality control samples.

Supplementary Table S4.

				Number of freeze-thaw cycles								
				1			2				3	
	N	Baseline	Material	N	Mean ± SD	Δ baseline (%)	N	Mean ± SD	N	Δ baseline (%)	Mean ± SD	Δ baseline (%)
Methylation	29	4.27 ± 0.17	Blood	29	4.24 ± 0.21	-0.03 (0.7%)	27	4.24 ± 0.21	29	-0.03 (0.7%)	4.24 ± 0.25	-0.03 (0.7%)
			DNA	29	4.29 ± 0.17	+0.02 (0.5%)	27	4.23 ± 0.20	29	-0.04 (0.9%)	4.27 ± 0.24	0.00 (0.0%)
Hydroxy-methylation	22	0.036 ± 0.007	Blood	23	0.034 ± 0.006	-0.002 (5.6%)	21	0.036 ± 0.008	22	0.00 (0.0%)	0.035 ± 0.006	-0.001 (2.8%)
			DNA	23	0.034 ± 0.007	-0.002 (5.6%)	23	0.034 ± 0.006	23	-0.002 (5.6%)	0.034 ± 0.005	-0.002 (5.6%)

SD= standard deviation. Temp.= storage temperature. Blood samples stored at 4°C were stored for only one month, thus (-) represents no sample quantified. Δbaseline = the absolute change from baseline, (%)=relative change from baseline, DNA methylation and hydroxymethylation were quantified simultaneously, thus DNA hydroxymethylation was assessed in the same blood – and DNA samples as in which DNA methylation was quantified. QC= quality control samples.



Chapter 3

Higher baseline global leukocyte DNA methylation is associated with MTX non-response in early RA patients

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ABSTRACT

Background: Low-dose methotrexate (MTX) is the first-line therapy in early rheumatoid arthritis (eRA). Up to 40% of eRA patients do not benefit from MTX therapy. MTX has been shown to inhibit one-carbon metabolism, which is involved in the donation of methyl groups. In this study, we investigate baseline global DNA methylation and changes in DNA methylation during treatment in relation to clinical non-response after 3 months of MTX treatment.

Methods: Two hundred ninety-four blood samples were collected from the Treatment in the Rotterdam Early Arthritis Cohort (tREACH, ISRCTN26791028), a multicenter, stratified single-blind clinical trial of eRA patients. Global DNA (hydroxy) methylation was quantified using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) and validated with a global DNA LINE-1 methylation technique. MTX response was determined as ΔDAS28. Additionally, patients were stratified into two response groups according to the European League Against Rheumatism (EULAR) response criteria. Associations between global DNA methylation and response were examined using univariate regression models adjusted for baseline DAS28, baseline erythrocyte folate levels, and body mass index (BMI).

Results: Higher baseline global DNA methylation was associated with less decrease of DAS28 ($\beta = 0.15, p = 0.013$) and with MTX non-response (OR = 0.010, 95% CI = 0.001–0.188). This result was validated in LINE-1 elements ($\beta = 0.22$, $p = 0.026$). Changes in global DNA (hydroxy)methylation were not associated with MTX response over 3 months.

Conclusions: These results show that higher baseline global DNA methylation in treatment naïve eRA patients is associated with decreased clinical response after 3 months of treatment of eRA patients and can be further evaluated as a predictor for MTX therapy non-response.

BACKGROUND

Rheumatoid arthritis (RA) is an autoimmune disease affecting about 1% of the world's population [1]. The disease onset is unknown; nevertheless, medication can restrain disease activity and permanent joint damage. The disease-modifying anti-rheumatic drug (DMARD) methotrexate (MTX) is the first-line therapy in early rheumatoid arthritis (eRA) [2] and is often prescribed in combination with sulfasalazine (SSZ), hydroxychloroquine (HCQ), and corticosteroids. Up to 40% of treated patients do not adequately respond to therapy and need to switch to expensive biologicals after 3 to 6 months of therapy, or withdraw because of severe adverse events [3]. Therefore, new biomarkers are required to distinguish non-responders prior to treatment.

MTX is a folate antagonist of which the underlying mechanism in RA is still not fully elucidated. MTX was originally designed for cancer therapy to inhibit DNA synthesis by inhibiting key intracellular enzymes in folate metabolism. These include dihydrofolate reductase (DHFR) and thymidylate synthase (TS). The anti-inflammatory mechanism of action of low-dose MTX treatment used in eRA probably relates to the inhibition of key enzymes in the purine de novo synthesis pathway and release of anti-inflammatory adenosine [4]. MTX also inhibits methionine S-adenosyltransferase (MAT), followed by the inhibition of S-adenosyl methionine (SAM) in vivo and in vitro [5, 6]. SAM is responsible for the donation of methyl groups required for global DNA methylation. MTX is therefore hypothesized to inhibit global DNA methylation, although elevated global DNA methylation was observed in peripheral blood mononuclear cells (PBMCs) of MTX-treated patients [7]. If the effect of MTX is related to a decrease in global DNA methylation through inhibition of MAT and SAM, then we hypothesize that higher baseline global DNA methylation might be more difficult to inhibit and therefore affects MTX responsiveness.

In the current prospective study, we investigated whether higher baseline global DNA (hydroxy)methylation in leukocytes of early RA patients is associated with MTX clinical non-response over the first 3 months of treatment. Furthermore, we assessed whether a lesser decrease in global DNA methylation during treatment or higher global DNA methylation at 3 months of MTX treatment was associated with MTX clinical non-response.

MATERIALS AND METHODS

Subjects and samples

Four hundred ninety-six subjects were eligible from the Treatment in the Rotterdam Early Arthritis Cohort (tREACH, ISRCTN26791028), a multicenter, stratified single-blind, randomized controlled trial of eRA patients, as previously described [8]. In brief, included patients were diagnosed with RA based on the American College of Rheumatology (ACR) 1987 classification criteria for RA [9] and were categorized in high, intermediate, or low

probability groups for persistent disease, according to the Visser prediction model [10]. All patients who received MTX mono or combination (MTX + corticosteroids and MTX + SSZ + HCQ + corticosteroids) therapy were enrolled in this study ($n = 336$). An escalating dose of MTX was prescribed in the first 3 weeks from 10 mg (week 1) up to 17.5 mg (week 2) and 25 mg (week 3). Additionally, all patients received weekly 10 mg folic acid, at least 24 h after MTX administration, as recommended [2]. Whole blood leukocytes were collected at baseline (T0) and after 3 months of MTX therapy (T3) and stored at −80 °C. This study was approved by the medical ethics committee of the Erasmus University Medical Center: MEC-2006-252. Medical ethics committees at each participating center approved the study protocol, and written informed consent was obtained for all patients.

LC-ESI-MS/MS

DNA digestion

Genomic DNA was isolated using the MagNA Pure Compact Nucleic Acid Isolation Kit (Roche Molecular Biochemicals®) according to the manufacturer's instructions. DNA concentration was quantified using a NanoDrop ND-1000 Spectrophotometer with DNA-50 default settings (NanoDrop Technologies), and 260/280 ratios ~1.8 were considered pure DNA. Samples were stored at −80 °C and diluted to 30 ng/µl 1 day prior to the start of the experiment. Six hundred nanograms of genomic DNA was added to the following digestion mixture: 1 µl DNA Degradase Plus™ enzyme (5 U/ml, Zymo Research®), 2.5 µl 10× DNA Degradase Reaction Buffer, and 1.5 µl Milli-Q, with a total reaction volume of 25 µl. The samples were centrifuged for 1 min at 3100 rpm and placed in a Thermo Mixer®C (Eppendorf) for 5 h at 37 °C, followed by an enzyme heat inactivation step for 20 min at 70 °C.

Quantification of global DNA (hydroxy)methylation

Following DNA degradation, the 25 µl reaction volume was 1:1 diluted with an Internal Standard mixture (IS, 19.2 nM 5-hmdc-d_3, 205 nM 5-mdc-d_3, 1.84 µM 2-dG-$^{15}N_5$). A calibration curve was made as follows: for each component, a calibrator was diluted to a final concentration of 10 nM (5-hmdC), 1000 nM (5-mdC), and 20,000 nM (2-dG), which were then serially 1:1 diluted to 0 in 5 steps. Of each dilution, 400 µl was added to 600 µl of diluted IS. Global DNA methylation and hydroxymethylation were measured using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) in the positive ionization mode. Twenty microliters was injected on a T3-high strength silica column (Acquity UPLC®, Waters, C18, 2.1 × 100 mm, 1.8 µm) at 35 °C. 0.1% formic acid in Milli-Q (A), and acetonitrile (B) was used as the mobile phase at a flow rate of 0.20 ml/min. The following gradient was used: 0–0.5 min (98% A and 2% B), 5 min (0% A and 100% B), 5.50 min (0% A and 100% B), 5.51 min (98% A and 2% B), and 7 min (98%A and 2%B), where all gradient steps were linear. An aliquoted DNA sample was measured as quality control

(QC) in every run to uncover potential errors during sample preparation and DNA (hydroxy) methylation quantification. The coefficient of variation (%CV) was calculated from all the QC measurements ($n = 24$) and was 2.4% for methylation and 7.7% for hydroxymethylation measurements. The percentage of (hydroxy)methylation was calculated in relation to the total guanine concentration, using the following formulas:

"%5mdC = (nM 5mdC/nM 2-dG) x 100"
"%5hmdC = (nM 5hmdC/nM 2-dG) x 100"

Sequenom EpiTYPER LINE-1 assay

LINE-1 global DNA methylation was determined in DNA from leukocytes, isolated using the Sequenom EpiTYPER® assay (Agena Bioscience™) as previously described [11, 12]. Briefly, 500 ng of purified genomic DNA was treated with sodium bisulfite to distinguish methylated from non-methylation cytosines using the EZ DNA MethylationTM Kit (Zymo Research®) according to the manufacturer's instructions. Converted DNA concentrations were quantified using a ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies Inc.) using the RNA-40 default settings, as recommended (Zymo Research®). Bisulfite-converted DNA was stored no longer than 1 month at – 80 °C or until the experiment was performed. A LINE-1 bisulfite-targeted PCR was performed on the C-1000 Touch Thermal Cycler™ (Bio-Rad) using the following primers: 5'aggaagagagGTGTGAGGTGTTAGTGTGTT TTGTT-3'and 3'cagtaatacgactcactatagggaggaaggctATATCCCACACCTAACTCAAAAAAT-'5. The PCR was followed by Shrimp Alkaline Phosphatase treatment, RNA transcription, and Sequenom analysis as previously described [12]. A mixture consisting of 100% enzymatically methylated DNA and 0% methylated DNA, due to a genetic knockout for methyltransferases, resulted in 50% DNA methylation and was used as a positive control during all steps. Milli-Q water was used as a negative control. DNA methylation was quantified using a Matrix-assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) MassARRAY® (Sequenom) analyzer according to the manufacturer's instructions. Methylation percentage was calculated using the following formula: % Methylation = (area methylate peak)/(area unmethylated peak + area methylated peak) × 100. All samples were measured in triplet and samples with a variation coefficient (CV) of > 10% were checked for outliers by means of Dixon's *Q* test. Outliers were removed, and if CV still exceeded 10% for the remaining duplicate, the sample was excluded. Twelve CpG sites were present within the LINE-1 PCR fragment of which CpG 6.7, CpG 8.9, and CpG 11.12 were combined, since these sites could not be separated. CpG 4 could not be analyzed because of a silent signal, and CpG 10 could not be analyzed due to a low mass fragment. Finally, the following seven CpG sites (CpG1, CpG2, CpG3, CpG5, CpG6.7, CpG8.9, and CpG11.12) were analyzed for differences in methylation [12].

Statistical analysis

We performed paired analysis to assess the change in global DNA (hydroxy)methylation over the first 3 months using paired-sample *t* tests. Associations between global DNA (hydroxy)methylation at baseline, at 3 months and over 3 months (Δ(hydroxy)methylation) with response (ΔDAS28-ESR) were first analyzed in a univariate linear regression model, after which the associations were adjusted for confounders. Baseline DAS28 score, baseline erythrocyte folate levels, BMI, age, sex, and smoking status (current versus former + never) are known to be associated with MTX response and DNA methylation and were therefore tested as confounders [13,14,15,16]. The presence of anti-citrullinated protein antibodies (ACPA) has previously been related to decreased MTX response [17]. ACPA positivity was therefore tested as potential covariate. Confounders and covariates were only considered important when the effect size (beta coefficient, B) changed with > 10% upon adjustment. In addition, the relation between baseline global DNA methylation and MTX response was assessed dichotomously (non-responders versus moderate/good responders), according to the EUropean League Against Rheumatism (EULAR) response criteria at 3 months [18]. Associations between global DNA methylation and response were assessed in a crude logistic regression model and in a model adjusted for baseline DAS28, baseline erythrocyte folate, and BMI. Results are expressed in odds ratios (OR) with 95% confidence interval (CI). Incomplete cases were excluded prior to the analysis. For the correlation analysis, distributions of the variables were tested for normality using the Shapiro-Wilkinson test, where $p > 0.05$ was considered normally distributed. The correlation between baseline erythrocyte folate and global DNA methylation was tested using Spearman's correlation due to the skewed distribution of erythrocyte folate, and the correlation between global DNA methylation determined by LC-ESI-MS/MS and LINE-1 was tested using Pearson's correlation test (normally distributed variables). All statistical analyses were conducted using R Studio Software (Version 1.1.423; RStudio Team 2015), and p values< 0.05 were considered significant. Models tested for both methylation and hydroxymethylation were corrected for multiple comparisons using the Bonferroni correction, where $p < 0.025$ ($0.05/2 = 0.025$) was considered significant. LINE-1 analysis was corrected using the Bonferroni correction for the 7 CpGs that were tested simultaneously; hence, $p < 0.007$ ($0.05/7 = 0.007$) was considered significant.

RESULTS

Subject baseline characteristics

Genomic DNA was available and isolated from leukocytes of 265 treatment-naive early RA patients and from 275 subjects at T3. A minimum of 600 ng was required for reliable (hydroxy)methylation measurements. Nine (T0) and five (T3) extracted DNA samples did not reach up to this minimum and were therefore excluded. Global DNA (hydroxy) methylation was successfully quantified in 294 patients, comprising 249 (T0) and 257

(T3) samples. Baseline characteristics of these 294 subjects are summarized in Table 1. The mean age was 53.4 ± 14.2 years, and 70.4% was female. Mean DAS28 at baseline was 4.7 ± 1.2 and decreased to 3.0 ± 1.2 over the first 3 months (Table 1). All patients were treatment naive at baseline and received MTX mono- or combination therapy for at least 3 months (Table 1).

Table 1. Baseline characteristics of early ra patients from the treach.

	Mean ± SD
Patients, n	294
Male, n (%)	87.0 (29.6%)
Age (years)	53.4 ± 14.2
Das28 score	4.7 ± 1.2
Das28 score three months *	3.0 ± 1.2
Erythrocyte – folate (nmol/l) *	936.0 ± 356.2
BMI (kg/m^2) *	26.3 ± 5.1
Smoking status *	
- Current, n (%	91.0 (31.0%)
- Never + former, n (%)	180.0 (61.2%)
Treatment groups	
- MTX, n (%)	54.0 (18.4%)
- MTX + prednisone p.o., N (%)	81.0 (27.6%)
- MTX + SSZ + HCQ + prednisone p.o., N (%)	83.0 (28.2%)
- MTX + SSZ + HCQ + corticosteroids i.m., N (%)	76.0 (25.9%)

Abbreviations: SD=standard deviation, SSZ=sulfasalazine, HCQ=hydroxychloroquine, BMI=body mass index, P.O.=per os, I.M.=intra muscular. * Data was missing for das28 score at T3 (N=10), Baseline erythrocyte folate (N=75), BMI (N=3) and smoking status (N=23).

Global DNA hydroxymethylation increases during three months of MTX therapy

Mean global DNA methylation at baseline was 4.41 ± 0.13% and did not change significantly over the first 3 months of therapy ($p = 0.454$) (Additional file 1: Table S1). Global DNA hydroxymethylation increased significantly with 0.0008% over the first 3 months ($p = 0.013$; Additional file 1: Table S1).

Higher baseline global DNA methylation is associated with MTX non-response at 3 months

Baseline global DNA methylation was associated with ΔDAS28 over the first 3 months when assessed in a crude univariate linear regression model ($B = 1.36$, $p = 0.044$). One percent difference in global DNA methylation at baseline corresponds to 1.41 difference in ΔDAS28 between patients, when adjusted for baseline DAS28, baseline erythrocyte folate, and BMI ($B = 1.41$, $p = 0.013$; Table 2).

Table 2. Associations between baseline global DNA (hydroxy)methylation and ΔDAS28 before and after 3 months of therapy.

Methylation		**Before MTX**			**After MTX**		
		B (SE)	**β**	**p**	**B(SE)**	**β**	**p**
1	Methylation	1.36 (0.67)	0.15	**0.044**	0.40 (0.55)	0.05	**0.471**
2	Methylation	1.41 (0.56)	0.15	**0.013**	0.44 (0.47)	0.06	**0.385**
	DAS28	-0.51 (0.06)	-0.49	**<0.001**	-0.50 (0.06)	-0.49	**<0.001**
	Erythrocyte-folate (nmol/L)	$-1.00x10^{-3}$	-0.17	**0.006**	$-4.00x10^{-4}$	-0.12	**0.063**
	BMI (kg/m^2)	($2.00x10^{-4}$)	0.14	**0.025**	($2.00x10^{-4}$)	0.18	**0.005**
	Age (years)	0.03 (0.02)	-		0.04 (0.02)	-	
	Gender		-			0.11	**0.098**
	Smoking (current)		-		0.27 (0.16)	0.11	**0.084**
	Observations		181		0.28 (0.16)	179	
Hydroxymethylation		**Before MTX**			**After MTX**		
		B (SE)	**β**	**p**	**B(SE)**	**β**	**p**
1	Hydroxymethylation	19.56 (18.38)	0.08	**0.288**	12.52 (19.26)	0.05	**0.517**
2	Hydroxymethylation	6.90 (15.89)	0.03	**0.664**	5.92 (16.75)	0.02	**0.724**
	DAS28	-0.54 (0.07)	-0.52	**<0.001**	-0.52 (0.07)	-0.51	**<0.001**
	Erythrocyte-folate (nmol/L)	$-1.00x10^{-3}$	-0.18	**0.007**	$-5.00x10^{-4}$	-0.14	**0.035**
	BMI (kg/m^2)	($2.00x10^{-4}$)	0.12	**0.062**	($2.00x10^{-4}$)	0.18	**0.006**
	Age (years)	0.03 (0.02)	0.10	**0.125**	0.04 (0.02)	0.13	**0.057**
	Gender	0.01 (0.01)	0.08	**0.176**	0.01 (0.01)	0.11	**0.087**
	Smoking (current)	0.22 (0.17)	-		0.28 (0.16)	0.11	**0.090**
	Observations		181		0.28 (0.16)	177	

Association between mean % global DNA (hydroxy)methylation and ΔDAS28 were tested in a crude univariate model (1) and adjusted for potential confounders (2). Potential confounders were: baseline DAS28 score, baseline erythrocyte – folate levels (nmol/L), BMI (kg/m^2), age (years), gender and smoking status (current smoker versus former + never smoker). Only biomarkers that changed the association with >10% were considered confounders. B= beta coefficient, SE=standard error, β =standardized beta coefficients. P<0.05 was considered significant.

In addition, we stratified subjects accordingly: non- and moderate/good responders according to the EULAR response criteria at 3 months.

Higher baseline global DNA methylation was associated with EULAR non-response in both a crude logistic model (OR = 0.027, 95% CI = 0.002–0.377) and when adjusted for baseline DAS28, baseline erythrocyte folate, and BMI (OR = 0.010, 95% CI = 0.001–0.188; Fig. 1).

Baseline global DNA hydroxymethylation was not significantly associated with ΔDAS28 in a crude univariate model ($B = 19.56$, $p = 0.288$), nor when adjusted for baseline DAS28, baseline erythrocyte folate, BMI, age, and sex ($B = 6.90$, $p = 0.664$; Table 2) and was therefore not further assessed between non-responders and moderate/good responders.

As folate is related to DNA methylation through one-carbon metabolism, we examined the correlation between baseline global DNA methylation and erythrocyte folate. We

did not observe a correlation between baseline global DNA methylation and baseline erythrocyte folate concentrations ($R = 0.084$, $p = 0.24$).

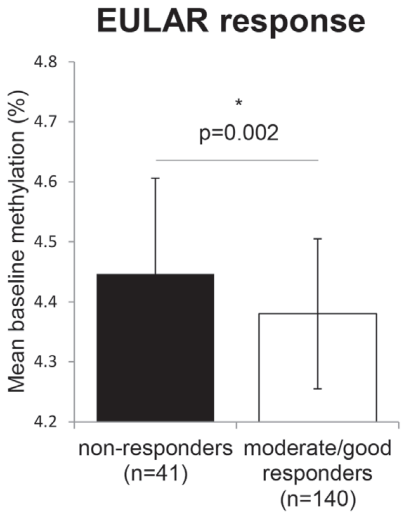

Figure 1. Higher mean (± SD) baseline global DNA methylation in EULAR non-responders compared to moderate/good responders. Response was determined according to the EULAR response criteria at 3 months. The p value is the result of a logistic regression analysis between baseline global DNA methylation and EULAR response criteria adjusted for baseline DAS28, baseline erythrocyte folate, and BMI. *$P<0.05$ was considered significant.

(Change in) global DNA methylation at 3 months is not associated with disease activity

Global DNA methylation at 3 months of therapy was not associated with ΔDAS28 ($B = 0.40$, $p = 0.471$), nor was global DNA hydroxymethylation at 3 months ($B = 12.52$, $p = 0.517$; Table 2). In addition, differences between DNA (hydroxy)methylation at baseline and after 3 months of therapy were not associated with changes in DAS28 (Δmethylation $B = -0.68$, $p = 0.182$, Δhydroxymethylation $B = -1.55$, $p = 0.925$; Table 3).

Table 3. Associations between changes in % (hydroxy)methylation and changes in DAS28 over the first three months (t3-t0) of mtx therapy.

Methylation		**Before MTX**			**After MTX**		
	Biomarkers	**B (SE)**	**β**	**p**	**B(SE)**	**β**	**p**
1	Δ(hydroxy)methylation	-0.50 (0.60)	-0.07	**0.403**	-9.32 (19.40)	-0.04	**0.632**
2	Δ(hydroxy)methylation	-0.68 (0.51)	-0.09	**0.182**	-1.55 (16.35)	-0.01	**0.925**
	DAS28	-0.51 (0.07)	-0.51	**<0.001**	-0.52 (0.07)	-0.51	**<0.001**
	Erythrocyte folate (nmol/L)	-1.00 x10-3	-0.15	**0.027**	-1.00 x10-3	-0.16	**0.024**
	BMI (kg/m^2)	(2.00 x10-4)	0.19	**0.005**	(2.00 x10-4)	0.18	**0.008**
	Age (years)	0.05 (0.03)	0.10	**0.134**	0.04 (0.02)	0.11	**0.130**
	Sex	0.01 (0.01)	-		0.01 (0.01)	0.08	**0.240**
	Smoking (current)		0.11	**0.086**	0.20 (0.17)	0.11	**0.101**
	ACPA status (positive)	0.29 (0.17)	-		0.28 (0.17)	-	
	Observations		163			161	

Associations were tested using crude univariate models (1) and adjusted for confounders (2). Potential confounders were: baseline das28 score, baseline erythrocyte – folate levels (nmol/l), bmi (kg/m2), age (years), gender and smoking status (current smoker versus former + never smoker) and acpa status. Only biomarkers that changed the effect size with >10% were considered confounders. B= beta coefficient, se= standard error, β=standardized beta coefficient. P<0.05 Was considered significant.

Higher LINE-1 methylation associated with decreased MTX response

LINE-1 global DNA methylation was determined in DNA isolated from leukocytes of 120 patients and was successfully quantified in 104 subjects. Seventy-eight individuals had no missing data in any of the variables needed in the analysis and were therefore used. LINE-1 methylation in CpG2 was not significantly associated with ΔDAS28 in a crude univariate model ($B = 0.09$, $p = 0.242$). However, it was associated with ΔDAS28 when adjusted for baseline DAS28, baseline erythrocyte folate, BMI, and smoking status ($B = 0.16$, $p = 0.026$; Table 4). Methylation at the other 6 CpG sites within LINE-1 was not associated with DAS28 nor was mean LINE-1 methylation (Additional file 1: Table S2).

Table 4. Validation of associations between global dna methylation with ΔDAS28 (T3-T0) in line-1 CPG2.

		Before MTX		
	Biomarkers	**B (SE)**	**β**	**p**
1	Methylation	0.09 (0.08)	0.13	0.242
2	Methylation	0.16 (0.07)	0.22	0.026
	DAS28	-0.49 (0.09)	-0.53	<0.001
	Erythrocyte folate	-1.00x10-3 4.00x10-4)	-0.12	0.197
	BMI	0.03 (0.02)	0.16	0.100
	Age	-	-	-
	Gender	-	-	-
	Smoking status	0.33 (0.23)	0.14	0.156
	ACPA status	-	-	-
	Observations		78	

Association between % baseline (T0) global DNA methylation in LINE-1 element CPG2 with ΔDAS28 (T3-T0), tested in a crude univariate model (1) and adjusted for potential confounders (2). Potential confounders were: baseline DAS28 score, baseline erythrocyte – folate levels (nmol/L), BMI (kg/m^2), age (years), gender and smoking status (current smoker versus former + never smoker). Only biomarkers that changed the association with >10% were considered confounders. B= beta coefficient, SE= standard error, β= standardized beta coefficient. P<0.05 was considered significant.

Furthermore, we examined the correlation between global DNA methylation obtained with LC-ESI-MS/MS and global DNA methylation in CpG2 assessed with the LINE-1 method. Here, we found a significantly positive correlation, although not strong ($R = 0.34$, $p = 0.00061$; Additional file 1: Fig. S1).

Association baseline global DNA methylation and non-response strongest in MTX monotherapy group

To assess whether the association between baseline global DNA methylation and disease activity was specific for MTX response and not due to combination therapy, subjects were stratified by therapy and univariate linear regression analyses were performed. The effect size was 1.8-fold higher in the MTX monotherapy group ($B = 2.06$, $p = 0.074$) compared to

the triple therapy group ($B = 1.12$, $p = 0.173$), although the associations were not significant (Table 5).

Table 5. Linear regression models for the association between global dna methylation before MTX and ΔDAS28 over three months stratified by treatment group.

Therapy	N	B (SE)	β	p
MTX	36	2.06 (1.12)	0.29	0.074
MTX + Corticosteroids	48	1.51 (1.08)	0.18	0.172
MTX + SSZ + HCQ + Corticosteroids	97	1.12 (0.81)	0.11	0.173

Associations were adjusted for baseline DAS28, baseline erythrocyte-folate and BMI. MTX=methotrexate, SSZ=sulfasalazine, HCQ=hydroxychloroquine, B=beta coefficient, SE=standard error, β=standardized beta coefficient. P<0.05 was considered significant.

DISCUSSION

In this study, we examined the association between global DNA (hydroxy)methylation, before-, at 3 months, and over 3 months of MTX therapy, in relation to changes in disease activity in leukocytes of eRA patients. We showed that higher baseline global DNA methylation is associated with clinical non-response, determined at 3 months of MTX treatment. This is in line with our hypothesis that higher baseline global DNA methylation levels are more difficult to inhibit and that this is associated with non-response. Furthermore, mean global DNA methylation did not change during MTX treatment, and global DNA methylation at and over 3 months was not associated with clinical efficacy. To our knowledge, we are the first to report an association between baseline global DNA methylation and early MTX response in eRA patients.

To predict response, associations prior to treatment are most suitable. Very few studies examined global DNA methylation status prior to treatment in relation to MTX response. Glossop and colleagues have identified 21 differentially methylated CpG sites in T-lymphocytes of 46 treatment-naive early RA patients. Of these, a combination of 1 hyper- and 1 hypomethylated CpG site gave the strongest predictive value [15]. A second study, in which 450k methylation arrays were performed, identified 2 baseline differentially methylated positions between 36 non-responders and 36 good responders that were associated with changes in c-reactive protein, but not with the complete DAS28 score [19].

Changes in global DNA methylation upon treatment were examined to give us more insight in the underlying mechanism. Despite the fact that MTX inhibits the universal methyl donor SAM, MTX administration has been shown to lead to increased global DNA methylation in peripheral blood mononuclear cells (PBMCs) of eRA patients [7, 20]. In contrast, we did not find significant methylation changes in leukocytes over the first 3 months. In our study, DNA was isolated from unsorted peripheral leukocytes. Leukocytes are a cell mixture of polymorphonuclear cells (PMN) and PBMCs, which have different methylomes. Changes in DNA methylation in PBMCs therefore might have been

overshadowed, which possibly explains the different results between these and our study. In addition, in our study, all subjects were supplemented with folic acid, which stimulates methyl-group donation. In contrast to global DNA methylation, we did observe a small, but significant increase in global DNA hydroxymethylation during the first 3 months of therapy. Future studies are necessary to assess this observed effect of MTX on global DNA hydroxymethylation.

Previously, we demonstrated that lower baseline erythrocyte folate concentration was associated with non-response at 3 months [14]. Assuming that erythrocyte folate concentrations reflect folate concentrations in leukocytes, and knowing that folate donates one-carbon groups required for methylation reactions, a correlation with baseline DNA methylation was expected, despite we did not observe a correlation between baseline erythrocyte folate and baseline global DNA methylation. Furthermore, from the adjusted beta values in our model, we observed that baseline erythrocyte folate and global DNA methylation both explained ~ 15% of variation in DAS28, although the associations were in opposing directions. In addition, upon adjustment of the model for confounders, which included erythrocyte folate, we showed that the positive association between global DNA methylation and DAS28 is independent from baseline erythrocyte folate concentration.

According to the EULAR response criteria, response to therapy is determined at 6 months. However, the tREACH study is designed to produce the greatest treatment differences during the first 3 months of therapy [8], which is why we examined response over the first 3 months of therapy. Upon stratification by treatment, the association between baseline global DNA methylation and DAS28 was strongest in the MTX monotherapy group, despite the fact that this group was the smallest. This suggests that the association is regulated through MTX treatment. The associations upon stratification were not significant, which was probably due to a loss of power.

Strength of this study is that all patients received the same MTX dose due to the prospective study design of the tREACH. Moreover, DNA methylation and hydroxymethylation were quantified for each patient simultaneously with the same technique. Furthermore, the association between global DNA methylation and changes in disease activity upon MTX treatment were validated with a second technique. Limitations are that the majority of the patients received MTX-combination therapy and that the group size for LINE-1 methylation was limited, thus replication in larger MTX monotherapy studies is required. In addition, it would be interesting to examine DNA methylation in sorted peripheral blood leukocytes.

CONCLUSIONS

In this paper, we showed that global DNA methylation is independently associated with disease activity over the first 3 months of MTX therapy. However, the underlying pathway, as well as the potential added value of global DNA methylation in a prediction model for MTX response requires further exploration.

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SUPPLEMENTAL MATERIAL

Additional Table 1. Global DNA methylation and hydroxymethylation levels before MTX and three months after MTX therapy.

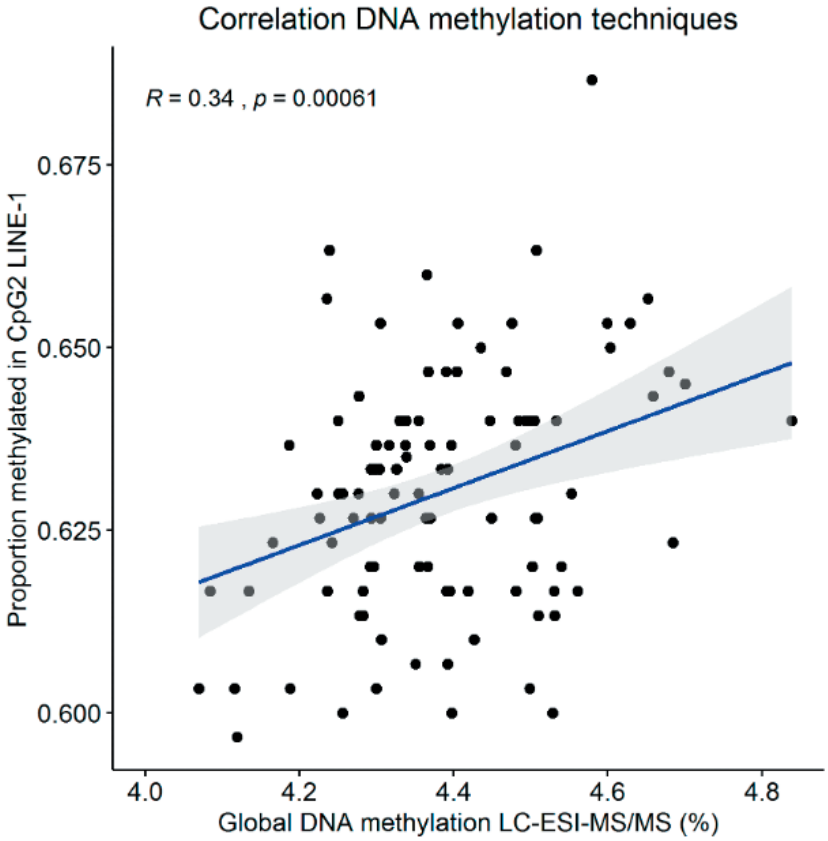
	N	Before MTX	After MTX	P
Methylation (%), mean ± SD	212	4.41 ± 0.13	4.40 ± 0.16	0.454
Hydroxymethylation (%), mean ± SD	210	3.64x10-2 ± 5.00x10^{-3}	3.72 x10-2 ± 5.00x10^{-3}	0.013

P-values are the result of a paired sample t-tests. P-values <0.05 were considered significant.

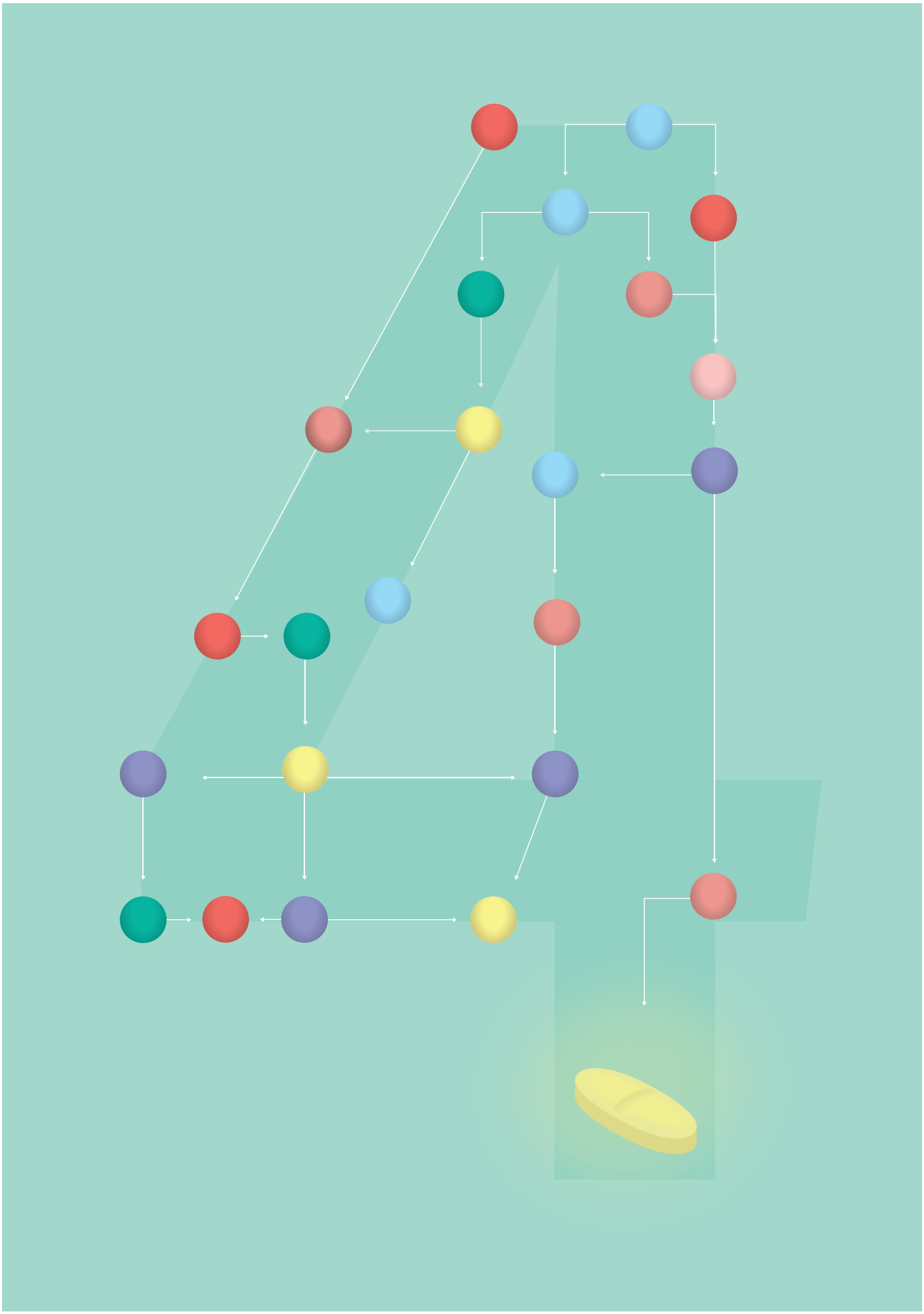
Additional Table 2. Linear regression models of %methylation in 6 LINE-1 CpG sites in relation to ΔDAS28 over three months of MTX therapy.

	CpG1	CpG3	CpG5	CpG6.7	CpG8.9	CpG11.12
N	79	78	78	79	79	79
Mean% ± SD	66.77 ± 0.03	74.08 ± 1.81	38.34 ± 1.65	70.81 ± 2.26	70.39 ± 1.70	84.31 ± 1.37
	β (p)	**β (p)**	**β (p)**	**β (p)**	**β (p)**	**β (p)**
Methylation	-0.02 (0.816)	0.09 (0.429)	0.07 (0.474)	0.13 (0.245)	0.14 (0.269)	0.13 (0.207)
DAS28	-0.50 <0.001)	-0.51 <0.001)	-0.52 <0.001)	-0.51 <0.001)	-	-0.54 <0.001)
Folate	-0.23 (0.021)	-0.22 (0.021)	-0.20 (0.034)	-0.22 (0.021)	-0.34 (0.003)	-
BMI	0.12 (0.226)	0.12 (0.208)	0.12 (0.225)	0.14 (0.148)	0.21 (0.064)	0.13 (0.178)
Age	-	-	-	-	-	-0.05 (0.620)
	-	-	-	-	-	-0.08 (0.391)
Gender	-	-0.01 (0.938)	-	0.01 (0.918)	-0.08 (0.521)	-
Smoking	0.10 (0.330)	0.11 (0.247)	-		0.15 (0.170)	-

Potential confounders were: baseline DAS28 score, baseline erythrocyte – folate levels (nmol/L), BMI (kg/m^2), age (years), gender and smoking status (current smoker versus former + never smoker). Only biomarkers that changed the association with >10% were considered confounders. β= standardized beta coefficient. P<0.05 were considered significant.



Additional Figure 1. Pearson correlation between global DNA methylation quantified and the LC-ESI-MS/MS and LINE-1 technique.



Chapter 4

Epigenome wide association study of response to methotrexate in early rheumatoid arthritis patients

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ABSTRACT

Introduction: To identify differentially methylated positions (DMPs) and regions (DMRs) that predict response to Methotrexate (MTX) in early rheumatoid arthritis (RA) patients.

Methods: DNA from baseline peripheral blood mononuclear cells was extracted from 72 RA patients. DNA methylation, quantified using the Infinium MethylationEPIC, was assessed in relation to response to MTX (combination) therapy over the first 3 months.

Results: Baseline DMPs associated with response were identified; including hits previously described in RA. Additionally, 1309 DMR regions were observed. However, none of these findings were genome-wide significant. Likewise, no specific pathways were related to response, nor could we replicate associations with previously identified DMPs.

Conclusion: No baseline genome-wide significant differences were identified as biomarker for MTX (combination) therapy response; hence meta-analyses are required.

INTRODUCTION

Methotrexate (MTX) is currently the anchor drug in the treatment of rheumatoid arthritis (RA), in agreement with the recommendations of the European league against rheumatism (EULAR)/ American college of rheumatology (ACR) [1]. However, treatment strategies are still trial and error due to the fact that treatment response is unknown until 3 to 6 months from initiation. While, about 30-40% of patients do not benefit from MTX. Clearly, there is a need for biomarkers to predict response prior to treatment in order to enable tight control of disease activity within the 'window of opportunity' and to restrain radiographic joint damage and functional disability [2].

Genomic DNA methylation at CpG dinucleotides has previously been associated with disease onset of RA [3–6] and therefore could possibly be utilized as a predictor for response to treatment with MTX (combination) therapy. Previously, we found an association between global DNA methylation and response to MTX in RA [7]. Other studies examined the relationship between differentially methylated positions (DMPs) in blood cells and DAS28 using Illumina's HumanMethylation450 BeadChip [8,9]. While Glossop *et al.* described that a combination of methylation levels at cg03018489 and cg14345882 in T-lymphocytes at baseline best predicts response to DMARD therapy at 6 months according to the EULAR criteria [8], Nair *et al*. did not find significant associations at baseline with changes in DAS28 over 6 months [9]. However, in the latter study, 4 DMPs at 4 weeks were associated with changes in single DAS28 components, such as swollen joint count and c–reactive protein, over 6 months [9]. Since a few years, Illumina has made available a new DNA methylation platform, the HumanMethylationEPICBeadChip array including >850,000 probes. This newly designed array is an extension of the Illumina HumanMethylation450 BeadChip, covering ~ 90% of previous sites and over 400,000 new probes of which the majority is positioned in potential enhancers [10].

In this study, we examine differentially methylated positions and regions in treatment naïve early RA patients in relation to treatment response to MTX assessed over the first 3 months of treatment initiation.

Materials and methods

Patients and materials

Patients were included from the treatment in early arthritis cohort Rotterdam (tREACH, registration number: ISRCTN26791028), a multicenter stratified single-blinded clinical trial of early rheumatoid arthritis (RA) patients [6]. Patient inclusion for current study was based on the availability of baseline Peripheral Blood Mononuclear Cells (PBMCs), which resulted in the inclusion of 83 patients. The tREACH was described earlier [6]. In short, inclusion criteria for the tREACH were the presence of arthritis in one or more joint(s), age ≥18 years and symptom duration < 1 year. This study was approved by the medical

ethics committee of the Erasmus MC, University Medical Center Rotterdam (MEC-2006-252). Medical ethics committees at each participating center (Erasmus MC, University Medical Center, Rotterdam; Sint Franciscus Gasthuis, Rotterdam; Maasstad Ziekenhuis, Rotterdam; Vlietland Ziekenhuis, Schiedam; Admiraal de Ruyter Ziekenhuis, Goes and Vlissingen; Zorgsaam Ziekenhuis, Terneuzen; Albert Schweitzer Ziekenhuis, Dordrecht) approved the study protocol and written informed consent was obtained for all including patients. Patients were recruited between July 2007 and April 2014 from outpatient clinics in participating centers in and near Rotterdam. The research for this manuscript took place in the Erasmus MC University Medical Center, Rotterdam. For current study, at baseline all patients were treatment naïve and were randomized to start methotrexate with corticosteroids as monotherapy or in combination with other disease modifying antirheumatic drugs (DMARDs): sulfasalazine (SSZ) and hydroxychlorqiuine (HCQ). In the tREACH, MTX dose was quickly increased (from 10 mg to 25 mg/week) within the first 3 weeks. Due to this aggressive treatment strategy in the tREACH, early response rates were expected at 3 months. If the target low disease activity (DAS28 <3.2) at 3 months was not reached, step-up treatment with biological or targeted synthetic DMARDs was prescribed. Additionally, patients weekly received 10 mg folic acid to reduce adverse events. PBMCs were extracted from whole blood using BD vacutainer ® CPT and stored in Roswell Park Memorial Institute (RPMI) 1640 Medium (R0883, with sodium bicarbonate, without L-glutamine, Merck) and 10% dimethylsulfoxide in liquid nitrogen.

DNA extraction

DNA extraction was performed for these 83 subjects using AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany) for simultaneous DNA and RNA isolation with a minimum input of 1 x 10^5 cells. DNA concentrations were assessed using a Nanodrop (NanoDrop Technologies, Wilmington, Germany). 72 samples with 260/280 ratios between 1.7 and 2.0 and of at least 500 ng were included in further analysis.

Human Methylation EPIC BeadChip

72 samples of 500 ng DNA were bisulfite treated using the Zymo EZ-96 DNA methylation kit (Zymo Research, Irvine, CA, USA). DNA methylation was quantified using the Infinium Human Methylation EPIC BeadChip Array according to manufacturer's protocol (Illumina, Inc., San Diego, CA, USA). Quality control and normalization was performed in R according to the incorporating Control Probe Adjustment and reduction of global CORrelation (CPACOR) workflow, as described previously [12]. In short, intensity values were stratified to autosomal and non-autosomal probes followed by quantile normalization for the six probe type categories separately (type I methylated red/green, type I unmethylated red/green and type II red/green). Beta values were calculated as a ratio between the fluorescence intensities of the methylated (M) and the M + unmethylated (U) probe intensity + a constant as follows: beta value= M/(M+U+100). Beta values below the

first quartile - 1.5 x interquartile range (IQR) or above the third quartile + 1.5 x IQR were considered outliers and were set to missing. Three samples did not pass quality control (N=2 due to unsuccessful bisulfite conversion, and N=1 due to unsuccessful hybridization) and were excluded. No gender mismatches or sample call rates below 98% were identified. Furthermore, probes with an intensity detection p-value ≥ 10^{-16} in > 5% of the samples were removed (N=14,184). The final dataset contained 846,415 probes and 69 patients.

Gene annotation

CpGs were annotated using the Illumina annotation file Version B.04. Missing gene names from the file were replaced by annotations using the Genomic Regions Enrichment of Annotations Tool (GREAT) (Human GRCh37 UCSC hg19, Feb/2009), where nearest basal regulatory regions within 5 kb upstream and 1 kb downstream of the transcription start site (TSS) with a maximum up to 1 MB were considered.

Epigenome-wide association study (EWAS)

Associations between baseline differentially methylated positions (DMP) and changes in disease activity over the first three months (ΔDAS28) were examined using MOMENT; a mixed-linear-model-based method using OmicS-data-based Complex train Analysis (OSCA) software [13]. This method tests for associations between baseline methylation and the linear outcome: ΔDAS28 and fits all distal probes in multiple random-effect components to account for unobserved confounders resulting in fewer false positive rates than other methods [13]. Prior to analysis, all beta values and the outcome were standardized to improve the comparison of effect sizes across probes. In our model the outcome was linear ΔDAS28 (i.e. 3 months DAS28 – baseline DAS28) as response rates were expected within 3 months due to the study design of the tREACH. We corrected for cell type composition using the Houseman method [14]. The association analysis in treatment naïve PBMCs was adjusted for the following covariates: baseline DAS28, gender, age, smoking and cell type composition (Houseman predicted: CD4T lymphocytes, CD8T lymphocytes, B lymphocytes, natural killer cells and monocytes). The smoking status of individuals included in our study was predicted using methylation profiles of targeted CpG sites known to be strongly associated with smoking, using the 'EpiSmokEr' package in R [15]. In addition, batch effects (plate and position) were treated as random effects to adjust for technical biases. Furthermore, MTX-polyglutamate concentrations that were previously determined in tREACH erythrocyte cell pellets [11] were used to assess treatment compliance in this study. With the aim of increasing the power of our study and with the rational that big differences between response groups would not be observed in probes with low biological methylation variance [16], association analyses were repeated filtering out lowly variable probes (probes with baseline methylation standard deviation <0.02) and excluding sex chromosomes. All tests were adjusted for multiple comparisons using Bonferroni correction.

Differentially methylated regions

To examine whether probes that lay in the same epigenomic region show the same relation to response to MTX (combination) therapy, differentially methylated regions (DMR) analyses were performed. DMRs were assessed using the DMRff package, which has been shown to be robust and control well for false positive rates [17]. Standard parameters of the DMRff package were applied to define genomic regions: at least two CpGs had to be present to form a region, the distance between probes within a DMR was maximum 500 base pairs, additionally CpGs had nominal EWAS p-values <0.05 and effect estimates of probes within a DMR were in the same direction [17].

Pathway analysis

Explorative gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using the top 1000 probes of DMP results, if nominal DMP p–values were <0.05, using the 'missMethyl' package in R.

RESULTS

Patient characteristics

14,184 probes out of 860,599 were removed during quality control. Hereafter, 69 subjects and 846,415 probes were included. The majority of patients were female (58%) with a mean age of 50.6 ± 15.4 years (Table 1). Mean DAS28 at baseline was 4.8 ± 1.3. 79.7% of the patients were positive for anti-citrullinated protein antibody (ACPA) and 82.6% were positive for rheumatoid factor. All patients received methotrexate with corticosteroids, of which 59.4% additionally received SSZ and HCQ (Table 1). Mean ± SD response determined over three months (ΔDAS28) was not significantly different for different treatment groups (High A: -1.9 ± 1.3, High B: -1.9 ± 1.1, High C: -1.8 ± 1.1, p= 0.963). Smoking status could not be determined for 1 subject; hence 68 subjects were included in the analysis.

Analysis complete probe set

The association between DMPs at baseline and changes in DAS28 over the first three months was assessed in a linear mixed model corrected for baseline DAS28, age, gender, smoking status and cell type composition. The Quantile-Quantile (QQ) plot with corresponding lambda, a measure to quantify the inflation in the test statistic, is shown in Fig 1. We did not observe genome-wide significant differences ($0.05/846{,}415 = 5.9 \times 10^{-8}$) nor DMPs located in certain chromosomes, as can be seen from the Manhattan plot (Fig 2). The top 10 DMPs with nominal p-values ≤ 1.0×10^{-4} are presented in Table 2.

Table 1. Baseline characteristics of subjects in the study.

	Mean ± SD
Subjects, N	69
Sex, Female (%)	40 (58.0)
Age (years)	50.6 (15.4)
Baseline DAS28	4.8 (1.3)
BMI (kg/m2)	26.8 (5.3) *
ACPA positive, N (%)	55 (79.7)
RF positive, N (%)	57 (82.6)
Smoking score, median (IQR)	3.5 (1.3)
Treatment, N (%)	
MTX + SSZ + HCQ + i.m. corticosteroids	21 (30.4)
MTX + SSZ + HCQ + p.o. corticosteroids	20 (29.0)
MTX + p.o. corticosteroids	28 (40.6)

*BMI: 1 missing value. MTX= methotrexate, SSZ= sulfasalazine, HCQ= hydroxychloroquine, i.m. = inter muscular, p.o.= per os. A smoking score was calculated using the EpiSmokEr package in R.

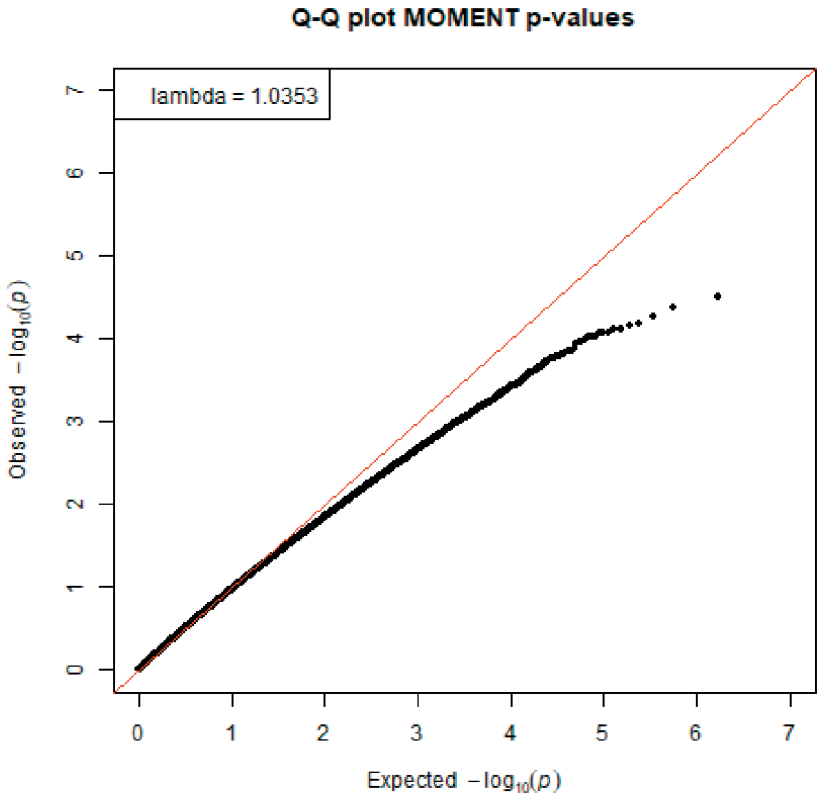

Figure 1. QQ-plot of linear mixed model for the association of DMPs and ΔDAS28.

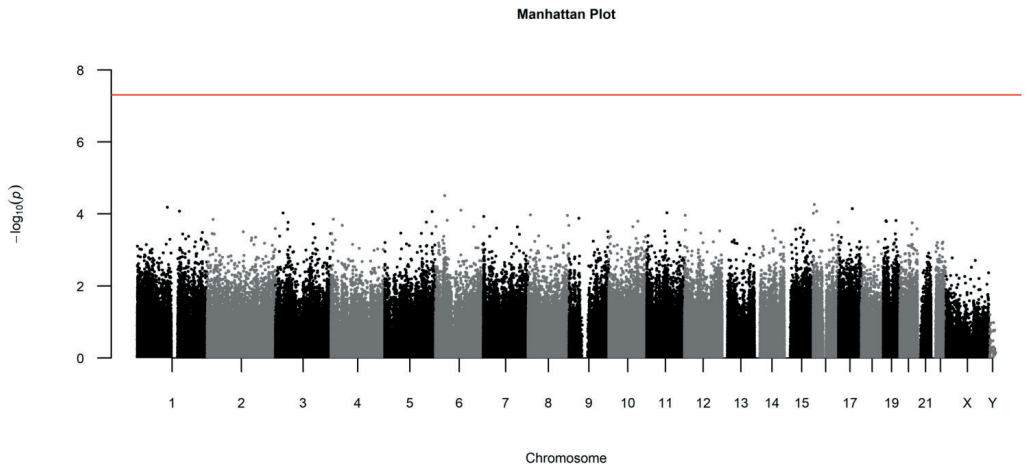

Figure 2. Manhattan plot of DMP analysis with all probes in association with ΔDAS28. Associations were adjusted for age, gender, smoking and cell type ratios. The red line indicates genome-wide significance according to Bonferroni correction (p-value= 5.91^{-8}).

Table 2. Top 10 DMP results from the analysis with complete probe set and the association with ΔDAS28.

Probe	Chr	Position	Gene	Relation to gene	450k loci	β	se	p
cg16944926	6	32940976	BRD2	Body	Yes	0.42	0.10	3.09×10^{-05}
cg11177738	3	193828742	HES1, OPA1*	-	No	0.45	0.11	4.42×10^{-05}
cg00519627	16	4466650	CORO7	TSS200	Yes	-0.45	0.11	5.43×10^{-05}
cg15697822	1	107684751	NTNG1	5'UTR	Yes	0.41	0.10	6.54×10^{-05}
cg02802788	17	49369718	UTP18	Body	No	-0.39	0.10	7.09×10^{-05}
cg14665002	12	64919341	RASSF3, TBK1*	-	No	0.40	0.10	7.80×10^{-05}
cg07248935	6	90643780	BACH2	Body	No	0.68	0.17	7.81×10^{-05}
cg11311263	16	11829117	TXNDC11	Body	No	-0.40	0.10	8.31×10^{-05}
cg00095674	1	150122654	PLEKHO1	Body	Yes	-0.42	0.11	8.36×10^{-05}
cg26426470	5	169181253	DOCK2	Body	Yes	-0.39	0.10	8.60×10^{-05}

β=standardized beta coefficient.*Genes annotated using the GREAT tool (Human GRCh37 UCSC hg19). Chr=chromosomes, TSS200= 0-200 bases from transcriptional start site (TSS), TSS1500= 200-1500 bp from TSS. Relation to gene was obtained from the Illumina annotation file version B4, a dash means that probes were not related to a gene by Illumina.

All adjusted p-values for multiple comparisons were ≥0.985. Results of the DMP analysis were used as input for the DMR analysis to examine whether closely located probes in certain regions show the same effect sizes and directions. We identified 1309 DMR regions, of which none were genome-wide significant. The top 10 DMRs at nominal p-values < 1.0×10^{-3} are presented in Table 3. Additionally, to examine if certain Gene Ontology (GO) terms were enriched, pathway analysis was performed on the top 1000 most significant DMP results, all at nominal p-values <2.5×10^{-3} and adjusted p-values of ≥ 0.960. The top

10 identified GO terms are presented in S1 Table, however, no genome-wide significantly enriched pathways were observed.

Table 3. Top 10 DMR results with complete probe set.

Chr	start	end	Nearest gene (distance to TSS)	N	b	se	p-value
8	**87355594**	87355773	*WWP1* (+717)	3	-0.40	0.10	1.05×10^{-04}
22	**41252959**	41253041	*XPNPEP3* (-102)	3	-0.31	0.08	1.17×10^{-04}
19	**50380748**	50380763	*TBC1D17* (+74)	2	-0.49	0.13	1.24×10^{-04}
7	**1610694**	1610747	*PSMG3* (-1092)	2	-0.06	0.02	1.35×10^{-04}
9	**99540409**	99540427	*ZNF510* (-80)	3	-0.44	0.11	1.39×10^{-04}
4	**59850015**	59850171	None	2	0.18	0.05	1.42×10^{-04}
6	**32829062**	32829208	*PSMB9* (+7197)	3	-0.14	0.04	1.47×10^{-04}
3	**49157911**	49158377	*USP19* (+120)	6	-0.33	0.09	1.64×10^{-04}
2	**98262546**	98262568	*COX5B* (+54)	2	-1.05	0.28	1.72×10^{-04}
3	**45017855**	45017955	*ZDHHC3* (-231)	2	-0.72	0.19	1.90×10^{-04}

Chr= chromosome, N= number of probes within DMR, b= change in DAS28 upon 1% difference in baseline methylation.

Analysis filtered probes

To increase power to detect significantly associated probes with treatment response, association analysis was also carried out on a restricted set of probes (N=393,282), after the removal of low variance probes and probes on sex chromosomes. Despite the strong reduction in the number of tested probes, we could not identify probes with genome-wide significance ($0.05/393{,}282 = 1.3 \times 10^{-7}$). Manhattan and QQ-plots are presented in Supporting Information file S1 and S2 Figs. The top 10 DMPs that reached nominal significance of $p < 1.82 \times 10^{-04}$ are presented in S2 Table. DMR analysis did not find genome-wide significant regions, however 359 nominally significant candidate regions were identified, of which the top 10 regions had a nominal p-value $<1.0 \times 10^{-3}$ (S3 Table).

Look up of previously identified loci

A study by Glossop et al described two CpGs (cg03018489 and cg14345882) in T lymphocytes of treatment naïve rheumatoid arthritis patients that could discriminate non-responder and moderate/good responders at baseline with an area under the curve of 0.85. We did a look up of these CpGs in our study. Cg03018489 was removed during the quality control steps in our study and could therefore not be assessed. Mean DNA methylation of cg14345882 was similar across good responders (mean= 0.23, sd= 0.09), moderate responders (mean = 0.23, sd= 0.09) and non-responders (mean= 0.19, sd= 0.06) as depicted in S3 Fig.

DISCUSSION

We present the first study that assesses baseline differential DNA methylation in relation to DAS28 in rheumatoid arthritis (RA) patients using Illumina's Human Methylation EPIC array. In this study, we did not identify genome-wide significant DMPs or DMRs in relation to changes in DAS28 over the first 3 months of treatment. However, some of the genes with p-values of $<10^{-4}$ have previously been associated with RA. Examples include *BRD2*[18], which binds to IL-6 promoters in macrophages where it stimulates IL-6 production, *PLEKHO1*, which regulates joint inflammation[19] and *BACH2*[20–22] and *DOCK2*[23,24] which are important in B cell differentiation and T cell regulation, both important events in the development of early RA. These probes are therefore interesting targets for future studies.

To examine whether the top 10 most significant probes in DMP analysis were part of a DMR, we compared the top 10 DMP and DMR results. However, we did not observe any overlap between the top 10 most significant findings. Moreover, explorative pathway analysis was performed on the top 1000 results, which did not suggest a specific pathway that was differentially methylated in relation to MTX (combination) therapy response. Importantly, the results from our pathway analysis should be interpreted with care, as the top 1000 DMP results were not genome-wide significant.

Furthermore, a look up of previously described baseline CpGs related to prediction of response to MTX did not show similar results in our data. These results may reflect differences in cell types assessed [8]. Our results were from a cell mixture, hence T cell specific differences that were previously observed could have been underestimated in our results. Another explanation could be that we show larger biological variance as our intermediate (n=28) and good responder groups (n=31) were slightly larger compared to the study of Glossop *et al.*

Next, we repeated our association analysis by filtering out low variance probes and probes on sex chromosomes as we postulated that these probes are expected to be less informative and their exclusion could increase the power of our study. This resulted in a shift of the top findings. Most significant hits in previous DMP analysis appeared to have been in lowly variable probes as they were no longer present after filtering. Only one of the previous top 10 findings (probe cg07248935 located in the *BACH2* gene) remained in the top 10 CpGs of this second EWAS. Genes listed in top 10 most significant DMPs in the second EWAS were all part of the top 30 most significant results of the first EWAS: including all the probes. As expected, effect sizes between the two EWAS studies were similar. Upon exclusion of lowly variable probes, probe cg07639783 in the top 10 DMP list, located in *PSMG3* promoter, overlapped with a top 10 most significant DMR regions. This region was also observed in the top 10 DMR analyses with all probes. This supports that is could be an interesting target. It should however be noted that this region only consisted of two DMPs and this finding was not genome-wide significant. Hence, this potential finding should be

further investigated in other studies.

On the one hand, the observation that the most significant findings were found for lowly variable probes may indicate false positive results due to the small biological variance (SD < 0.02). On the other hand, differences in methylation related to RA response have been shown to be small; hence our findings may still be clinically relevant. To find genome-wide significant results for small differences (2%) in case – control studies, very large sample sizes (>1000) are required [25]. We calculated the power of our study based upon two equal groups and this showed that we had 80% power to detect a mean difference of 8% in 77.5% of all genomic sites with recommended significance threshold of 9.42×10^{-8}. If we assume that the power calculation for two equal groups is at least comparable to our linear analysis, we would have enough power to find large differences (>8%) in the majority of probes. However, we did not observe such large differences. This power calculation also indicates that we may have missed smaller differences. Therefore, meta-analyses are required to increase statistical power and investigate whether smaller differences in DNA methylation profiles are clinically relevant. Thus far, other studies assessing response to MTX have been conducted using the 450k array. In principle, such studies could be meta-analyzed with our study, however, challenges when combining 450k and EPIC array results exist. Not all probes have been shown to replicate well across the two platforms and several probes are not common to the two arrays [26]. Also, differences in cell types used for the experiments complicate combining studies. Therefore, more studies using the EPIC array in PBMCs are required prior to perform meta-analysis in order to assess whether smaller mean differences are related to response to MTX (combination) therapy.

Strengths of this study are that it was performed in a prospective cohort where subjects received controlled treatment of similar dosages of MTX (combination) therapy. Despite that the majority received combination therapy, which could potentially influence the outcome (ΔDAS28), no significant differences in ΔDAS28 between treatment groups were observed. Moreover, erythrocyte methotrexate-polyglutamate levels at 3 months were quantified in all patients [11,27]. In the majority of the samples (66/68), MTX polyglutamate levels were present, supporting treatment compliance. Another strength is that results were acquired from PBMCs consisting of monocytes and lymphocytes but not granulocytes. This is important, as the methylome of granulocytes is very different compared to that of cells of the lymphoid lineage [19]. The downside of using PBMCs is that it is still a cell mixture and that it is more labor intensive to extract them first from whole blood. However, in this study we assessed the cell type composition using the Houseman method [14] and included cell type percentages as covariates. Furthermore, RA is an infiltrating disease; hence for future studies DNA methylation statuses from leukocytes in the synovial fluid may be more predictive in relation to disease activity. Another weakness is that we may have missed small differences due to the limited group size. Furthermore, what could have influenced the relationship between DNA methylation

and DAS28 was the presence of anti-citrullinated protein antibody (ACPA) [20]. However, in our study 80% of the patients were positive for ACPA, accordingly we did not correct for ACPA positivity in the models.

In conclusion, we performed the first DNA methylation association analysis using the Illumina MethylationEPIC array to test for treatment response in naïve early rheumatoid arthritis patients. We did not observe genome-wide significant DMPs or DMRs in relation to changes in DAS28 over the first 3 months of treatment. Larger studies are required to demonstrate or rule out the use of DNA methylation sites as predictive marker for response to MTX. Potential biomarkers could be combined with other clinical and laboratory predictors to improve prediction to response and personalize treatment in early RA.

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SUPPORTING INFORMATION

Table S1. Top gene ontology (GO) terms from pathway analysis of 1000 most significant probes in DMP analysis with full probe set.

Gene Ontology term			Nr. of genes in GO term	Nr. of genes differentially methylated	P-value
GO:0050872	BP	white fat cell differentiation	13	5	3.15×10^{-04}
GO:1904798	BP	positive regulation of core promoter binding	7	3	1.96×10^{-03}
GO:0051965	BP	positive regulation of synapse assembly	61	9	2.56×10^{-03}
GO:0002268	BP	follicular dendritic cell differentiation	2	2	2.72×10^{-03}
GO:0033257	CC	Bcl3/NF-kappaB2 complex	2	2	2.72×10^{-03}
GO:0007416	BP	synapse assembly	59	9	2.77×10^{-03}
GO:0005592	CC	collagen type XI trimer	2	2	3.21×10^{-03}
GO:0033523	BP	histone H2B ubiquitination	9	3	3.23×10^{-03}
GO:0042351	BP	'de novo' GDP-L-fucose biosynthetic process	2	2	3.34×10^{-03}
GO:0035252	MF	UDP-xylosyltransferase activity	8	3	4.19×10^{-03}

BP= biological process, CC= cellular component, MF= molecular function.

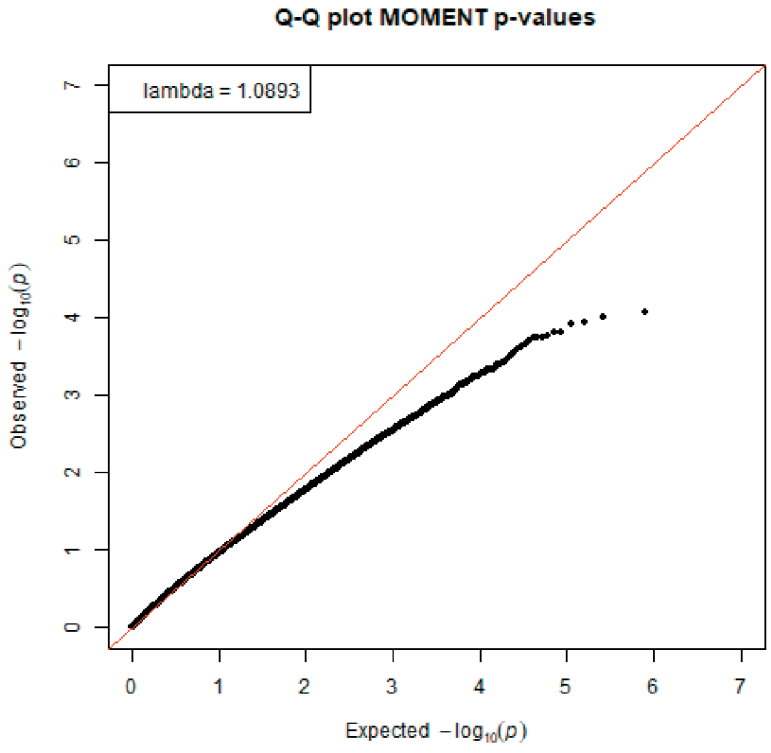


Figure S1. QQ-plot of DMP analysis with selected probe set.

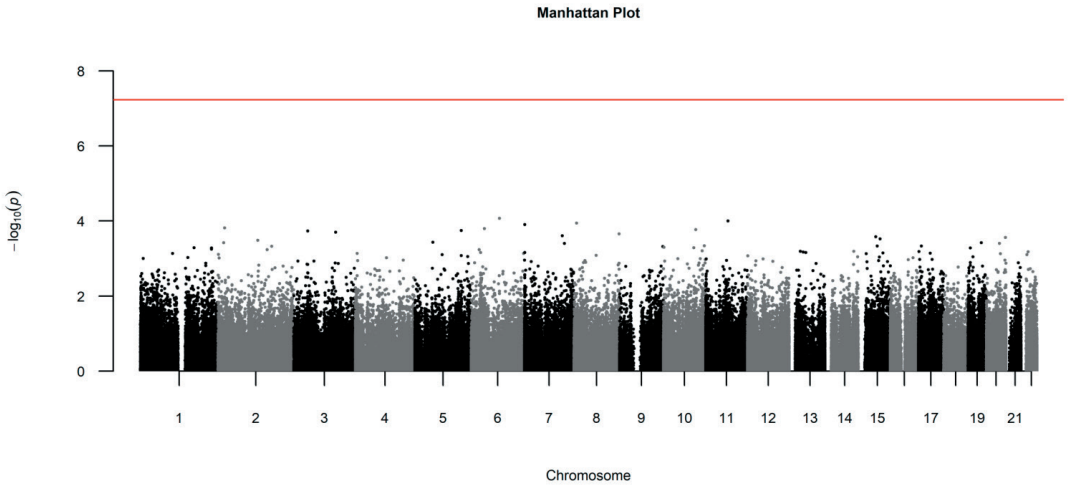


Figure S2. Manhattan plot of DMP analysis with selected probe set. Low variance probes (baseline methylation SD<0.02) and probes on sex chromosomes were excluded. Associations were adjusted for age, gender, smoking and cell type ratios.

Table S2. Top DMPs from selected probe set and the association with ΔDAS28.

Probe	Chr	bp	Gene	Relation to gene	450k loci	b	se	p
cg07248935	6	90643780	BACH2	Body	No	0.68	0.17	8.40×10^{-05}
cg22457050	11	71790060	LRTOMT; NUMA1	TSS1500,Body; 5'UTR	No	0.42	0.11	9.92×10^{-05}
cg05062393	8	8861278	ERI1	Body	No	0.40	0.10	1.13×10^{-04}
cg07639783	7	1610747	PSMG3; KIAA1908	TSS1500; Body	Yes	-0.43	0.11	1.24×10^{-04}
cg09835161	2	21023915	LDAH	TSS1500	No	0.38	0.10	1.51×10^{-04}
cg02711899	6	42410890	TRERF1	5'UTR	Yes	-0.40	0.11	1.59×10^{-04}
cg18288462	10	103986268	ELOVL3	1stExon; 5'UTR	Yes	0.41	0.11	1.71×10^{-04}
cg14152587	5	148612561	ABLIM3	Body	No	-0.41	0.11	1.80×10^{-04}
cg21672276	3	44754072	ZNF502	TSS200	Yes	0.41	0.11	1.83×10^{-04}
cg17445273	13	23489472	SGCG*	TSS-265kb	Yes	-0.43	0.11	1.84×10^{-04}

Effect=beta coefficient; the change in DAS28 corresponding to an increase in methylation of 1%. *Genes annotated using the GREAT tool (Human GRCh37 UCSC hg19). Chr=chromosomes, TSS1500= 200-1500 base pairs from transcriptional start site (TSS). Relation to gene was obtained from the Illumina annotation file version B4.

Table S3. Top 10 DMR results with selected probe set.

Chr	start	end	Nearest gene (distance from TSS)	N	b	se	p-value
7	1610694	1610747	PSMG3 (-1092)	2	-0.06	0.02	1.45 x 10-04
7	887534	887678	GET4 (-28583), SUN1 (+15468)	2	-0.12	0.03	3.68 x 10-04
1	152087889	152088085	TCHH (-1431)	2	0.07	0.02	3.70 x 10-04
6	32829062	32829208	PSMB9 (+7197), HLA-DMB (+79712)	2	-0.15	0.04	4.89 x 10-04
12	130908778	130909093	PIWIL1 (+86504), RIMBP2 (+93474)	2	-0.14	0.04	5.52 x 10-04
5	78281819	78281983	ARSB (-293)	5	-0.06	0.02	7.04 x 10-04
7	994666	994742	ADAP1 (-371)	3	0.10	0.03	7.96 x 10-04
8	107459398	107460168	OXR1 (-369)	9	-0.05	0.02	8.66 x 10-04
17	39204767	39204882	KRTAP2-1 (-1257)	2	0.12	0.04	8.82 x 10-04
15	78632109	78632144	CRABP1 (-539)	2	-0.07	0.02	9.44 x 10-04

Chr= chromosome, distance from transcriptional start site (TSS) is reported in ase pairs. N= number of probes, b= change in DAS28 upon 1% difference in baseline methylation.

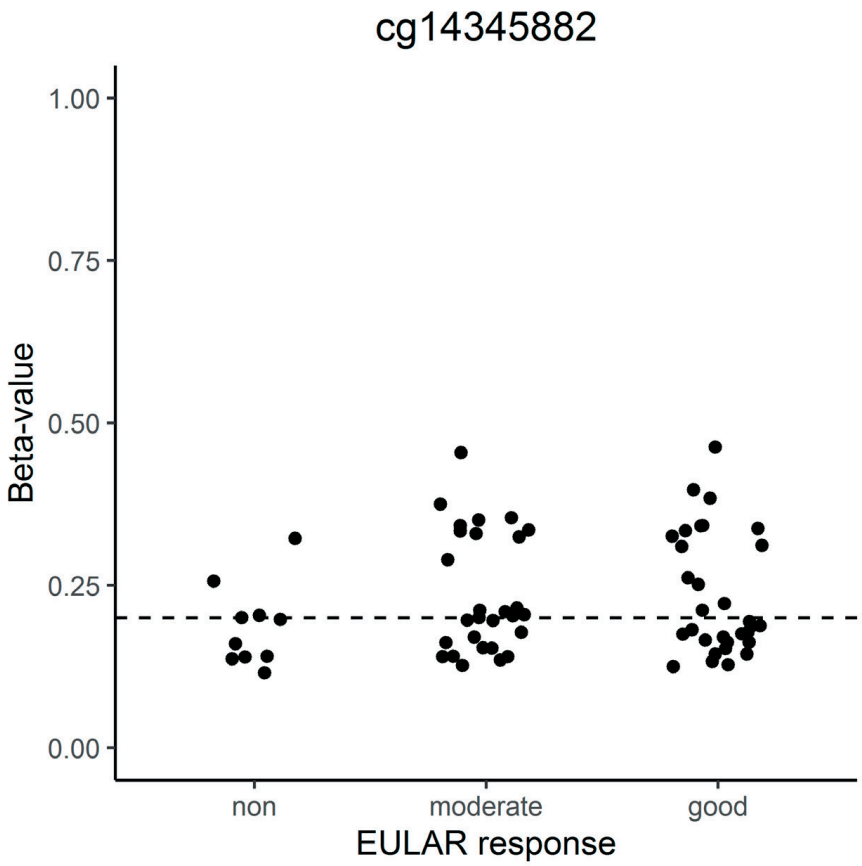
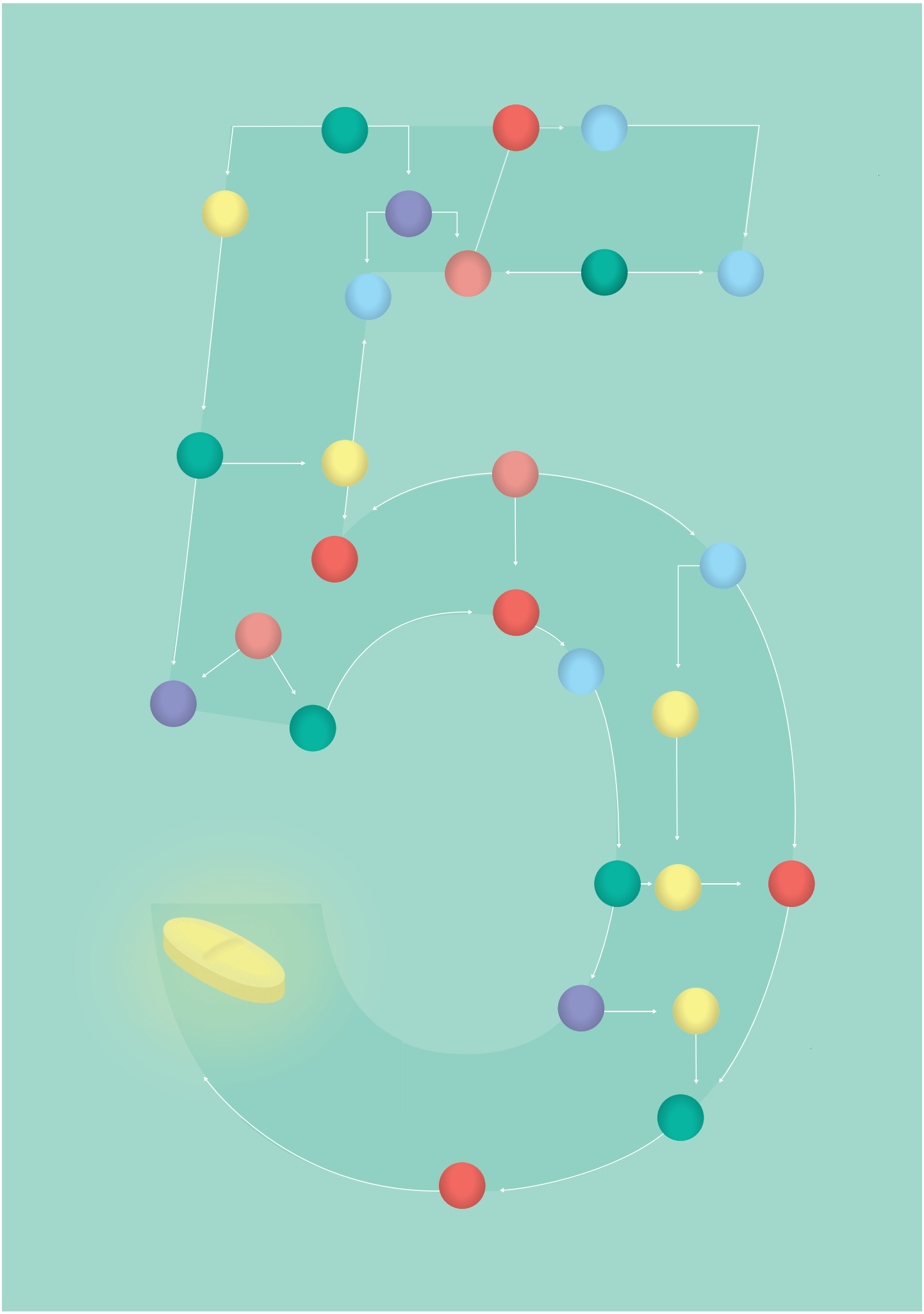


Figure S3. Look up study of CpG finding from Glossop et al. Dashed horizontal line represents previously established cut-off value. Response was categorized in non–responders (n=10), moderate responders (n=28) and good responders (n=31) according to the EULAR criteria at 3 months.



Chapter 5

Identification of Metabolic Biomarkers in Relation to Methotrexate Response in Early Rheumatoid Arthritis

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ABSTRACT

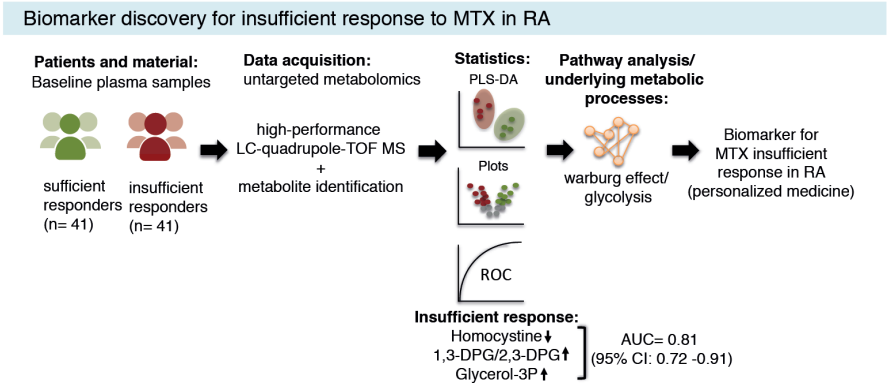
Introduction: This study aimed to identify baseline metabolic biomarkers for response to methotrexate (MTX) therapy in rheumatoid arthritis (RA) using an untargeted method.

Methods: In total, 82 baseline plasma samples (41 insufficient responders and 41 sufficient responders to MTX) were selected from the Treatment in the Rotterdam Early Arthritis Cohort (tREACH, trial number: ISRCTN26791028) based on patients' EULAR response at 3 months. Metabolites were assessed using high-performance liquid chromatography-quadrupole time of flight mass spectrometry. Differences in metabolite concentrations between insufficient and sufficient responders were assessed using partial least square regression discriminant analysis (PLS-DA) and Welch's *t*-test. The predictive performance of the most significant findings was assessed in a receiver operating characteristic plot with area under the curve (AUC), sensitivity and specificity. Finally, overrepresentation analysis was performed to assess if the best discriminating metabolites were enriched in specific metabolic events.

Results: Baseline concentrations of homocystine, taurine, adenosine triphosphate, guanosine diphosphate and uric acid were significantly lower in plasma of insufficient responders versus sufficient responders, while glycolytic intermediates 1,3-/2,3-diphosphoglyceric acid, glycerol-3-phosphate and phosphoenolpyruvate were significantly higher in insufficient responders. Homocystine, glycerol-3-phosphate and 1,3-/2,3-diphosphoglyceric acid were independent predictors and together showed a high AUC of 0.81 (95% CI: 0.72–0.91) for the prediction of insufficient response, with corresponding sensitivity of 0.78 and specificity of 0.76. The Warburg effect, glycolysis and amino acid metabolism were identified as underlying metabolic events playing a role in clinical response to MTX in early RA.

Conclusions: New metabolites and potential underlying metabolic events correlating with MTX response in early RA were identified, which warrant validation in external cohorts.

Graphical abstract:



INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting joint linings, resulting in pain and inflammation [1]. Methotrexate (MTX) is the first-line therapy in rheumatoid arthritis (RA); however, treatment strategies still consist of trial and error [2]. MTX is an antifolate with a long background in cancer chemotherapy acting as a potent inhibitor of folate metabolism impacting numerous targets in one-carbon metabolism, nucleotide and amino acid biosynthesis [3]. The mechanism of MTX in RA is still not fully understood, which is why it is still unknown why some patients respond better than others to MTX [4,5]. Response to MTX-based therapy can be determined after 3 to 6 months according to changes in disease activity score 28 (DAS28) and insufficient responders require step-up treatment with biologic disease modifying anti rheumatic drugs (bDMARDs; e.g., TNF-alpha inhibitors, IL-6 inhibitors [6]) or targeted synthetic DMARDs (tsDMARDs, e.g., Janus-kinase inhibitors [7,8]) as described in the EULAR recommendations for the management of RA [2]. To enable quicker treatment adjustments, earlier identification of insufficient responders to MTX will be of great clinical importance in personalized medicine.

Several studies investigated baseline biomarkers to predict clinical response to MTX at 3 and/or 6 months in a targeted way [9]. We have previously developed [10] and externally validated [11] a baseline clinical prediction model for insufficient response to MTX. Apart from clinical predictors, this prediction model includes biomarkers such as erythrocyte folate and adenosine triphosphate (ATP) binding cassette (ABC) transporter polymorphisms. Applying an untargeted approach might reveal new and overlooked biomarkers and provide new insights into the etiology of non-response to MTX. Others have shown that RA patients have a different serum metabolite signature compared to healthy controls [12,13,14,15]. Study results from a literature review showed that essential amino acids (citric acid, isoleucine, methionine, valine) and non-essential amino acids (threonine, histidine and alanine) were consistently lower in RA patients compared to healthy controls [16]. Additionally, differences in metabolic profiles have been associated with different stages of disease [14,15] as well as in relation to treatment response [17,18,19,20]. The aim of the current study was to identify potential baseline biomarkers in treatment-naive patients for the prediction of insufficient response to MTX at 3 months in RA patients using an untargeted approach.

MATERIALS AND METHODS

Materials and Subjects

Baseline plasma samples of 82 early RA patients were selected from the treatment in the Rotterdam early arthritis cohort (tREACH; ISRCTN registered trial, number: ISRCTN26791028) [21], based on plasma availability and their European League Against Rheumatism (EULAR) response at 3 months, including 41 insufficient responders and 41

sufficient responders. Insufficient response was defined as: 3-month DAS28-ESR > 5.1 and improvement of DAS28-ESR ≤ 1.2. Sufficient response was defined as: 3-month DAS28-ESR ≤ 3.2 and improvement in DAS28-ESR > 1.2 over the first 3 months. All subjects received MTX (combination) therapy (see Table 1) and all accomplished the American College Rheumatism (ACR)/EULAR 2010 classification criteria for rheumatoid arthritis (RA) [22].

Table 1. Characteristics of RA patients with insufficient response versus sufficient response to MTX (combination) therapy.

	Insufficient responders (DAS28-ESR > 3.2) N=41	**Sufficient responders (DAS28-ESR ≤ 3.2) N=41**	**P–value**
Baseline DAS28, mean ± SD	4.3 ± 1.3	5.6 ± 1.0	<0.001
Age, mean ± SD	50.0 ± 11.9	52.6 ± 16.9	0.41
Sex, Male, N (%)	8 (20)	15 (37)	0.14
BMI (kg/m^2), mean ± SD	28.1 ± 5.4	24.3 ± 4.1[#]	<0.001
RF positive, N (%)	26 (63)	33 (80)	0.14
ACPA positive, N (%)	25 (61)	31 (76)	0.24
Treatment			
MTX + SSZ + HCQ + corticosteroids i.m.	8 (20)	15 (37)	0.14
MTX + SSZ + HCQ + corticosteroids per os	11 (27)	15 (37)	0.48
MTX + corticosteroids per os	13 (32)	7 (17)	0.20
MTX	9 (22)	4 (10)	0.23

#BMI, N=1 missing value, BMI= body mass index, MTX= Methotrexate, SSZ= Sulfasalazine, HCQ= hydroxychloroquine, i.m. = intramuscular,

After blood collection in ethylenediamine tetraacetic acid (EDTA) tubes, samples were immediately placed on ice, followed by centrifugation for 10 min at 1700× *g* at a temperature of 4 °C. Plasma samples were stored at −80 °C, as previously described [23]. This study was approved by the medical ethics committee of Erasmus Medical Center (MEC-2006-252) and written informed consent was obtained for included patients. All procedures performed were in accordance with the 1964 Helsinki Declaration and its later amendments.

2.2. Metabolomics Study

Metabolomics analysis was performed using a semi-quantitative analysis at the Core Facility Metabolomics of the Amsterdam UMC as described previously [24]. In short, a mixture of 75 µL of the following internal standards in water was added to 25 µL plasma: adenosine-$^{15}N_5$-monophosphate (100 µM), adenosine-$^{15}N_5$-triphosphate (100 µM), D_4-alanine (100 µM), D_7-arginine (100 µM), D_3-aspartic acid (100 µM), D_4-citric acid (100 µM), $^{13}C_1$-citrulline (100 µM), $^{13}C_6$-fructose-1,6-diphosphate (100 µM), guanosine-$^{15}N_5$-monophosphate (100 µM), guanosine-$^{15}N_5$-triphosphate (100 µM), $^{13}C_6$-glucose (1 mM), $^{13}C_6$-glucose-6-phosphate

(100 µM), D_3-glutamic acid (100 µM), D_5-glutamine (100 µM), $^{13}C_6$-isoleucine (100 µM), D_3-leucine (100 µM), D_4-lysine (100 µM), D_3-methionine (100 µM), D_6-ornithine (100 µM), D_5-phenylalanine (100 µM), D_7-proline (100 µM), $^{13}C_3$-pyruvate (100 µM), D_3-serine (100 µM), D_5-tryptophan (100 µM), D_4-tyrosine (100 µM), D_8-valine (100 µM). Subsequently, 425 µL water, 500 µL methanol and 1 mL chloroform were also added and the samples were mixed and centrifuged for 10 min at 14,000 rpm. The polar phase was dried using a vacuum concentrator at 60 °C. Subsequently, dried samples were reconstituted in 100 µL methanol/water (6/4; *v/v*). Then, 5 µL metabolic extract was injected onto a SeQuant 100 × 2.1 mm ZIC-cHILIC column, 3 µm particle diameter (Merck, Darmstadt, Germany). The column temperature was maintained at 30 °C and samples at 12 °C during analysis. An impact II quadrupole time of flight (QTOF) (Bruker Daltoniks) mass spectrometer (MS) was used in the negative and/or positive electrospray ionization mode where mass spectra of the metabolites were obtained by continuous scanning from *m/z* 50 to *m/z* 1200 with a resolution of 50,000 full half-maximum width (FHMW). Data were analyzed using Bruker TASQ software version 2.1.22.3. All reported metabolite intensities were normalized to internal standards with comparable retention times and response in the MS. Metabolite identification was based on a combination of accurate mass, (relative) retention times and fragmentation spectra, compared to the analysis of a library of standards. Statistical analysis and visualization of the acquired data were done in a R environment using the ggplot2, ropls and mixOmics packages [25,26,27]. Identified metabolites were classified according to the Human Metabolome Database [28].

2.3. Statistics

Mean and standard deviation (± SD) between baseline group characteristics were compared using a two-sample *t*-test. Proportions in baseline characteristics were compared using a two-proportion test in R. To identify metabolites that could discriminate insufficient responders from sufficient responders, we used partial least square regression discriminant analysis (PLS-DA). Variable Importance Projection (VIP) scores were examined to select best discriminating variables, where a VIP score of ≥1 was considered important [29]. Furthermore, to investigate differences in mean concentrations between response groups at baseline, a Welch's *t* test was performed and fold changes were calculated, which were together visualized in a volcano plot. We corrected for multiple comparisons using the Benjamini–Hochberg method. A multivariable model was built with metabolites that were significantly different between insufficient and sufficient responders and had a VIP score >1. As highly correlated variables could influence logistic regression, correlations between metabolites were first assessed using Pearson's correlation in a correlation matrix using the "corrplot" package in R. In the same analysis, the relation between metabolites and inflammatory factors (erythrocyte sedimentation rate [ESR] and C-reactive protein [CRP]) was assessed to examine whether the metabolites were a surrogate for inflammation.

Metabolites with a Pearson's correlation coefficient of >0.6 were considered strongly correlated. In case two metabolites were strongly correlated, only the metabolite with the highest VIP score in relation to response was included in the model. From the model, a receiver operating characteristic (ROC) curve with area under the curve (AUC) was produced. Sensitivity and specificity were calculated using the "pROC" package in R. In addition, non-linear relationships between metabolites and the outcome were examined in a random forest analysis, which is an ensemble classification method. For the random forest analysis, a random seed was set to 415 to make the analysis reproducible. Mean decrease in accuracy (how well the model performs) and decrease in Gini score (how pure the nodes are at the end of the tree) were assessed to evaluate variable importance upon removal of each variable. Hence, the larger the decrease in accuracy and Gini score, the more important the variable.

To obtain a better understanding of which metabolic pathways were enriched between insufficient and sufficient responders to MTX, an overrepresentation analysis (ORA) was performed using the online "Metabolite Set Enrichment Analysis" (MSEA) tool as integrated in the MetaboAnalyst software 4.0 [30]. Compound names of metabolites with a VIP score >1 produced by the PLS-DA analysis were used as input. Small molecule pathway database (SMPDB) was selected as reference library containing 99 metabolite sets based on normal human metabolic pathways. A hypergeometric test was performed to evaluate if combinations of differentially expressed metabolites were represented more than expected by chance, providing a one-tailed *p*-value. *p*-values were adjusted for multiple testing using the Holm–Bonferroni method and false discovery rate (FDR) according to the Benjamini–Hochberg method.

RESULTS

Baseline Comparisons

Mean baseline DAS28 was lower in RA patients with insufficient response to MTX therapy (4.3 ± 1.3) compared to RA patients with sufficient response to therapy (5.6 ± 1.0, $p < 0.001$; Table 1), while BMI was higher in the insufficient responder group ($p < 0.001$; Table 1). Other characteristics such as age, sex, rheumatoid factor (RF) positivity, anti-citrullinated protein antibody (ACPA) positivity and medication were similar between both groups.

3.2. Metabolite Analysis

Metabolites were examined as a potential biomarker for response to MTX. A list of the 50 most important variables was created according to their VIP scores from to the PLS-DA analysis (Supplementary Figure S1) and *p*-values acquired from Welch's *t*-test, which is presented in Supplementary Table S1. Moreover, 1,3-diphosphoglyceric acid (DPG)/2,3-

DPG and homocystine had the largest VIP scores (2.439 and 1.927, respectively) and were most significantly different between insufficient responders and sufficient responders (p = 0.001 and p = 0.004, respectively; Table S1). Homocystine, taurine, adenosine triphosphate (ATP), guanosine diphosphate (GDP) and uric acid concentrations were significantly lower in insufficient responders versus sufficient responders, while 1,3-diphosphoglyceric acid (1,3-DPG) and 2,3-diphosphoglyceric acid (2,3-DPG), glycerol-3-phosphate and phosphoenolpyruvate (PEP) were significantly higher in insufficient responders versus sufficient responders (Table S1 and Figure 1).

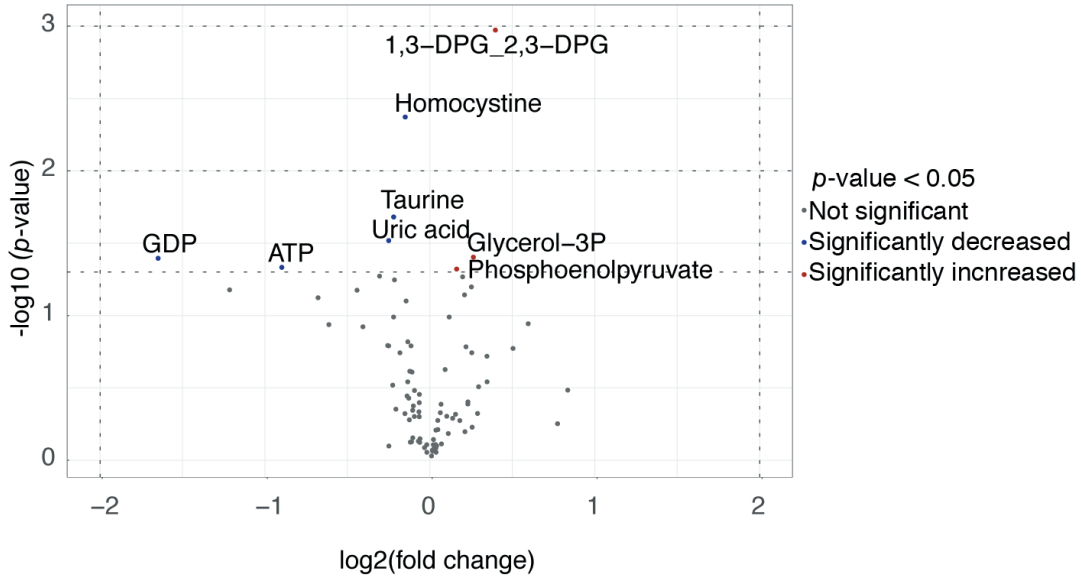


Figure 1. Volcano plot of significantly different metabolites in insufficient responders (DAS28-ESR > 3.2) and sufficient responders (DAS28-ESR ≤ 3.2).

From the significantly different metabolites, GDP had the largest log2 fold change (1.647) as depicted in a volcano plot (Figure 1). No significant differences were observed after correction for multiple testing.

It should be noted that, in this study, we aimed to find a new biomarker for response and not another surrogate marker for inflammation, such as the erythrocyte-sedimentation rate (ESR) or C-reactive protein (CRP). To examine whether the most promising candidate metabolites were independent of inflammation, we examined their correlation with ESR and CRP (Figure 2).

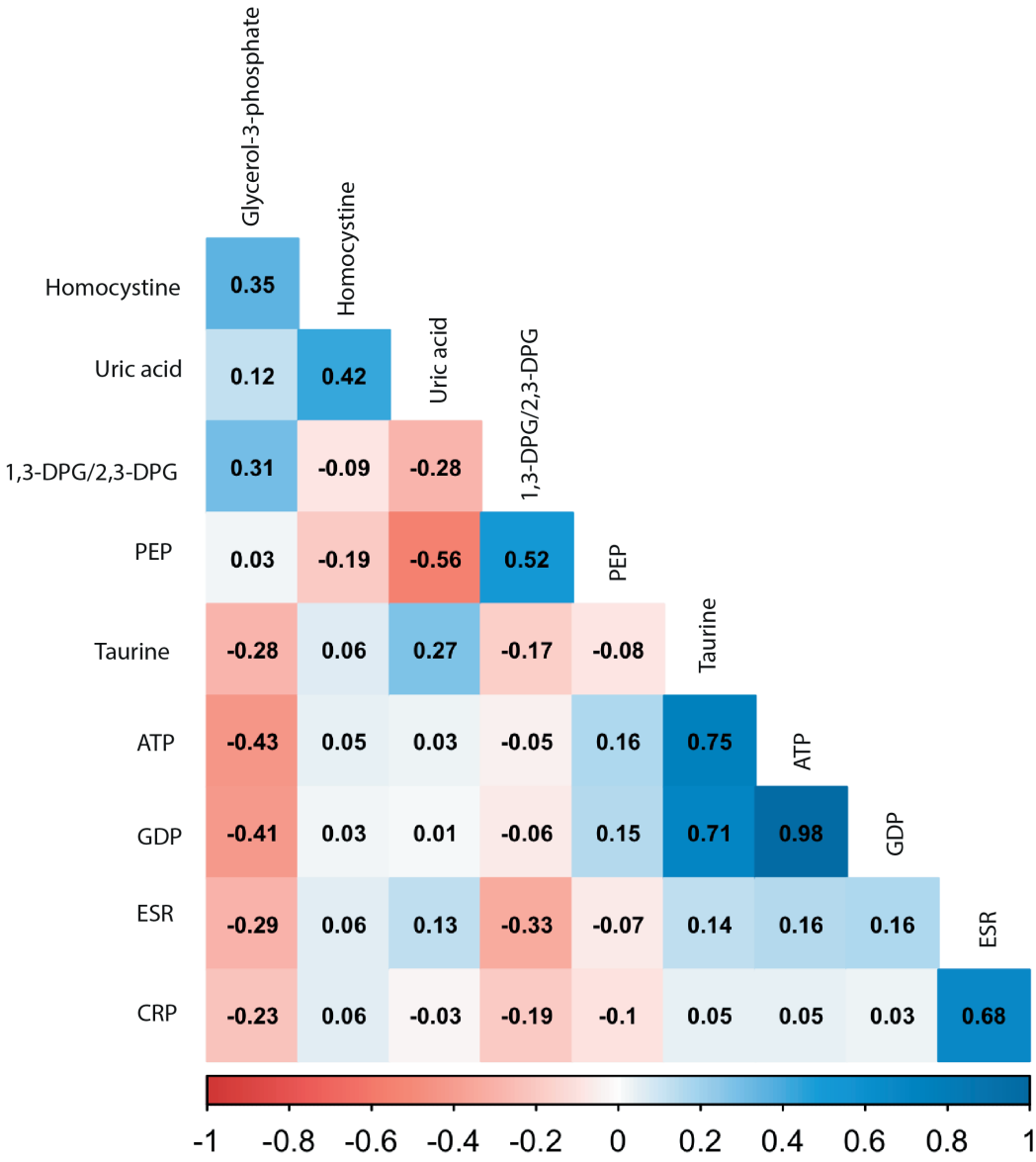


Figure 2. Correlation matrix between significantly different metabolites at baseline and inflammatory factors. Included metabolites shown were significantly different in relation to response at 3 months according to results of a Welch's *t*-test. The color indicates the strength of the correlation: dark red indicates a strong negative correlation and dark blue a strong positive correlation. The Pearson's correlation coefficient is printed in the squares. Erythrocyte-sedimentation rate (ESR) and C-reactive protein (CRP) were added as a proxy for inflammation.

All correlations with ESR and CRP were weak (Pearson's correlation coefficient r < 0.33), indicating that these metabolites do not reflect inflammation. The most significant metabolites were analyzed together in a multivariable logistic regression model to assess their performance as biomarkers in predicting insufficient response to MTX, including: homocystine, PEP, glycerol-3-phosphate, 1,3-DPG/2,3-DPG, uric acid and taurine. ATP and GDP concentrations were also significantly different between response groups; however, these were highly correlated with taurine (Figure 2). Of this model, a receiver-operating characteristic (ROC) plot was constructed with an area under the curve (AUC) of 0.82 (95% CI: 0.73–0.91). From all predictors in the model, only homocystine ($p = 0.007$) and glycerol-3-phosphate ($p = 0.020$) were significant independent predictors, while 1,3-/2,3-DPG was borderline significant ($p = 0.080$), for which reason we reduced the model to these three predictors. Using the combination of these predictors, a new ROC curve was constructed with an AUC of 0.81 (95% CI: 0.72–0.91; Figure 3) and corresponding sensitivity of 0.78 and specificity of 0.76.

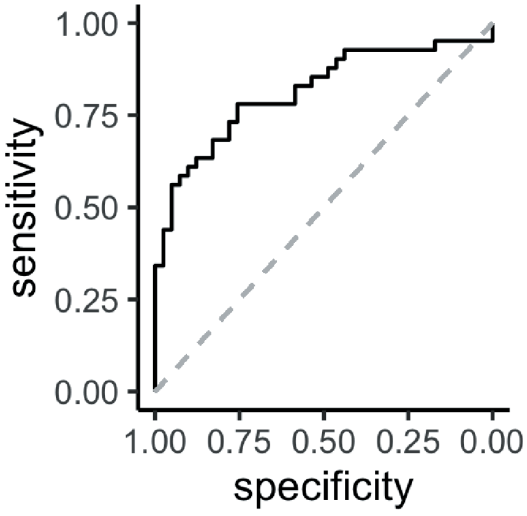


Figure 3. Receiver operating characteristic (ROC) curve (black solid line) of prediction of insufficient response (DAS28-ESR > 3.2) including significantly different metabolites in relation to response at 3 months. Predictors included in the model were: baseline homocystine, glycerol-3-phosphate and 1,3-diphosphoglyceric acid/2,3-diphosphoglyceric acid. The grey dotted line represents "the line of no discrimination".

Random Forest Analysis

Additionally, non-linear relationships between metabolites and response were tested using a random forest analysis. Variable importance was determined according to the decrease in accuracy and Gini score upon removal of variables from the models tested. The most significant variables again were homocystine and 1,3-DPG/2,3-DPG (Figure 4).

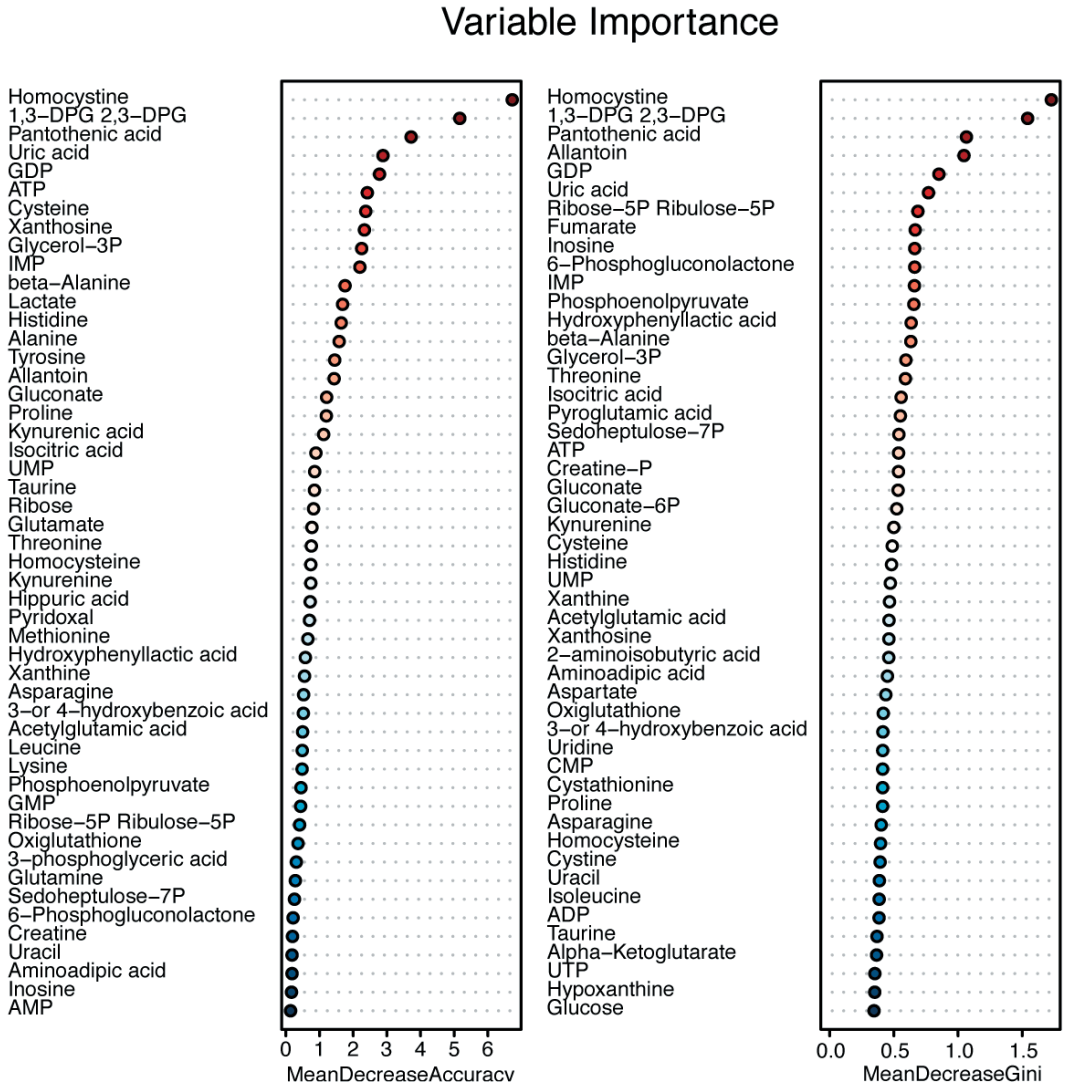


Figure 4. Variable importance plot from random forest analysis. Variable importance was determined using the mean decrease in accuracy and mean decrease in Gini score upon removal of the variable.

Enrichment Analysis

Finally, to examine whether certain cellular processes were overrepresented in insufficient versus sufficient responders, we performed an overrepresentation analysis (ORA). Metabolites with a VIP score >1 (Table S1) were included in the analysis. The most significantly enriched metabolic events were related to cellular respiration: Warburg effect ($FDR_{padjust} = 5.59 \times 10^{-5}$), gluconeogenesis ($FDR_{padjust} = 1.38 \times 10^{-4}$), glycolysis ($FDR_{padjust} = 5.69 \times 10^{-4}$), lactose synthesis ($FDR_{padjust} = 8.22 \times 10^{-4}$), pentose phosphate pathway ($FDR_{padjust} = 8.22 \times 10^{-4}$), urea cycle ($FDR_{padjust} = 8.22 \times 10^{-4}$) and to amino acid metabolism (Figure 5 and Supplementary Table S2).

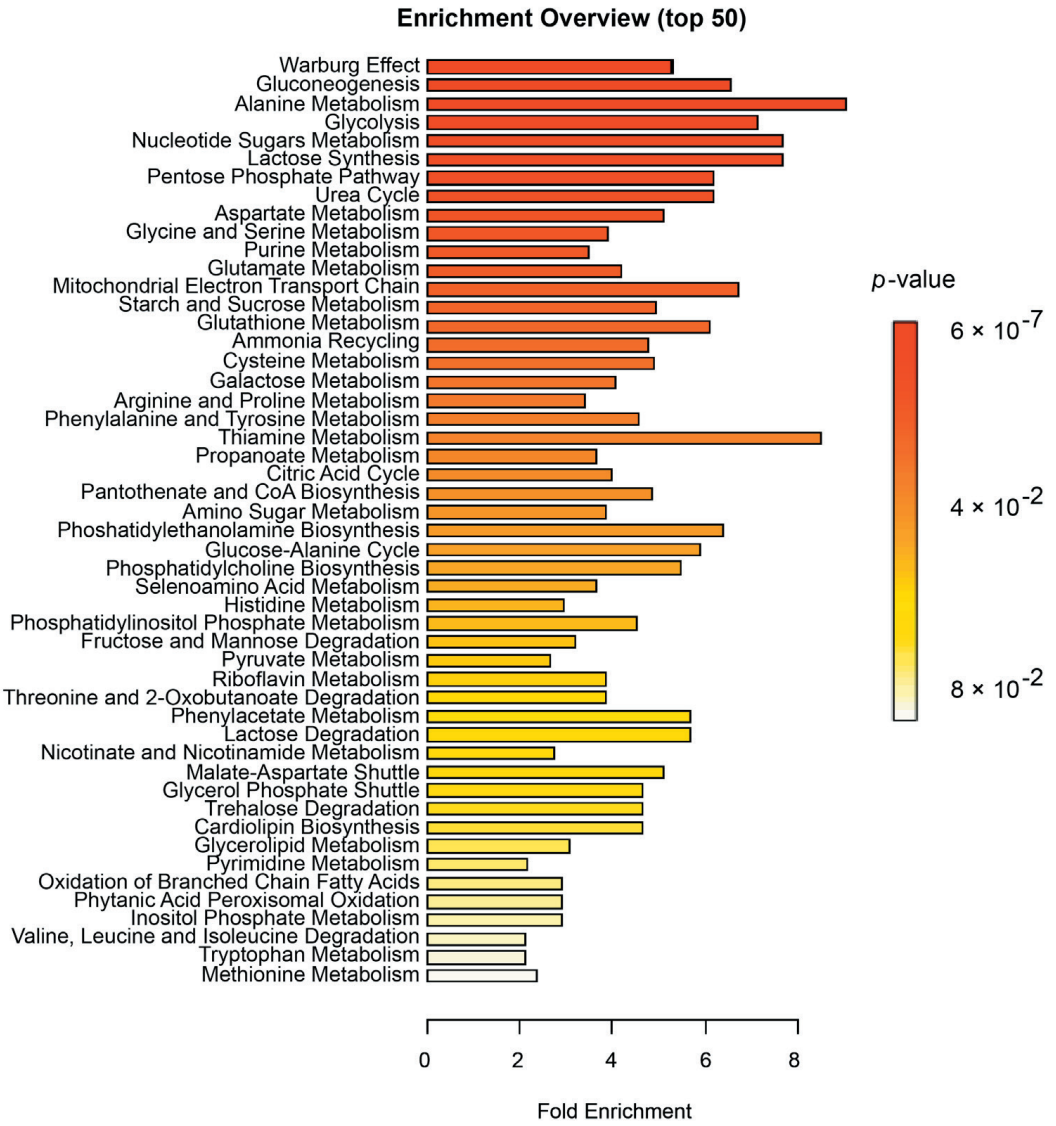


Figure 5. Results of overrepresentation analysis (ORA) between insufficient and sufficient responders to MTX. Summary of overrepresentation analysis results at baseline in relation to response to MTX at 3 months. The X-axis shows the fold enrichment between response groups and the color indicates the significance level, where red is most significant. *p*-values < 0.05 were considered significant. For details on the number of metabolites per pathway, see Supplementary Table S2.

DISCUSSION

In this study, we examined metabolite profiles prior to treatment initiation in early RA patients to identify potential biomarkers for response to MTX. At baseline, significantly different concentrations were observed between insufficient responders and sufficient responders in eight metabolites. Homocystine, taurine, ATP, GDP and uric acid concentrations were significantly lower in insufficient responders, while glycolytic intermediates 1,3-DPG/2,3-

DPG, glycerol-3-phosphate and phosphoenolpyruvate (PEP) were significantly higher in sufficient responders. The most promising biomarkers, homocystine, glycerol-3-phosphate and 1,3-DPG/2,3-DPG, together constructed a ROC with high AUC of 0.81 (95% CI: 0.72–0.91) and sensitivity of 78% and specificity of 76%. Furthermore, overrepresentation analysis indicated that metabolic processes related to cellular respiration and amino acid metabolism at baseline were potentially associated with treatment response, which might be interesting pathways to further explore in MTX-based therapies for RA.

In this study, lower baseline plasma levels of uric acid and taurine were related to insufficient response to MTX. Uric acid concentrations should be interpreted with caution in this study, as the analytical variation for this metabolite exceeded 25%. Uric acid was also previously quantified in 226 patients receiving MTX in the tREACH dataset, measured using a routine chemistry method on a Roche Cobas 8000 system (Roche, Almere, Netherlands) [10]. In this set, uric acid was borderline insignificant in a crude logistic regression model (OR = 0.04, 95% CI: 0.00–1.66, $p = 0.09$) and when adjusted for baseline DAS28 (OR = 0.02, 95% CI: 0.00–1.16, $p = 0.06$). Although not significant, the effect sizes pointed in the same direction as findings in the current study, suggesting that uric acid might play a role in response to MTX. This result is also in agreement with a study by Wang et al., who assessed 38 early RA patients on MTX monotherapy (13 insufficient responders versus 25 sufficient responders) at baseline and at 24 weeks [31].

The same trend was observed for taurine in the present study and the one by Wang et al. [31]. Interestingly, for taurine, the opposite was observed in serum samples of established RA patients, where taurine levels were lower in sufficient responders prior to TNFα inhibitor initiation after insufficient response to DMARD therapy [20]. Although these studies support taurine as a potential biomarker to choose between therapies, it has to be considered that the latter study was performed in a group of established RA patients from whom it was not clear what the effect of previous DMARD use was on the metabolite concentrations. In the same study [20], glycerol-3-phosphate was lower in sufficient responders at the start of TNFα inhibitor initiation, which is consistent with our findings that glycerol-3-phosphate was higher in insufficient responders at the start of MTX combination therapy, suggesting that insufficient responders to MTX with low glycerol-3-phosphate may be insufficient responders to TNFα inhibitors as well. Sasaki and colleagues [19] also observed higher glycerol-3-phosphate levels in the plasma of RA patients versus non RA controls; however, they did not observe differences in relation to response to MTX and/or corticosteroid therapy. This may be due to the small group sizes of patients receiving MTX ($n = 27$ sufficient responders versus $n = 12$ insufficient responders). Plasma amino acid metabolites that were previously described in relation to DAS28 by Smolenska et al. [17], such as threonine, tryptophan (positive correlation) and histidine and phenylalanine (negative correlation), could also separate insufficient and sufficient responders in our study (Figure 4). However, we did note that the Gini score was largely unaltered upon removal of threonine, tryptophan, histidine and phenylalanine

compared to other metabolites in the variable importance plots (e.g., homocystine and 1,3-DPG; Figure 4). This means that threonine, tryptophan, histidine and phenylalanine were less important in discriminating insufficient responders compared to metabolites ranked higher in the variable importance plots. However, the intercorrelation between metabolites can influence their contribution to the model and their ranking in Figure 4. This may, for instance, apply to taurine, which seems to have only minor importance in the random forest analysis but was significantly different between response groups at baseline (0.021) and had a VIP score of (1.607; *Ta*ble S1). However, taurine is highly correlated to GDP and ATP (Figure 2); hence, the inclusion of GDP and ATP in the model in the random forest analysis made taurine redundant in this case (Figure 4).

From the most successful, 1,3-DPG/2,3-DPG has not been previously described in relation to response to MTX treatment. Homocystine consists of two homocysteine molecules connected by a disulfide bond [32]. Previous studies showed that homocysteine concentrations increase upon MTX treatment in RA, while concentrations are reduced again by supplementation with folic acid [33,34], which is prescribed to RA patients to avoid adverse events. Total homocysteine is quantified as a mixture of all bound and unbound homocysteine molecules, including homocystine, which is first reduced to free homocysteine components. Higher total homocysteine levels could therefore be influenced by higher homocystine levels. Total homocysteine was also previously quantified in the plasma samples of 285 individuals from the tREACH study [23]; however, no relation was observed between homocysteine and response to MTX. Moreover, homocystine from the current metabolomic study and previously observed total homocysteine levels in the same individuals did not correlate ($R = 0.03$, $p = 0.77$). The precise role of homocystine in relation to response to MTX warrants further investigation.

Under normal physiological circumstances, phosphorylated metabolites are usually maintained intracellularly. There could be several reasons that phosphorylated metabolites were identified in plasma samples analyzed in this study. Inflammatory/oxidative stress conditions related to the pathogenesis of RA have been reported to trigger the extracellular release of lactate, ATP, ADP and AMP [14]. These extracellular adenine nucleotides represent a potential pro-inflammatory metabolite during the early stages of RA [35]. However, ectophosphatases CD73 and CD39 on immune-competent cells, or alkaline phosphatase, can convert extracellular ATP, ADP and AMP into adenosine, which acts as an anti-inflammatory regulator via interaction with adenosine receptors on leukocytes [36,37]. Accordingly, low CD39 expression on regulatory T-cells has been identified as a biomarker for MTX resistance in RA [38,39].

Furthermore, parallel changes in glycerol-3-phosphate, 2,3-DPG and PEP in good and poor responders point to alterations in glycolysis at the level of the regulatory enzyme pyruvate kinase (PK). In fact, RBC enzymopathies due to PK deficiency are characterized by increased levels of glycerol-3-phosphate, 2,3-DGP and PEP (and low ATP/GTP) [40,41] whereas enzymopathies due to hyperactive PK activity feature marked downregulation

of the three glycolytic intermediates (but high ATP/GTP) [42].

To better understand the biological relevance of our findings, an overrepresentation analysis was performed, of which the results should be considered as exploratory given that solely metabolites with VIP > 1 were included and not all metabolites were significantly different at baseline. From this perspective, results from the overrepresentation analysis showed that differences in baseline metabolites in relation to MTX response were primarily involved in the Warburg effect and glycolysis. These findings are consistent with recent studies in the field of "immunometabolism", describing alternate metabolic signatures during the activation of immune cells and autoimmune pathogenesis [15,43]. Especially the Warburg effect, describing a shift towards inefficient energy production through aerobic glycolysis, and well recognized for its impact on drug response in cancer cells [44], has been extensively described in RA patients, as well as the upregulation of glycolysis [45,46,47,48,49]. As these processes have been associated with a proinflammatory state, targeting the Warburg effect or glycolysis has been suggested as a potential RA therapy [50,51,52,53]. However, these processes have, to date, not been linked to the response to existing therapies in RA. The results of our study suggest that there may be a subgroup within early RA patients prior to treatment in which the Warburg effect and enhanced glycolysis could play a role in relation to response to MTX combination therapy. Moreover, MTX is a metabolite inhibitor itself, with primary targets in the folate/one-carbon metabolism pathway (e.g., dihydrofolate reductase (DHFR), thymidylate synthase (TYMS) and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC)), which have many downstream effects, varying per immune cell type. For instance, as reviewed by Cronstein and Aune [5], MTX indirectly inhibits NF-κB activity in T-cells through the induction of long intergenic non-coding RNA p21 (lncRNA-p21). Interestingly, lncRNA-p21 also promotes HIF1-α upregulation under hypoxic circumstances, which regulates the Warburg effect [54]. This might be an interesting link between response to MTX and the Warburg effect that deserves further investigation.

Strengths of this study were that it consisted of two equal groups with extremes in responses to MTX, which allowed us to identify the largest differences between response groups. Secondly, we used an untargeted approach, which led to new insights into possible metabolic biomarkers and pathways involved in the response to MTX. Furthermore, the study was performed on blood plasma samples, which are easily accessible for routine biomarker purposes. Limitations to this study were that it was performed using a semi-quantitative assay, meaning that metabolite concentrations cannot be directly compared with measurements by other methods and in other studies, but only between response groups in the same study. Moreover, our sample size was limited; thus, validation using other methods is warranted. Finally, correlations between top findings and BMI were low (Pearson's $r < 0.3$), and due to the low number of patients per group, we did not take into consideration other factors such as comorbidity, food intake and lifestyle factors, such as smoking, which may have influenced metabolic profiles [55].

For future studies, it would also be interesting to examine metabolite samples longitudinally. As a predictor for response, baseline samples are most suitable, as treatment adjustments can be made from the start of treatment initiation when appropriate. However, to obtain a better understanding of MTX's mechanism(s) of action and, in particular, its effect on metabolic processes, it would be interesting to follow metabolites longitudinally before and after MTX in relation to treatment response. This approach may reveal certain biomarkers that could possibly also serve as early markers for response during the first few months of treatment. A decrease in uric acid, for instance, has been observed in good responders to MTX in RA patients [56]. This, together with our results demonstrating that lower uric acid levels in insufficient responders were observed at baseline, could indicate that MTX acts better when certain pathways are upregulated prior to treatment. Nevertheless, both results first require validation.

Up to now, many other baseline variables have been assessed in relation to MTX response without much success, as previously reviewed [5,9,57,58]. Ideally, biomarkers should be combined in prediction models, including clinical, laboratory and lifestyle parameters [11,59,60]. Conceivably, metabolomic biomarkers for MTX response could be used as standalone or in addition to such a prediction model to identify insufficient responders prior to treatment and enabling prescription of step-up treatment from the start.

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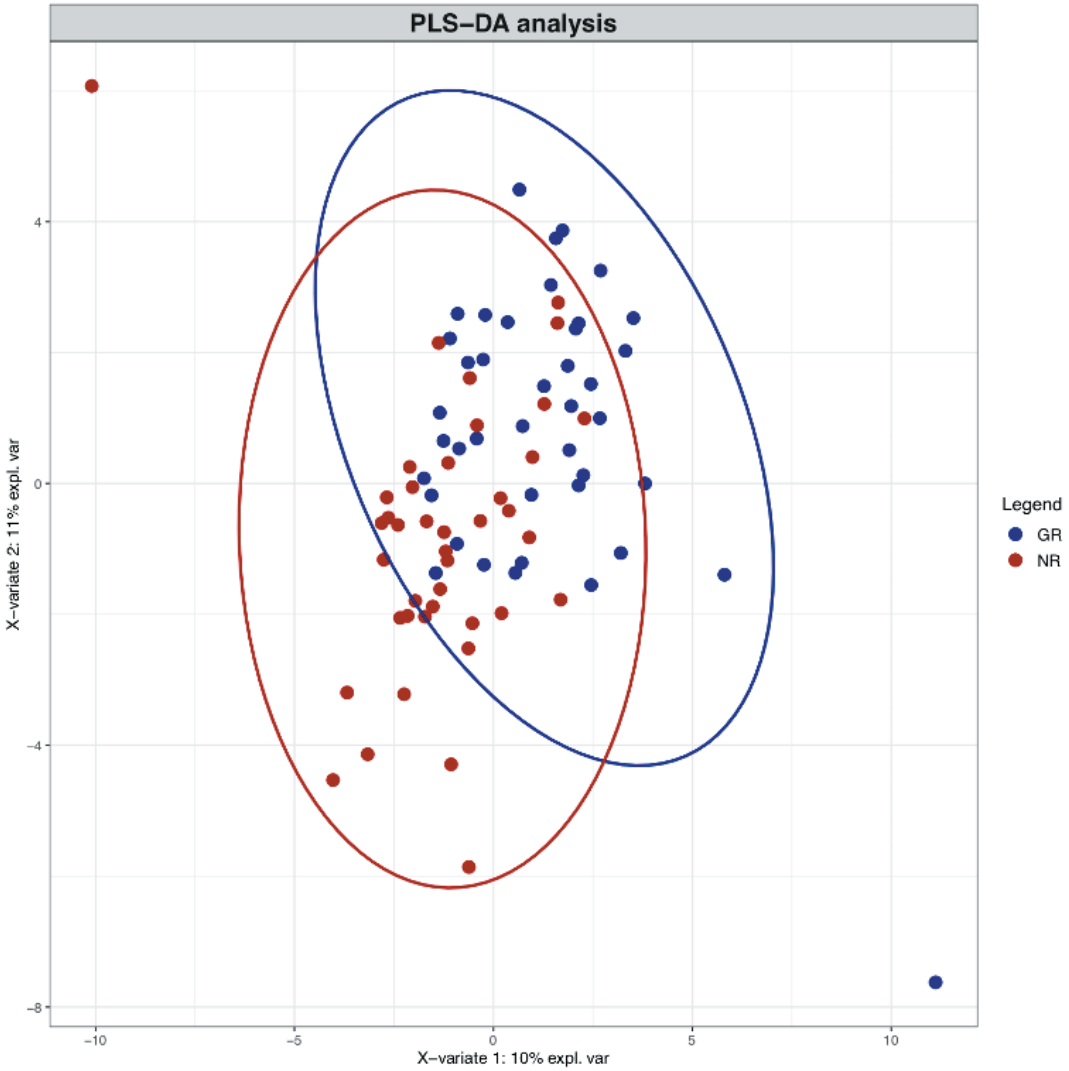
SUPPLEMENATRY FILES



Supplementary Figure S1.

Table S1. Results of overrepresentation analysis at baseline in relation to response to MTX at 3 months in early RA patients.

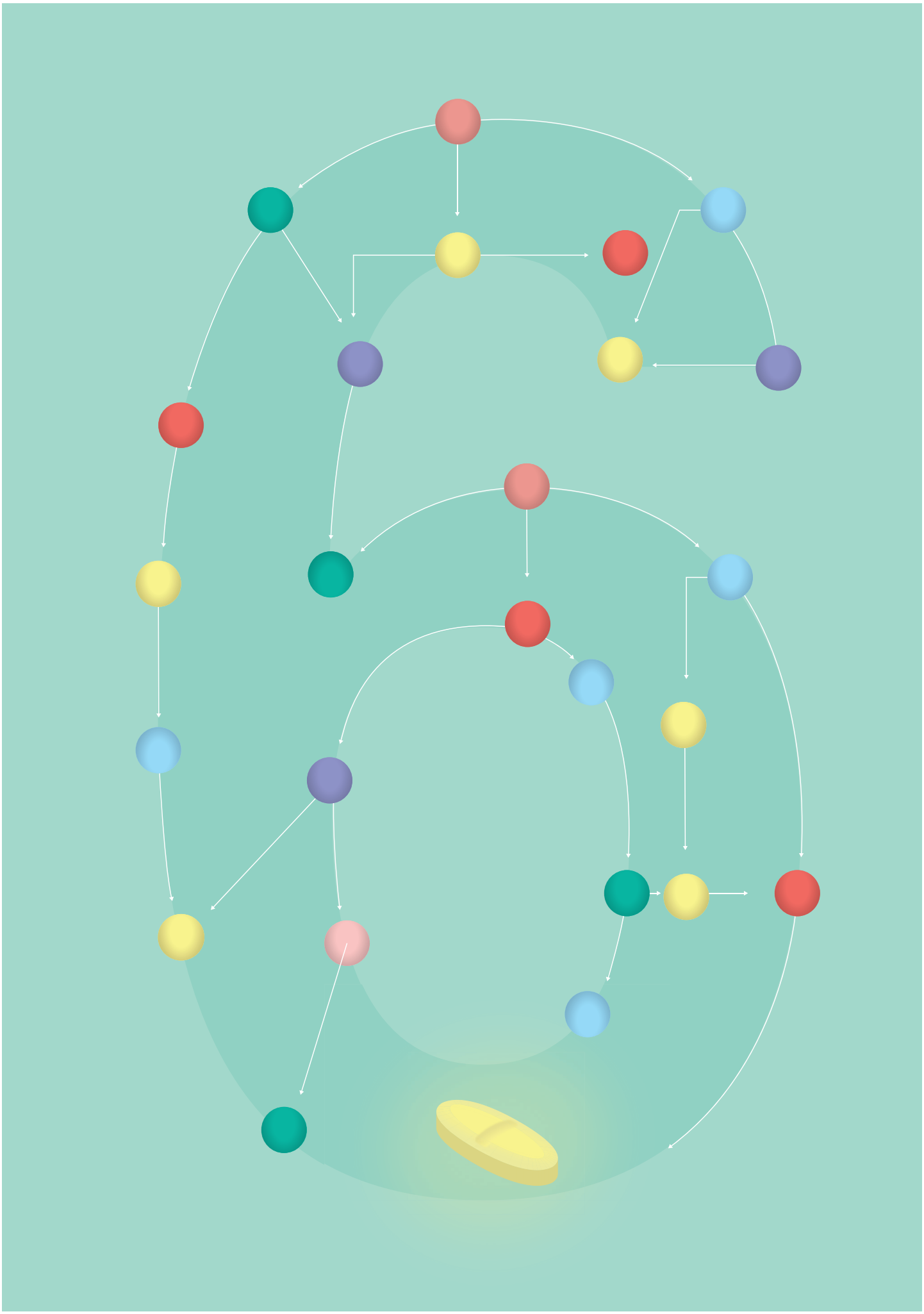
	Total	Exp.	Hits	Raw p	Holm p	FDR
Warburg Effect	58	2.27	12	5.71E-07	5.59E-05	5.59E-05
Gluconeogenesis	35	1.37	9	2.82E-06	2.74E-04	1.38E-04
Alanine Metabolism	17	0.66	6	2.19E-05	2.10E-03	5.69E-04
Glycolysis	25	0.98	7	2.32E-05	2.21E-03	5.69E-04
Nucleotide Sugars Metabolism	20	0.78	6	6.27E-05	5.90E-03	8.22E-04
Lactose Synthesis	20	0.78	6	6.27E-05	5.90E-03	8.22E-04
Pentose Phosphate Pathway	29	1.13	7	6.71E-05	6.17E-03	8.22E-04
Urea Cycle	29	1.13	7	6.71E-05	6.17E-03	8.22E-04
Aspartate Metabolism	35	1.37	7	2.43E-04	2.19E-02	2.49E-03
Glycine and Serine Metabolism	59	2.30	9	2.54E-04	2.26E-02	2.49E-03
Purine Metabolism	74	2.89	10	3.07E-04	2.70E-02	2.73E-03
Glutamate Metabolism	49	1.91	8	3.67E-04	3.19E-02	3.00E-03
Mitochondrial Electron Transport Chain	19	0.74	5	5.48E-04	4.72E-02	4.13E-03
Starch and Sucrose Metabolism	31	1.21	6	8.65E-04	7.35E-02	5.92E-03
Glutathione Metabolism	21	0.82	5	9.06E-04	7.61E-02	5.92E-03
Ammonia Recycling	32	1.25	6	1.03E-03	8.58E-02	6.33E-03
Cysteine Metabolism	26	1.02	5	2.53E-03	2.08E-01	1.44E-02
Galactose Metabolism	38	1.48	6	2.64E-03	2.14E-01	1.44E-02
Arginine and Proline Metabolism	53	2.07	7	3.29E-03	2.63E-01	1.70E-02
Phenylalanine and Tyrosine Metabolism	28	1.09	5	3.57E-03	2.82E-01	1.75E-02
Thiamine Metabolism	9	0.35	3	3.94E-03	3.08E-01	1.84E-02
Propanoate Metabolism	42	1.64	6	4.47E-03	3.44E-01	1.99E-02
Citric Acid Cycle	32	1.25	5	6.52E-03	4.95E-01	2.78E-02
Pantothenate and CoA Biosynthesis	21	0.82	4	7.39E-03	5.55E-01	2.92E-02
Amino Sugar Metabolism	33	1.29	5	7.46E-03	5.55E-01	2.92E-02
Phosphatidylethanolamine Biosynthesis	12	0.47	3	9.52E-03	6.95E-01	3.59E-02
Glucose-Alanine Cycle	13	0.51	3	1.20E-02	8.67E-01	4.37E-02
Phosphatidylcholine Biosynthesis	14	0.55	3	1.49E-02	1.00E+00	5.22E-02
Selenoamino Acid Metabolism	28	1.09	4	2.07E-02	1.00E+00	7.01E-02
Histidine Metabolism	43	1.68	5	2.27E-02	1.00E+00	7.40E-02
Phosphatidylinositol Phosphate Metabolism	17	0.66	3	2.57E-02	1.00E+00	8.12E-02
Fructose and Mannose Degradation	32	1.25	4	3.25E-02	1.00E+00	9.96E-02
Pyruvate Metabolism	48	1.88	5	3.49E-02	1.00E+00	1.04E-01
Riboflavin Metabolism	20	0.78	3	3.97E-02	1.00E+00	1.11E-01
Threonine and 2-Oxobutanoate Degradation	20	0.78	3	3.97E-02	1.00E+00	1.11E-01
Phenylacetate Metabolism	9	0.35	2	4.50E-02	1.00E+00	1.19E-01

Table S1. Continued

	Total	Exp.	Hits	Raw p	Holm p	FDR
Lactose Degradation	9	0.35	2	4.50E-02	1.00E+00	1.19E-01
Nicotinate and Nicotinamide Metabolism	37	1.45	4	5.19E-02	1.00E+00	1.34E-01
Malate-Aspartate Shuttle	10	0.39	2	5.49E-02	1.00E+00	1.38E-01
Glycerol Phosphate Shuttle	11	0.43	2	6.55E-02	1.00E+00	1.53E-01
Trehalose Degradation	11	0.43	2	6.55E-02	1.00E+00	1.53E-01
Cardiolipin Biosynthesis	11	0.43	2	6.55E-02	1.00E+00	1.53E-01
Glycerolipid Metabolism	25	0.98	3	7.00E-02	1.00E+00	1.58E-01
Pyrimidine Metabolism	59	2.30	5	7.44E-02	1.00E+00	1.58E-01
Oxidation of Branched Chain Fatty Acids	26	1.02	3	7.70E-02	1.00E+00	1.58E-01
Phytanic Acid Peroxisomal Oxidation	26	1.02	3	7.70E-02	1.00E+00	1.58E-01
Inositol Phosphate Metabolism	26	1.02	3	7.70E-02	1.00E+00	1.58E-01
Valine, Leucine and Isoleucine Degradation	60	2.34	5	7.88E-02	1.00E+00	1.58E-01
Tryptophan Metabolism	60	2.34	5	7.88E-02	1.00E+00	1.58E-01
Methionine Metabolism	43	1.68	4	8.20E-02	1.00E+00	1.61E-01
Folate Metabolism	29	1.13	3	9.99E-02	1.00E+00	1.92E-01
Inositol Metabolism	33	1.29	3	1.34E-01	1.00E+00	2.53E-01
Beta Oxidation of Very Long Chain Fatty Acids	17	0.66	2	1.40E-01	1.00E+00	2.59E-01
Beta-Alanine Metabolism	34	1.33	3	1.43E-01	1.00E+00	2.60E-01
Methylhistidine Metabolism	4	0.16	1	1.48E-01	1.00E+00	2.63E-01
Butyrate Metabolism	19	0.74	2	1.68E-01	1.00E+00	2.88E-01
Ethanol Degradation	19	0.74	2	1.68E-01	1.00E+00	2.88E-01
Sulfate/Sulfite Metabolism	22	0.86	2	2.11E-01	1.00E+00	3.50E-01
Transfer of Acetyl Groups into Mitochondria	22	0.86	2	2.11E-01	1.00E+00	3.50E-01
Biotin Metabolism	8	0.31	1	2.74E-01	1.00E+00	4.47E-01
Mitochondrial Beta-Oxidation of Short Chain Saturated Fatty Acids	27	1.05	2	2.85E-01	1.00E+00	4.50E-01
Mitochondrial Beta-Oxidation of Medium Chain Saturated Fatty Acids	27	1.05	2	2.85E-01	1.00E+00	4.50E-01
Mitochondrial Beta-Oxidation of Long Chain Saturated Fatty Acids	28	1.09	2	3.00E-01	1.00E+00	4.56E-01
Homocysteine Degradation	9	0.35	1	3.02E-01	1.00E+00	4.56E-01
De Novo Triacylglycerol Biosynthesis	9	0.35	1	3.02E-01	1.00E+00	4.56E-01
Lysine Degradation	30	1.17	2	3.29E-01	1.00E+00	4.89E-01
Taurine and Hypotaurine Metabolism	12	0.47	1	3.82E-01	1.00E+00	5.58E-01
Sphingolipid Metabolism	40	1.56	2	4.70E-01	1.00E+00	6.72E-01
Bile Acid Biosynthesis	65	2.54	3	4.73E-01	1.00E+00	6.72E-01
Fatty acid Metabolism	43	1.68	2	5.09E-01	1.00E+00	7.11E-01
Spermidine and Spermine Biosynthesis	18	0.70	1	5.15E-01	1.00E+00	7.11E-01

Tyrosine Metabolism	72	2.81	3	5.44E-01	1.00E+00	7.41E-01
Steroid Biosynthesis	48	1.88	2	5.70E-01	1.00E+00	7.56E-01
Betaine Metabolism	21	0.82	1	5.71E-01	1.00E+00	7.56E-01
Carnitine Synthesis	22	0.86	1	5.88E-01	1.00E+00	7.68E-01
Plasmalogen Synthesis	26	1.02	1	6.50E-01	1.00E+00	8.38E-01
Phospholipid Biosynthesis	29	1.13	1	6.90E-01	1.00E+00	8.78E-01
Arachidonic Acid Metabolism	69	2.70	2	7.67E-01	1.00E+00	9.64E-01

Compound names of metabolites with VIP score >1 were used as input. The small molecule pathway database was selected as reference library. Hits= number of metabolites that were enriched in the pathway. Expected is number of hits expected in the pathway due to chance. FDR=False Discovery Rate. FDR- adjusted p-values <0.05 were considered significant.



Chapter 6

Validation of a Prognostic Multivariable Prediction Model for Insufficient Clinical Response to Methotrexate in Early Rheumatoid Arthritis and Its Clinical Application in Evidencio

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Johanna M. W. Hazes and R. de Jonge equally contributed to this manuscript as senior authors

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ABSTRACT

Introduction: Methotrexate (MTX) constitutes the first-line therapy in rheumatoid arthritis (RA), yet approximately 30% of the patients do not benefit from MTX. Recently, we reported a prognostic multivariable prediction model for insufficient clinical response to MTX at 3 months of treatment in the treatment in the Rotterdam Early Arthritis Cohort (tREACH), including baseline predictors: Disease activity score 28 (DAS28), Health Assessment Questionnaire (HAQ), erythrocyte folate, single-nucleotide polymorphisms (SNPs; ABCB1, ABCC3), smoking, and BMI. The purpose of the current study was (1) to externally validate the model and (2) to enhance the model's clinical applicability.

Methods: Erythrocyte folate and SNPs were assessed in 91 early disease-modifying antirheumatic drug (DMARD)-naïve RA patients starting MTX in the external validation cohort (U-Act-Early). Insufficient response (DAS28 > 3.2) was determined after 3 months and non-response after 6 months of therapy. The previously developed prediction model was considered successfully validated in the U-Act-Early (validation cohort) if the area under the curve (AUC) of the receiver operating characteristic (ROC) was not significantly lower than in the tREACH (derivation cohort).

Results: The AUCs in U-Act-Early at three and 6 months were 0.75 (95% CI 0.64–0.85) and 0.71 (95% CI 0.60–0.82) respectively, similar to the tREACH. Baseline DAS28 > 5.1 and HAQ > 0.6 were the strongest predictors. The model was simplified by excluding the SNPs, while still classifying 73% correctly. Furthermore, interaction terms between BMI and HAQ and BMI and erythrocyte folate significantly improved the model increasing correct classification to 75%. Results were successfully implemented in Evidencio online platform assisting clinicians in shared decision-making to intensify treatment when appropriate.

Conclusions: We successfully externally validated our recently reported prediction model for MTX non-response and enhanced its clinical application thus enabling its evaluation in a clinical trial.

INTRODUCTION

Methotrexate (MTX) is the first-line therapy in rheumatoid arthritis (RA) [*1*]. Although efficacious in a large proportion of patients, MTX is poorly effective in approximately 30% of early RA patients [2,3]. Patients on MTX who do not show improvement at 3 months (insufficient responders) or do not reach the treatment target of low disease activity/remission at 6 months (non-responders) are switched to biologic disease-modifying anti-rheumatoid drug (bDMARD) therapies or novel targeted synthetic DMARD (tsDMARD) therapies, including, e.g., Janus kinase (JAK) inhibitors [1,4,5], with or without concomitant MTX treatment. To ensure that only patients unresponsive to MTX receive early (additional) treatment with b/tsDMARDs and those responsive to MTX are spared costly biologics or synthetic drugs, we and others have constructed models to predict MTX (non)-response [6,7,8,9]. Our prognostic multivariable prediction model for the prediction of insufficient response, defined as: disease activity score 28 (DAS28 > 3.2) at 3 months of MTX therapy, was constructed in the treatment in the Rotterdam Early Arthritis Cohort (tREACH) and included clinical predictors (DAS28 and Health Assessment Questionnaire [HAQ]), life-style predictors (smoking and BMI) and laboratory parameters involved in MTX metabolism (erythrocyte folate and single-nucleotide polymorphisms: SNPs) [9]. This model classified 80% of patients correctly (area under the curve [AUC] of the receiver operating characteristic [ROC]: 0.80 [95% CI 0.73–0.86]) and was externally validated in the MTX-Rotterdam cohort showing a similar prognostic performance (AUC 0.80 [95% CI 0.69–0.91]) even though BMI and smoking predictors were absent from this validation cohort [9]. The aim of the current study was to validate the complete prediction model, including BMI and smoking status predictors, in an external early RA cohort (U-Act-Early) from a different geographic region and to enhance the model's applicability in clinical practice [10].

METHODS

The methodology of this study followed transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD) guidelines [11].

Patients

The external validation cohort consisted of 91 patients from the U-Act-Early cohort, a multicenter, double-blind, placebo-controlled strategy trial, registered at ClinicalTrials.gov (number: NCT01034137) [10]. DMARD- and glucocorticoid (GC)-naïve early RA patients were eligible for inclusion once classified as RA patients according to the 1987 America College of Rheumatology (ACR) [12] ($n = 7$, 8%) or the 2010 ACR/EULAR classification criteria ($n = 84$, 92%) [13], and had a disease duration < 12 months and active disease at baseline (disease activity score 28; DAS28 ≥ 2.6). Patients were randomly assigned to a treatment strategy

with tocilizumab (TCZ) + placebo, MTX + placebo or their combination (TCZ + MTX) and treated to the target of sustained remission (i.e., a DAS28 < 2.6 and swollen joint count of ≤ 4 joints of the 28 joints assessed, during ≥ 24 weeks). All 91 patients included were derived from the initial MTX + placebo strategy arm. The starting MTX dose was 10 mg/week orally and increased stepwise 5 mg every 4 weeks up to 30 mg/week until remission or the maximum tolerable dose. During the trial, GC use was not permitted. The tREACH (n = 285) cohort was described earlier [9]. Importantly, in the tREACH, the optimal MTX dose of 25 mg/week was reached within 3 weeks (combined with other conventional synthetic (cs) DMARDs and/or GCs) and therapy was targeted to low disease activity (DAS28 ≤ 3.2) at 3 months. If this failed, step-up treatment with additional csDMARDs (sulfasalazine and/or hydroxychloroquine) or bDMARDs (i.e., TNF-alpha inhibitor) was initiated. In both cohorts, folic acid (10 mg/week) was prescribed during MTX treatment. This study was approved by the medical ethics committee of the University Medical Center Utrecht (ML22497) and the medical ethics committee of Erasmus Medical Center (MEC-2006-252). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained for all patients.

Outcome and Clinical Predictors

The primary outcome was insufficient MTX response after 3 months of treatment start, defined as DAS28 > 3.2, where DAS28 was based on the erythrocyte sedimentation rate (DAS28-ESR). All predictors were dichotomized prior to analyses. Cut-off values were defined previously in the tREACH cohort as: DAS28 > 5.1, HAQ > 0.6, erythrocyte folate < 750 nmol/l, current smoking, BMI > 25 kg/m^2, *ABCB1* rs1045642 (GG/GA vs. AA) genotype, and *ABCC3* rs4793665 (TC/CC vs. TT) genotype [9]. As erythrocyte-folate levels were slightly higher in U-Act-Early, new cut-off points were examined for erythrocyte-folate (deciles) and BMI (> 30 kg/m^2) in U-Act-Early and tested for improvement of the model. The secondary outcome measure was non-response to MTX after 6 months of treatment, defined as DAS28-ESR > 3.2.

Erythrocyte Folate and Genetic Variants

In U-Act-Early, erythrocyte folate and genetic variants included in the original prediction model [9] were determined from EDTA whole blood samples stored at −80 °C, as described elsewhere [14,15]. DNA was obtained from whole blood using a MagNAPure Compact (Roche Life Science, Almere, The Netherlands) and genotypes were determined for *ABCB1* rs1045642 and *ABCC3* rs4793665 using real-time PCR with Taqman, as described previously [15]. Samples were tested for deviation from the Hardy–Weinberg equilibrium (HWE). SNPs were determined in the same lab and according to the same protocols as the tREACH study [9]. Importantly, there were methodological differences in the measurement

of folate between the tREACH and U-Act-Early cohorts. The Elecsys® Folate III assay (Ref 7027290190; Roche Diagnostics) has been re-standardized since 2017 in accordance with the WHO International Standard NIBSC Code 03/178. This resulted in 10% lower erythrocyte folate levels (U-Act-Early) compared to those quantified using the previous assay (tREACH). Furthermore, serum folate levels, required for folate correction in whole blood, were not available in U-Act-Early. As serum folate levels take up only a small part of the total folate concentrations, whole blood folate levels were corrected for the average serum folate concentration in the tREACH (25 nmol/l).

Statistical Analysis

Clinical and laboratory parameters for the tREACH (derivation) and U-Act-Early (validation) cohorts were compared. The difference in DAS28 at 3 months (compared to baseline) was expressed as a mean with standard deviation (± SD) and assessed using a paired-sample *t* test. Differences between cohort variables were tested using an independent two-group *t* test, if the assumptions of normal distribution (visual inspection) and equal variances (tested using the Levene's test) were met. If these assumptions were not met, non-parametric Mann–Whitney *U* test was performed. Differences in proportions were tested using a two-sample proportion test. Due to missing informed consent at the start of this validation study, eight subjects from the tREACH (derivation) cohort were excluded from analyses in this study. This is why the model as described previously was first re-analyzed on the tREACH data excluding these eight subjects, resulting in negligible differences in effect sizes compared to previous study [9]. Next, to validate the prediction model in the external validation cohort (U-Act-Early), the predictors, DAS28 > 5.1, HAQ > 0.6, erythrocyte folate < 750 nmol/l, current smoking, BMI > 25 kg/m^2, *ABCB1* rs1045642 genotype and *ABCC3* rs4793665 genotype were entered into a multivariable logistic regression and the probability for insufficient response was calculated using the pROC package in R according to the following formula:

$$P\ insufficient\ response\ (\%) = \frac{e^{(\beta 0+\beta 1\cdot x1+\beta 2\cdot x2+\cdots+\beta n\cdot xn)}}{1+e^{(\beta 0+\beta 1\cdot x1+\beta 2\cdot x2+\cdots+\beta n\cdot xn)}}\ x\ 100,$$

where β_0 represents the constant, β, β_2, and β_n represent the regression coefficients for each of the predictors x_1, x_2, x_n. Subsequently, an ROC curve with AUC was constructed using the predicted probabilities and compared with results in the tREACH. The previously developed prediction model in tREACH (derivation) was considered successfully validated in the U-Act-Early (validation) if the area under the curve (AUC) of the receiver operating characteristic (ROC) was not significantly lower than in the tREACH. Goodness of fit between the predicted probabilities and observed values was tested using the Hosmer–Lemeshow test, where $p > 0.05$ indicated that a model fit the data well. All analyses were performed in R studio (Version: 3.5.3, "2019-03-11"). *P* values < 0.05 were considered statistically significant. Subjects with missing data ($N = 104$) were excluded from analyses (complete-case analysis).To enhance the model's clinical applicability and thus facilitate

its clinical implementation, we applied the prediction model on the combined dataset of tREACH and U-Act-Early ($N = 264$) using the step-up approach. Therefore, the model could be simplified using fewer predictors and possible two-way interactions could be examined in a combined cohort with more power. Statistically significant interactions ($p < 0.05$) were added to the model. To simplify the model, we assessed individual contribution of variables to the predictive power of the model by sequential addition of predictors. Model fits were compared using the likelihood ratio test. Probability for insufficient response was calculated for each patient as well as the corresponding specificity, positive predictive value (PPV), sensitivity, and negative predictive value (NPV) were determined, using the "pROC" package in R. The final prediction model was uploaded onto the online platform 'Evidencio' providing a tool for clinicians to decide whether to start MTX combination therapy.

RESULTS

Cohort Comparisons

In U-Act-Early (validation cohort), mean DAS28 decreased from 5.0 (± 1.1) to 3.6 (± 1.6) during the first 3 months ($p < 0.001$). Mean DAS28 in tREACH (derivation cohort) was 5.0 (± 1.1), which decreased to a mean DAS28 of 3.1 (± 1.2, $p < 0.001$). Baseline DAS28 in U-Act-Early was comparable to that of tREACH ($p = 0.613$; Table 1). In U-Act-Early, 58 patients (64%) were categorized as insufficient MTX responders (i.e., DAS28 > 3.2 at 3 months) compared to 114 (43%) in tREACH ($p = 0.006$). In U-Act-Early, 39 (44%) patients were classified as MTX non-responders (i.e., DAS28 > 3.2 at 6 months), which was not significantly different from 38% in tREACH (Table 1). Additionally, U-Act-Early consisted of significantly more rheumatoid factor positive patients (81%) compared to tREACH (65%) ($p = 0.007$; Table 1), whereas no significant differences were found for anti-citrullinated protein antibody (ACPA) positivity ($p = 0.214$; Table 1). Despite the lower folate levels due to (international) re-standardization of the method, the erythrocyte-folate levels were still significantly higher in U-Act-Early compared to tREACH ($p = 0.006$; Table 1) and genotype GG/GA for *ABCB1* was significantly more frequent in tREACH ($p = 0.016$; Table 1), while genotypes for *ABCC3* were similar between cohorts. Importantly, besides MTX, co-medication was prescribed in tREACH (derivation) but not in U-Act-Early (validation; Table 1).

Table 1. Descriptives of the derivation (tREACH) and external validation (U-Act-Early) cohorts.

Clinical parameters	tREACH Derivation cohort N=277	U-Act-Early Validation cohort N=91	P-value
DAS28>3.2 at 3 months	43%	64%	0.006**
DAS28>3.2 at 6 months	38%	44%	0.417
Gender, male	30%	36%	0.300
Age, mean ± SD	54 ± 14	53 ± 13	0.498
Baseline DAS28, mean ± SD	5.0 ± 1.1	5.0 ± 1.1	0.613
HAQ>0.6	76%	70%	0.330
Laboratory parameters			
Erythrocyte folate, median (IQR)#	862 (665 – 1163)	1020 (795 – 1221)	0.006**
ABCB1 GG/GA	73%	58%	0.016*
ABCC3 TC/CC	66%	67%	0.909
Rheumatoid factor positive	65%	81%	0.007**
ACPA positive	71%	79%	0.214
Life style parameters			
BMI, median (IQR)	25 (23 - 29)	25 (23 - 29)	0.950
Current smokers, N (%)	84 (33)	28 (31)	0.820
Co-medication			
Other DMARDs	56%	0%	<0.001***
Oral corticosteroids	58%	0%	<0.001***
Parental corticosteroids	28%	0%	<0.001***
Subcutaneous	0%	0%	

Missing values tREACH: DAS28 at 3 months N=13, DAS28 at 6 months, N= 28, erythrocyte folate N=78, rheumatoid factor N=35, BMI N=3, smoking status N=21, HAQ N=18, *ABCB1* N=21, *ABCC3* N=20. Missing values U-Act-Early: DAS28 at 6 months, N=2, rheumatoid factor N=1, ACPA status N=1. Percentages shown are of valid data points. *P<0.05 was considered significant, **P<0.01, ***P<0.001.

Expressed in nmol/L

Validation of Prediction Model at 3 Months

The model combining all predictors accomplished an AUC of 0.75 (95% CI 0.64–0.85) (Fig. 1) in U-Act-Early (validation), which means that 75% of the patients could be classified correctly. The strongest predictor for insufficient response to MTX in U-Act-Early was baseline DAS28 > 5.1 ($p = 0.008$; Table 2). Odds ratios (ORs) for the predictors DAS28, HAQ, erythrocyte folate, BMI and smoking in U-Act-Early (validation) were in the same direction as in the tREACH (derivation; Table 2). The OR of the *ABCC3* SNP was however in the opposite direction in U-Act-Early (OR = 0.6, 95% CI 0.23–1.79) compared to tREACH (OR = 3.1, 95% CI 1.39–6.94).

Table 2. Validation of multivariable logistic regression models for insufficient response to MTX (DAS28 >3.2) at 3 months of treatment in an external validation cohort (U-Act-Early).

	tREACH derivation cohort N= 173	**U-Act-Early validation cohort N= 91**
Predictors	**OR (95% CI)**	**OR (95% CI)**
DAS28 >5.1	3.7 (1.62 – 8.38)**	4.1 (1.44 – 11.82)**
HAQ >0.6	2.8 (1.15 – 7.00)*	2.1 (0.67 – 6.35)
ABCB1 GG/GA	2.4 (1.06 – 5.23)*	1.0 (0.37 – 2.75)
ABCC3 TC/CC	3.1 (1.39 – 6.94)**	0.6 (0.23 – 1.79)
Folate <750 nmol/L	2.1 (0.97 – 4.40)	3.4 (0.88 – 12.79)
Smoker	4.2 (1.91 – 9.42)**	1.3 (0.44 – 4.00)
BMI >25 kg/m^2	3.3 (1.52 – 7.21)**	1.6 (0.62 – 4.23)
AUC (95% CI)	0.81 (0.74 – 0.87)	0.75 (0.64 – 0.85)

Left column presents data from the derivation cohort (tREACH) and right of the external validation cohort (U-Act-Early). OR= odds ratio, CI= confidence interval. Predictors that contributed significantly to the model were indicated with an asterisk, where *P<0.05 and **P<0.01.

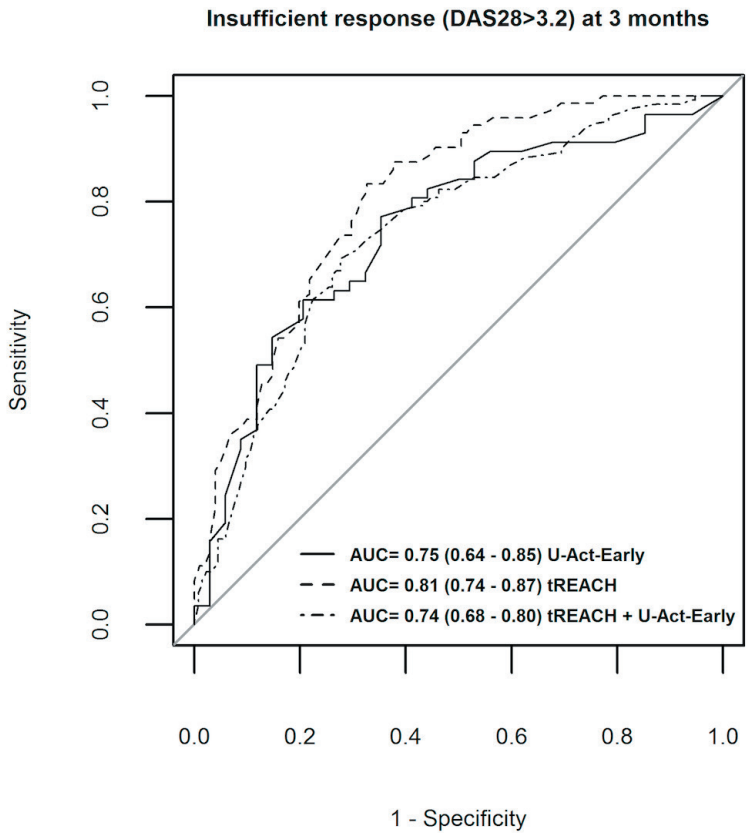

Figure 1. ROC curve for the prediction of insufficient response (DAS28 > 3.2) to MTX after 3 months of treatment. Area under the curve (AUC) is reported as follows: AUC (95% confidence interval). Predictors were: baseline DAS28 > 5.1, baseline HAQ > 0.6, *ABCB1* genotype, *ABCC3* genotype, baseline erythrocyte folate, BMI > 25 kg/m^2 and current smoking.

Similar results were found for the prediction of non-response at 6 months. An ROC curve was constructed with an AUC of 0.71 (95% CI 0.60–0.82; Supplementary Figure S1) in U-Act-Early (validation), which is comparable to the predictive value of the tREACH model (derivation) at 6 months (AUC 0.75, 95% CI 0.67–0.83; Supplementary Figure S1).

Enhancement of the Model's Clinical Applicability

Next, the U-Act-Early and tREACH cohort were combined to increase power and enhance the model's clinical applicability. In this combined cohort, the ORs for all predictors were greater than 1 and all predictors, except for the SNPs were significant (Supplementary Table S1). The combined model reached an AUC of 0.74 (95% CI 0.68–0.80; Supplementary Table S1 and Fig. 1) at 3 months.

Additionally, in this combined set, we investigated whether all predictors were required to reach 74% predictive power or whether the model could be further simplified. To do so, we analyzed changes in AUC upon sequential addition of predictors to the model. We started with the most readily available clinical predictors DAS28 > 5.1 and HAQ > 0.6, which generated an ROC with an AUC of 0.67 (95% CI 0.61–0.74; Table 3).

Table 3. Logistic model building in combined datasets: U-Act-Early + tREACH.

Model	Predictors	Log likelihood	Chisquare	P	AUC (95% CI)
1	DAS28 + HAQ	-168.68			0.67 (0.61 – 0.74)
2	DAS28 + HAQ + smoking	-165.52	6.32	0.01*	0.70 (0.64 – 0.76)
3	DAS28 + HAQ + smoking + BMI	-162.98	5.08	0.02*	0.72 (0.66 – 0.78)
4	DAS28 + HAQ + smoking + BMI + erythrocyte folate	-160.43	5.11	0.02*	0.73 (0.67 – 0.79)
5	DAS28 + HAQ + smoking + BMI + erythrocyte folate + *ABCC3*	-158.57	3.71	0.05	0.74 (0.68 – 0.80)
6	DAS28 + HAQ + smoking + BMI + erythrocyte folate + *ABCB1*	-160.15	0.57	0.45	0.74 (0.68 – 0.80)
7	DAS28 + HAQ + smoking + BMI + erythrocyte folate + *ABCC3* + *ABCB1*	-158.29	4.28	0.12	0.74 (0.68 – 0.80)

Each model was compared to the previous model. Model 6 and 7 were compared to model 4. *P-value<0.05 was considered significant. DAS28= DAS28 >5.1, HAQ = HAQ>0.6, smoking= current smoking, BMI= BMI >25kg/m^2, erythrocyte folate= erythrocyte folate <750 nmol/L, *ABCC3*= genotype TC or CC, *ABCB1*= genotype GG or GA.

Upon addition of smoking to the model, the AUC significantly increased ($p = 0.01$) to 0.70 (95% CI 0.64–0.76), followed by BMI, upon which the AUC further improved to 0.72 (95% CI 0.66–0.78, $p = 0.02$). Upon addition of erythrocyte folate to the model the AUC reached 0.73 (95% CI 0.67–0.79, $p = 0.02$). Addition of *ABCB1* and *ABCC3* genotypes did not significantly improve the model (AUC = 0.74, 95% CI 0.68–0.80, $p = 0.12$; Table 3). Hence, the model could be simplified to a model where SNP genotypes were excluded resulting in a model with predictive power of 73%.

To fine-tune the model, all two-way interaction terms between predictors were tested. An interaction term between HAQ and BMI (OR = 3.68 95% CI 1.07—13.14) significantly contributed to the model. This means that a BMI > 25 kg/m^2 was associated with worse disease activity when HAQ values were > 0.6. Furthermore, an interaction term between HAQ and erythrocyte folate (OR = 0.23, 95% CI 0.06–0.86) also significantly contributed to the model, indicating that low erythrocyte folate concentrations (< 750 nmol/l) significantly predicted insufficient response when HAQ values were < 0.6. Hence, interaction terms for HAQ and BMI and HAQ and erythrocyte folate were added to the model. Upon addition of these interaction terms to the model, the AUC of the final model, shown in Table 4, increased to 0.75 (95% CI 0.69–0.81). As mentioned in the Methods section, we generated new cut-off values for erythrocyte folate and the BMI in the U-Act-Early cohort which, when included, did not result in higher AUCs.

Table 4. Final prediction model enhanced for clinical implementation.

	β	**OR (95% CI)**	**p**
Intercept	-1.67	0.19 (0.07 – 0.44)	<0.001***
Baseline DAS28 > 5.1	1.34	3.81 (2.12 – 6.99)	<0.001***
HAQ >0.6	0.44	1.56 (0.58 – 4.33)	0.383
BMI >25 kg/m^2	-0.34	0.71 (0.24 – 2.04)	0.528
Erythrocyte folate <750nmol/L	1.79	5.98 (2.00 – 19.09)	0.002**
Smoking (current smoker)	0.81	2.26 (1.25 – 4.16)	0.008**
HAQ >0.6 x BMI >25 kg/m^2	1.30	3.68 (1.07 – 13.14)	0.040*
HAQ >0.6 x Erythrocyte folate <750nmol/L	-1.46	0.23 (0.06 – 0.86)	0.031*
AUC		.75 (95% CI: 0.69 – 0.81)	
Hosmer – Lemeshow test			0.634

β= beta coefficient of the final logistic regression model. OR (95% CI)= odds ratio with 95% confidence interval. The model was constructed in the combined dataset (tREACH + U-Act-Early, N=264). *P-values < 0.05, **P-values < 0.01, ***P-values<0.001. AUC= area under the curve. The multiplication sign indicates that there is an interaction between two predictors.

Model Translation to the Clinic

In order to apply the model in clinical practice, the prediction model was integrated into an online platform "Evidencio" [16]. Using this tool, clinicians can easily enter DAS28, HAQ, erythrocyte folate, smoking, and BMI for individual patients using sliding scales and buttons. The model then automatically calculates and presents a probability of insufficient response to MTX for this specific patient. Specificity, positive predictive value (PPV), sensitivity, and negative predictive values (NPV) were calculated for different cut-off values of these probabilities for insufficient response and are presented in Supplementary File S1.

Our online model can be found in Evidencio using the following link: *https://www.evidencio.com/models/show/2191*. A patient-specific report presenting the results and model interpretation can be downloaded from Evidencio (Supplementary File S1). An example is shown in Fig. 2, where a patient with DAS28 score = 4.9, HAQ = 0.4, BMI = 22 kg/m^2, erythrocyte-folate = 720 nmol/l, and current smoking status = yes, has a probability of insufficient response of 71.7%, with corresponding specificity of 86% and PPV of 75%.

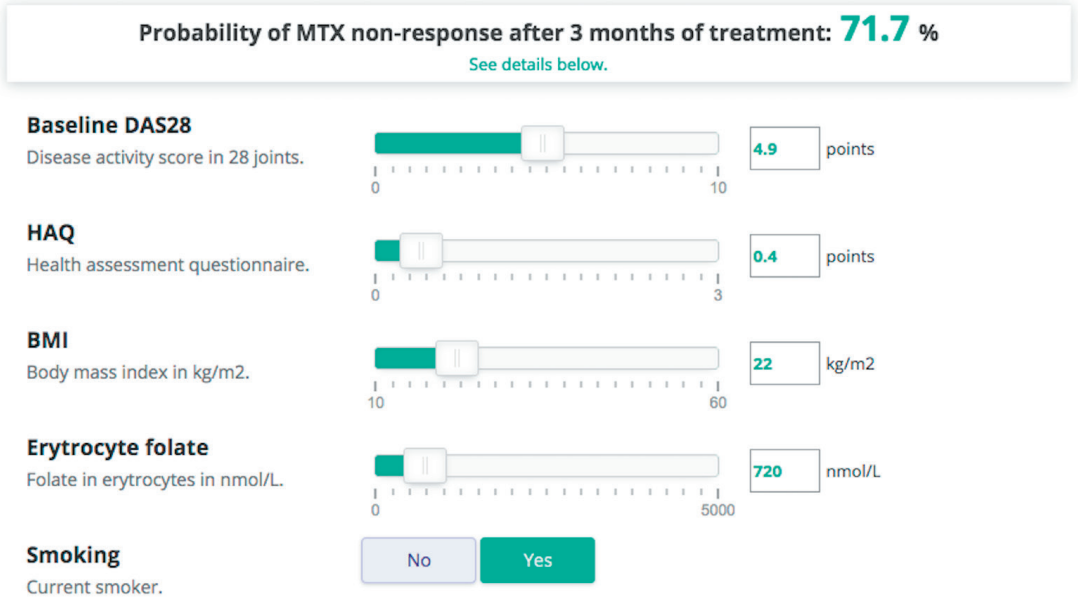

Figure 2. Example of online platform Evidencio for the implementation of the prediction model. Values for each individual patient can be filled out using the buttons and slides. Corresponding probability for insufficient response is automatically calculated using the prediction model.

DISCUSSION

We externally validated our previously developed prediction model for insufficient response to MTX therapy at 3 and 6 months after treatment initiation in early RA patients including all predictors DAS28 > 5.1, HAQ > 0.6, *ABCB1* rs1045642 genotype, *ABCC3* rs4793665 genotype, erythrocyte folate < 750 nmol/l, current smoking and BMI > 25 kg/m^2. To enhance clinical applicability and facilitate implementation, the validated model was applied in the combined derivation and validation cohort. This model, without *ABCB1* rs1045642 genotype and *ABCC3* rs4793665 genotype, had an AUC of 0.75, meaning that it classified 75% of the insufficient responders correctly. Currently, according to the EULAR treatment guidelines, MTX is the first-line therapy in RA. Treatment is only up-scaled after 3 to 6 months of insufficient response to MTX, despite evidence supporting a 'window of

opportunity' for targeted treatment [1, 17]. This window of opportunity is a limited period between diagnosis and RA progression in which the disease could still be modified, radiographic damage/functional disability could be limited, and progression could be slowed down upon early control of disease activity, for which sufficient treatment is required [17]. Our prediction model could assist in identification of insufficient responders at diagnosis: for those with high probability of insufficient response to MTX, additional biologics or JAK inhibitors could be prescribed, while for patients with low probabilities of insufficient response these expensive treatments could be spared. This distinction at diagnosis could save precious time for insufficient responders, allowing earlier control of disease activity resulting in better long-term outcomes.

We externally validated the model for the first time in its entirety as, besides the clinical and laboratory predictors, the lifestyle predictors (BMI and smoking) were also examined in the U-Act-Early cohort (as opposed to the initial validation in the MTX-Rotterdam cohort, which lacked the life-style predictors) [9]. The strongest predictor was high disease activity at baseline (DAS28 > 5.1) confirming previous findings [7, 18]. Due to differences in treatment intensities (i.e., MTX dose and co-medication) between the derivation and the validation cohort, we investigated whether the model was applicable at 6 months despite step-up treatments after the 3-month mark. Indeed, 71% of the non-responders to MTX were classified correctly, which was similar to the 75% in the tREACH derivation cohort at 6 months. In the combined cohort, all predictors except for *ABCB1* and *ABCC3* genotypes significantly contributed to the predictive power of the model. Addition of *ABCB1* and *ABCC3* genotypes to the model showed only minimal improvement, resulting in an absolute change in AUC of 0.01, which was not statistically significant. A meta-analysis on the relationship between *ABCB1* genotype and response to MTX in 2014 RA patients showed an association between this genotype and response to MTX, yet our patient group was too small to validate this result [9]. Another recent GWAS study did not show a relationship between *ABCB1* or *ABCC3* and treatment response [19]. Since the differences in predictive power were minor and the effect of *ABCC3* genotype pointed in opposite directions in the two cohorts, possibly indicating a spurious finding, we excluded both genotypes from the model.

In agreement with our study, increased BMI (obese > 30 kg/m^2) was previously found to be associated with insufficient response to MTX in RA patients [20, 21]. It is postulated that the effect of BMI on non-response to MTX could be due to the release of proinflammatory adipokines [e.g., leptin, interleukin-6, and or tumor-necrosis factor alpha (TNF-α)] from the adipose tissue [20]. Also, the predictive power of smoking was is in accordance with previous studies [6, 7, 20, 22].

So far, several prediction models for MTX non-response have been proposed, which resulted in an AUC between 0.65 and 0.85 [6,7,8, 22, 23]. Different outcome measures at different time points and combination therapies complicate comparison between models and their validation. However, the best-performing models all included clinical parameters and

laboratory parameters, which is in line with our findings [6, 8]. We also showed that clinical predictors (DAS28 > 5.1 and HAQ > 0.6) alone classified fewer insufficient responders correctly (67%) compared to the model combining clinical, life-style (BMI and smoking) and laboratory predictors (erythrocyte folate), which classified 73% of patients correctly. Most clinical predictors and life-style predictors are easy to assess. Erythrocyte folate may not be available in every laboratory, however the assay is relatively easy to assess [24].

Strengths of this study are that both derivation and validation studies were prospectively designed and that patients in the external validation cohort were included from different districts in the Netherlands. Limitations are that the size of the external validation cohort was limited, however the number of cases in both the internal (tREACH) and external (U-Act-Early) cohort were similar. In addition, the model was validated in an MTX monotherapy group, while it was designed in a combination (GC and csDMARD) therapy group. Commonly, however MTX is co-prescribed with a short course of glucocorticoids (prednisone) as MTX's optimal effect ensues after 8–12 weeks [*1*]. Despite differences in co-medication between the cohorts, the prediction model had similar predictive value and OR for predictors were in the same direction in both cohorts, indicating that co-medication did not affect the prediction of response to MTX. Another limitation is that smoking status was assessed using questionnaires; possibly biasing the results as smoking behavior could be underreported or underestimated. In future studies, cotinine, the degradation product of nicotine, could be quantified as an objective measure for smoking status, which can easily be determined in serum [25].

Furthermore, we showed that the online platform Evidencio provides an easy tool for implementation of the prediction model in clinical practice. Evidencio is freely available so that the data can be uploaded to automatically validate the model in specific cohorts. In addition, using the Evidencio platform, clinicians can directly use the model in their practice. When a new patient is diagnosed with RA, patient's information on DAS28, HAQ, erythrocyte-folate, BMI, and smoking status can be provided to Evidencio. Subsequently, a probability of insufficient response to MTX with corresponding specificity, positive predictive value (PPV), sensitivity, and negative predictive value (NPV) are provided by the tool and may help clinicians and patients in shared decision-making on step-up treatment with bDMARDs or tsDMARDs. The choice of a cut-off depends on the clinical goal. Taking into consideration the "window of opportunity" [17] for optimal treatment, we consider it crucial to adequately treat insufficient MTX responders with additional bDMARDs/tsDMARDs. Therefore, our goal for this prediction model was to identify as many insufficient responders as possible, while at the same time attempting to restrict the use of bDMARDs/tsDMARDs to those patients who really need them, hence to avoid misclassification of sufficient responders. Considering this, a cut-off probability of 70% (of insufficient response) could be chosen. At this cut-off, 75% of patients classified as insufficient responder match actual insufficient responders (PPV) and could be treated with additional bDMARDs/tsDMARDs. Additionally, at this cut-off, 86% of all sufficient

responders would be correctly classified as such (specificity) and could be spared additional treatment.

The importance of erythrocyte-folate for the predictive power of the model implies that this model is specific in predicting insufficient response to MTX, as MTX is structurally similar to folate. Hence, low erythrocyte folate levels are possibly a surrogate measure for poor MTX absorption, transportation, and MTX accumulation in the cell, as described previously [14]. However, it is possible that a certain proportion of insufficient responders to MTX are difficult-to-treat RA patients who are poorly responsive to various b/ts DMARDs [26, 27]. So far, we cannot identify difficult-to-treat RA patients in advance. Furthermore, as recently argued, treatment strategies could be more important than specific drugs, implying that these patients could still benefit from quicker and more aggressive treatment to reach a certain treatment target when earlier identified as insufficient responders [28, 29].

CONCLUSIONS

We successfully externally validated our previously published prognostic prediction model of insufficient response to MTX, which correctly classified 75% of insufficient responders at 3 months and 71% of non-responders at 6 months of treatment. The model can be used in clinical practice to identify insufficient responders to MTX with the goal of treating them with additional biologic or JAK inhibitors as early as possible to reduce disease activity and limit joint damage. Application of the tool by means of a clinical trial is warranted.

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SUPPLEMENTAL FILES

EVIDENCIO
MEDICAL PREDICTION MODELS

Model-id: 2191 | Version: 1.28 | Revision date: 2020-06-09

PREDICTION OF 3 MONTHS MTX NON-RESPONSE (DAS28>3.2) IN EARLY RHEUMATOID ARTHRITIS

Validated prediction model to identify DMARD-naïve rheumatoid arthritis patients with high risk of insufficient response to MTX.

Research authors: *Gosselt HR, Verhoeven MMA, de Rotte MCFJ, Pluijm SMF, Muller IB, Jansen G, Tekstra J, Bulatović-Ćalasan M, Heil SG, Lafeber FPJG, Hazes JMW, and de Jonge R.*

RESULT

2020-06-30 11:22

Probability of MTX non-response after 3 months of treatment: 71.7 %

Based on the following parameters:

Baseline DAS28	4.9 points
HAQ	0.4 points
BMI	22 kg/m2
Erytrocyte folate	720 nmol/L
Smoking	Yes

Disclaimer: Calculations alone should never dictate patient care, and are no substitute for professional judgement.

Supplementary File S1. Model performance given different risk cut-offs for insufficient response (Gosselt et al, 2020) Calculations alone should never dictate patient care, and are no substitute for professional judgement. See our full disclaimer at: https://www.evidencio.com/disclaimer.

OUTCOME STRATIFICATION

Result interval 60 to 80

Based on a probability cut-off of **70%** risk of insufficient respons to methotrexate, calculated sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) were:

Sensitivity: 43% Specificity: 86% PPV: 75% NPV: 61%

Result interval 70 to 90

Based on a probability cut-off of **80%** risk of insufficient respons to methotrexate, calculated sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) were:

Sensitivity: 8% Specificity: 96% PPV: 67% NPV: 52%

CONDITIONAL INFORMATION

An interaction term was acitivated and added to the model:
Based on the provided input, a two-way interaction term between HAQ and erythrocyte folate (OR = 0.23, 95% CI 0.06 – 0.86) was automatically added to the model. This interaction term was found to significantly contribute to the model, meaning that low erythrocyte folate concentrations (<750 nmol/L) significantly predicted insufficient response when HAQ values were <0.6.

RESULT INTERPRETATION

How this model should be used:
This prediction model could assist in identification of insufficient responders at diagnosis. For patients with high probability of insufficient response to MTX, additional biologics or JAK-inhibitors could be prescribed. For those with low probabilities of insufficient response, these expensive treatments could be spared. This distinction at diagnosis could save precious time for insufficient responders, allowing earlier control of disease activity resulting in better long-term outcomes.

Model performance:
Discriminative power of the model was assessed through evaluating the area under the receiver operating characteristic curve (AUC). The AUC of the model was 0.75 (95% CI: 0.69 – 0.81), indicating that the model correctly classified patients in 75% of the cases.

Goodness-of-fit between the predicted probabilities and observed values was tested using the Hosmer-Lemeshow test. The associated P-value was 0.634, indicating good model fit.

Decisions on appropriate risk cut-offs:
Taking into consideration the “window of opportunity” for optimal treatment we consider it crucial to adequately treat insufficient MTX responders with additional bDMARDs/tsDMARDs. Therefore, our goal for this prediction model was to identify as many insufficient responders as possible, while at the same time attempting to restrict the use of bDMARDs/tsDMARDs to those patients who really need them, hence to avoid misclassification of sufficient responders. Considering this, a cut-off probability of 70% (of insufficient response) could be chosen.

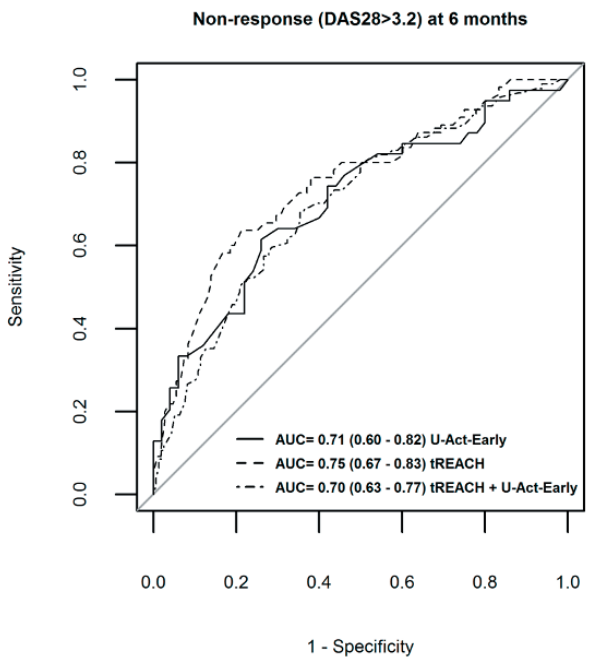
At this cut-off, 75% of patients classified as insufficient responder match actual insufficient responders (PPV) and could be treated with additional bDMARDs/tsDMARDs. Additionally, at this cut-off 86% of all sufficient responders would be correctly classified as such (specificity) and could be spared additional treatment.

Supplementary File S1. Continued

Cut-off values for probabilities of Insufficient response (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
10	1	0	49	NA
20	96	15	62	80
30	88	48	62	81
40	87	51	63	80
50	73	71	71	73
60	58	79	73	66
70	43	86	75	61
80	8	96	67	52
90	1	100	1	51

Probabilities of insufficient response were calculated according to the prediction model. Model performance measures were calculated for different cut-off values (column 1). PPV = positive predictive value, NPV = negative predictive value.

Supplementary File S1. Continued

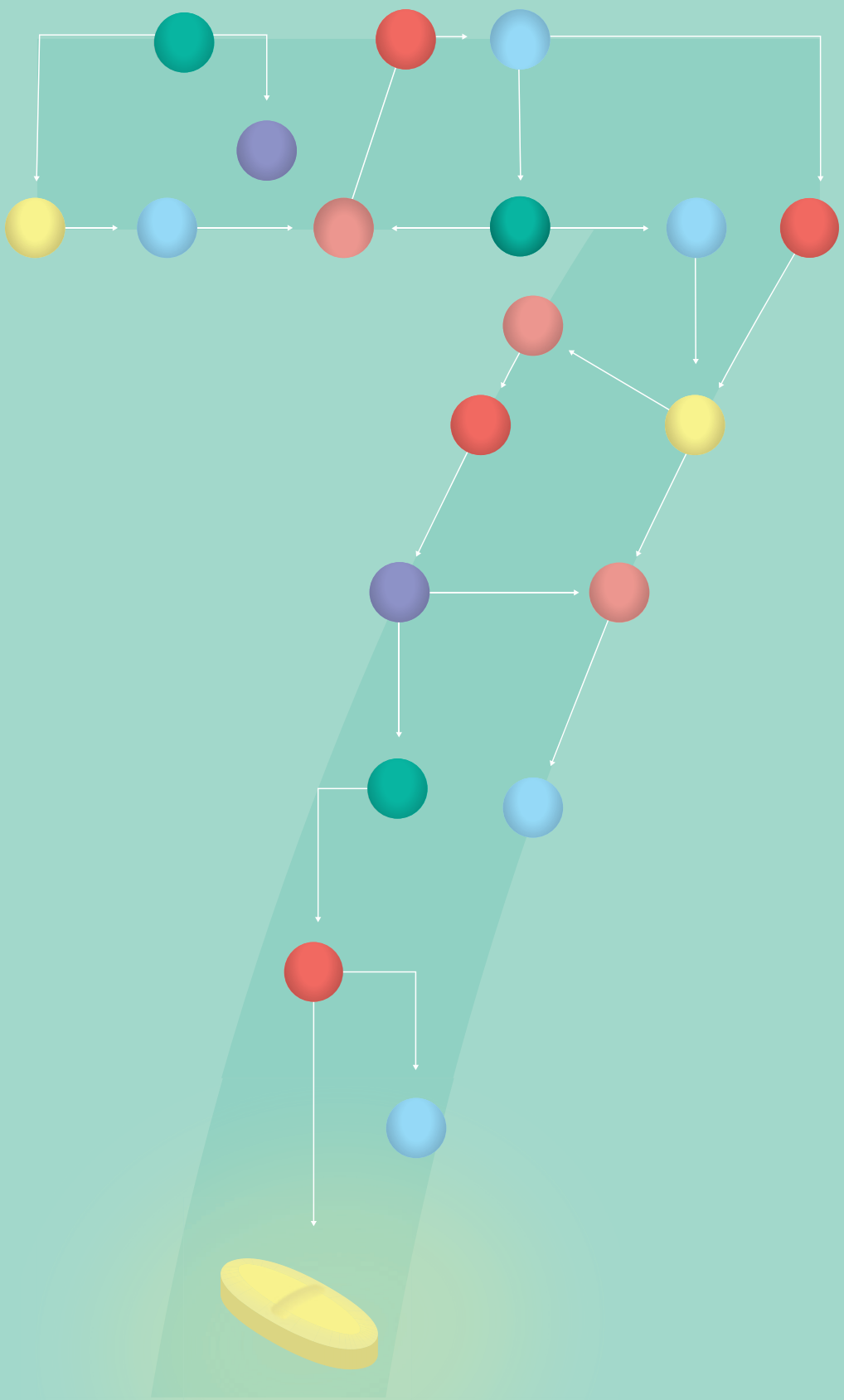


Supplementary Figure S1. ROC curve for the prediction of non-response (DAS28>3.2) to MTX after six months of treatment. Area under the curve (AUC) is reported as follows: AUC (95% confidence interval). Predictors were: baseline DAS28 >5.1, baseline HAQ >0.6, ABCB1 genotype, ABCC3 genotype, baseline erythrocyte folate, BMI >25kg/m2 and current smoking.

Supplementary Table S1. Effect sizes of multivariable logistic regression models for insufficient response to MTX (DAS28 >3.2) at 3 months of treatment in the combined data set.

	U-Act-Early + tREACH Combination cohorts N=264
Predictors	**OR (95% CI)**
DAS28 >5.1	3.6 (1.98 – 6.47)**
HAQ >0.6	2.1 (1.08 – 3.90)*
ABCB1 GG/GA	1.2 (0.71 – 2.17)
ABCC3 TC/CC	1.8 (0.99 – 3.11)
Folate <750 nmol/L	1.9 (1.06 – 3.50)*
Smoker	2.2 (1.23 – 4.00)**
BMI >25 kg/m^2	1.9 (1.11 – 3.31)*
AUC (95% CI)	0.74 (0.68 – 0.80)

Model shown is from the combined data set (U-Act-Early + tREACH). OR= odds ratio, CI= confidence interval. Predictors that contributed significantly to the model were indicated with an asterisk, where *P<0.05 and **P<0.01.



Chapter 7

Complex machine learning algorithms and multivariable logistic regression on par in prediction of insufficient clinical response to methotrexate in rheumatoid arthritis

Helen R. Gosselt, Maxime M.A. Verhoeven, Maja Bulatović - Ćalasan, Paco M. Welsing, Maurits C.F.J. de Rotte, Johanna M.W. Hazes, Floris P.J.G. Lafeber, Mark Hoogendoorn and Robert de Jonge

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ABSTRACT

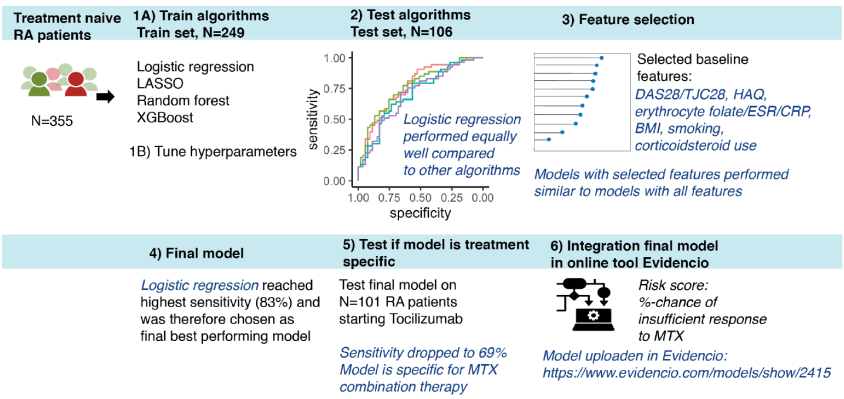
Introduction: To examine whether machine-learning algorithms outperform multivariable logistic regression in prediction of insufficient response to MTX. Secondly, to examine which features are essential for correct prediction. Finally, to investigate whether the best-performing model specifically identifies insufficient responders to MTX (combination) therapy.

Methods: Prediction of insufficient response (3-month DAS28-ESR>3.2) was assessed using: logistic regression, least absolute shrinkage and selection operator (LASSO), random forest and extreme gradient boosting (XGBoost). Baseline features of 355 rheumatoid arthritis (RA) patients from the "treatment in the Rotterdam Early Arthritis CoHort" (tREACH) and U-Act-Early trial were combined for analyses. Model performances were compared using area under the curve (AUC) of receiver operating characteristic (ROC) curves, 95% confidence intervals (95% CI), sensitivity and specificity. Finally, the best performing model following feature selection was tested on 101 RA patients starting tocilizumab (TCZ)-monotherapy.

Results: Logistic regression (AUC=0.77 95% CI:0.68 – 0.86) performed equally well to LASSO (AUC=0.76, 95% CI:0.67 – 0.85), random forest (AUC=0.71, 95% CI:0.61=0.81) and XGBoost (AUC=0.70, 95% CI:0.61–0.81), yet logistic regression reached highest sensitivity (81%). Most important features were baseline DAS28 (components). For all algorithms, models with 6 features performed similarly to those with 16. When applied to the TCZ-monotherapy group, logistic regression's sensitivity significantly dropped from 83% to 69% (p=0.03).

Conclusions: In current dataset, logistic regression performed equally well compared to machine-learning algorithms in prediction of insufficient response to MTX. Models could be reduced to 6 features, which are more conducive for clinical implementation. Interestingly, the prediction model was specific for MTX (combination) therapy response.

Graphical abstract:



INTRODUCTION

Methotrexate (MTX) is the anchor drug in the treatment of rheumatoid arthritis (RA) patients. Currently, every early RA patient receives MTX (combination) therapy for at least 3-6 months, which is the interval to build-up dose and reliably determine response to MTX [1]. A substantial proportion of patients do not benefit from MTX treatment due to inefficacy or adverse events and require step-up treatment with targeted synthetic - or biologic disease modifying antirheumatic drugs (ts/bDMARDs) [1]. Preferably, personalized medicine is implemented; allowing predicted insufficient responders to MTX to step-up treatment from the start. To enable personalized medicine we and others have previously proposed prediction models to identify insufficient responders to MTX prior to treatment initiation [2–6]. We recently externally validated our model and implemented it in the online clinical tool *Evidencio* [7]. Until now, these clinical prediction models have been developed using multivariable logistic regression. In recent years, the use of machine-learning algorithms has gained popularity in healthcare due to their flexibility in handling large complex datasets and non-linear relationships [8,9]. Also, in the RA healthcare domain there are many opportunities for the application of machine-learning algorithms: for instance categorization of different arthritis subtypes or prediction of treatment response [10–12]. Others already successfully examined whether machine-learning algorithms could be used to predict response to MTX therapy in JIA patients [13] and to Anti–Tumor Necrosis Factor in RA patients [14]. However, it is unclear whether these algorithms outperform multivariable logistic regression models in prediction of insufficient response, as these statistical techniques have not been examined in parallel.

To facilitate clinical implementation, an insightful model using least number of variables, referred to as "features", is preferred. Several feature selection methods exist to determine the essential features, of which some automated feature selection methods are embedded within machine-learning algorithms (e.g., least absolute shrinkage and selection operator [LASSO]) [15]. Furthermore, it is essential to predict insufficient response specifically to MTX (combination) therapy, because these patients would benefit from a step-up treatment, while other strategies are required for RA patients that are also irresponsive to non-conventional DMARDs (e.g. toculizumab [TCZ]) [16].

Based on the points described above, the primary aim of this study is to assess the performance of machine-learning algorithms compared to multivariable logistic regression in prediction of insufficient response to MTX (combination) therapy in RA patients. Secondly, feature selection is performed to examine which features are essential to predict insufficient response in RA. Lastly, to investigate whether the model identifies insufficient responders specifically to MTX (combination) therapy, the best-performing model will also be assessed on a similar group of RA patients starting TCZ-monotherapy.

MATRIALS AND METHODS

Patients

355 subjects were included in current study. 264 patients randomized to start MTX-monotherapy or MTX combination therapy with conventional DMARDs (i.e. sulfasalazine [SSZ], hydroxychloroquine [HCQ]) and corticosteroids, satisfying the 2010 American college of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for RA and of whom DAS28 was available at 3 months were eligible from the treatment in the Rotterdam Early Arthritis Cohort (tREACH, registered retrospectively at ISRCTN, registry number: ISRCTN26791028 on 23 August 2007) and 91 of the U-Act-Early trial registered at ClinicalTrials.gov (number: NCT01034137).

The tREACH, described previously [17] was designed to achieve early response rates (within 3 months), by quickly increasing MTX dosage up to 25 mg/week within the first 3 weeks. U-Act-Early, also previously described [18], consisted of three treatment arms: MTX + placebo, TCZ + MTX and TCZ + placebo. MTX dosage was increased 5 mg per 4 weeks up to 30 mg/week with a starting dose of 10 mg/week and the use of corticosteroids was not permitted. 91 patients of the MTX-monotherapy arm and 101 RA patients from the TCZ-monotherapy arm of U-Act-Early were included in current study. Two patients from the total TCZ arm (N=103) were excluded from the analyses due to missing DAS28 scores at 3 months.

U-Act-Early was approved by the medical ethics committee of the University Medical Center Utrecht (ML22497) and tREACH by the medical ethics committee of Erasmus Medical Center Rotterdam (MEC-2006-252). Written informed consent was obtained for all included patients.

Features and outcome

Features related to RA pathogenesis (RF, ACPA status and DAS28 components) or to MTX metabolism (e.g. SNPs in ABC transporter genes and erythrocyte folate) that were available in both the tREACH and U-Act-Early were included (Table 1). The outcome 'insufficient response' was defined as DAS28 >3.2, based on the erythrocyte sedimentation rate (ESR) and determined at 3 months, because after that time point treatment could be intensified with a bDMARD in tREACH.

Table 1. List of baseline features that were included in the study.

ABCB1 genotypes AA vs AG/GG
ABCC3 genotypes TT vs TC/CC
Age, years
Alcohol (Never consumed: 0, Ever consumed: 1)
Anti-citrullinated protein antibody (ACPA, negative/positive)
Body mass index (BMI), kg/m^2
C-reactive protein (CRP), mg/l
Disease activity score 28 (DAS28)
DMARD and/or corticosteroid use (no use: 0, use: 1)
Erythrocyte folate (nmol/L packed erythrocytes)
Erythrocyte-sedimentation rate (ESR), mm/first hour
Gender (male/female)
Health assessment questionnaire (HAQ)
Rheumatoid Factor (RF, negative/positive)
Smoking, never/former vs current
Tender joint count 28 (TJC28)

List of features included in the study based on availability and clinical rationale. *ABCB1* = ATP binding cassette subfamily B member 1, *ABCC3* = ATP binding cassette subfamily C member 3, DMARD= disease modifying antirheumatic drug.

Train and test data

The total dataset contained 355 subjects and 16 features (Table 1). The data was first split into a train (70%, N=249) and a test set (30%, N=106). A stratified split was applied, meaning that the ratio between insufficient and sufficient responders was kept similar to the ratio in the complete data set. Hence, the train set contained N=124/249 insufficient responders (50%) and the test set N=53/106 insufficient responders (50%) at 3 months. Moreover, train and test set were fixed upfront using a random seed. To prevent data leakage, preprocessing steps were performed on the train and test set separately. At the start, all features contained <20% missing values. Missing values were imputed using K-nearest neighbors, a widely used technique where imputation is based on values of other patients (neighbors) with most similar data [19]. To prevent ties in imputation of categorical features only odd numbers (K=3, 5, 7, 9, 11) were tested. K=5 was initially randomly chosen en showed comparable results to the other K values and was therefore used for imputation. All analyses were performed in RStudio Version 1.3.1056.

Algorithms, preprocessing and statistics

Mean baseline characteristics between insufficient and sufficient responders to MTX (combination) therapy in the complete dataset were compared using a Welch's two-sample t-test and proportions were compared using the two-sample test for equality of proportions. The following random selection of popular supervised classification

algorithms were tested and compared to logistic regression for the prediction of insufficient response: least absolute shrinkage and selection operator (LASSO) [20], random forest and extreme gradient boosting (XGBoost) [21,22]. The latter two algorithms are based on decision trees. Pre-processing for LASSO included centering and scaling of the features. We performed 10-fold stratified cross-validation to tune the hyperparameters to avoid overfitting. Hyperparameters were automatically tuned [23] and best hyperparameters of final models were: random forest (mtry=4, ntree=500), LASSO (alpha=1, 0.017) and XGBoost (eta=0.3, max_depth=1, gamma=0, colsample_bytree=0.6, min_child_weight=1, subsample=0.67).

First, model performances on the train set (70%) were assessed using the area under the curve (AUC) of the receiver-operating characteristic (ROC) curves. Second, performances of tuned models were examined on the test set. Again, a random seed was set to make model assessments reproducible. Differences between two ROC curves were tested using the DeLong's test. Additionally, accuracy, precision, sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) were assessed using the pROC package [24]. A cut-off was chosen based on highest possible sensitivity and specificity of ≥0.60. The rationale behind this step was correct identification of as many insufficient responders as possible (sensitivity), while maintaining correct classification of sufficient responders (specificity). Differences in sensitivity were tested using a 2-sample test for equality of proportions with continuity correction. Third, feature selection was performed in order to simplify the models for clinical application. To determine the essential features for prediction of insufficient response, feature importance plots were created based on their regression coefficients (logistic/LASSO) or decrease in accuracy/Gini score (random forest/XGBoost). Additionally, feature correlations were examined using Pearson's correlation test. In case of two highly correlated features ($r > 0.60$), the feature that was easiest to clinically assess was included. Finally, best-performing model was applied to a TCZ-monotherapy group and its performance was compared to that on the MTX (combination) therapy group (for which it was developed). First, power calculation for the AUCs were performed using the pROC package in R to assure that enough cases were included [25]. Next, calibration curves were generated for the two treatment groups (i.e., MTX combination therapy or TCZ-monotherapy) in order to examine concordance between calculated (using model) and observed probability of insufficient response. Furthermore, to compare the model's fit on the MTX (combination) therapy group and TCZ-monotherapy group, a risk score for insufficient response was calculated according to the logistic model coefficients (intercept + β_1 x pred1 + β_2 x pred2, etc). To compare differences between the two calibration curves, the main effects "risk score" and "treatment group" and their interaction term were assessed in relation to prediction of insufficient response in a logistic regression model on the total dataset (MTX combination therapy + TCZ, N=435), excluding cases with incomplete values for any feature.

RESULTS

Baseline comparisons

Our data was balanced with 49.9% insufficient responders (DAS28 >3.2) after 3 months of treatment and 50.1% sufficient responders (Table 2). The majority received combination therapy with SSZ, HCQ and/or corticosteroids. Significantly more patients on MTX-monotherapy (p=0.01) and on MTX combination therapy with intra-muscular corticosteroids (p=0.04) were insufficient responders.

Table 2. Baseline comparisons between sufficient and insufficient responders.

	Insufficient responders (3-month DAS28 >3.2)	**Sufficient responders (3-month DAS28 ≤3.2)**	**P-value**
N (%)	**177 (49.9%)**	**178 (50.1%)**	
Age, mean ± SD	54 ± 13	53 ± 15	0.35
Gender, male	50 (28.2%)	63 (35.4%)	0.18
Rheumatoid factor, positivity	108 (67.1%)	113 (70.6%)	0.57
ACPA positivity	122 (69.3%)	136 (76.4%)	0.17
Behandeling			
MTX + SSZ + HCQ + i.m. cortico	28 (15.8%)	45 (25.3%)	0.04*
MTX + SSZ + HCQ + cortico per os	31 (17.5%)	45 (25.3%)	0.10
MTX + cortico per os	41 (23.2%)	36 (20.2%)	0.63
MTX	77 (43.5%)	52 (29.2%)	0.01*

*p-value < 0.05 was considered significant. MTX= methotrexate. SSZ= Sulfasalazine.
HCQ= hydroxychloroquine. i.m. = intramuscular. Cortico = corticosteroids. Missing values: erythrocyte folate N=71, *ABCB1* N=16, *ABCC3* N=15, RF N=34, ACPA N=1, BMI N=3, HAQ N=15, smoking N=14, alcohol use N=14, CRP N=1.

Model performances on test set – including all features

Performances between tuned algorithms on the train set were comparable with AUCs ranging from 0.71 to 0.73 (Supplemental Table S1). Next, trained models were tested on the test set (N=106). The highest AUC of 0.77 (95% CI: 0.68 – 0.86) was reached with logistic regression (Table 3).

Table 3. Results model performances on test set (N = 106).

	AUC (95%CI)	**Sensitivity**	**Specificity**	**Accuracy**	**PPV**	**NPV**
Logistic regression	0.77 (0.68 – 0.86)	0.81	0.60	0.71	0.67	0.76
LASSO	0.76 (0.67 – 0.85)	0.79	0.60	0.70	0.67	0.74
Random forest	0.71 (0.61 – 0.81)	0.66	0.64	0.65	0.65	0.65
XGBoost	0.70 (0.61 – 0.81)	0.75	0.60	0.68	0.66	0.71

The threshold was chosen according to the highest sensitivity where specificity was at least 0.60. Baseline features included in the model: *ABCB1* genotype, *ABCC3* genotype, age, alcohol use, ACPA status, BMI, CRP, DAS28, DMARD/cortico use, erythrocyte-folate, ESR, gender, HAQ, RF positivity, smoking, tender joint count 28 (TJC28). Abbreviations: LASSO= least absolute shrinkage and selection operater, XGBoost= extreme gradient boosting, PPV= positive predictive value, NPV= negative predictive value.

Largest differences in AUCs were observed between logistic regression and random forest (Figure 1), although these were not significantly different (p=0.09).

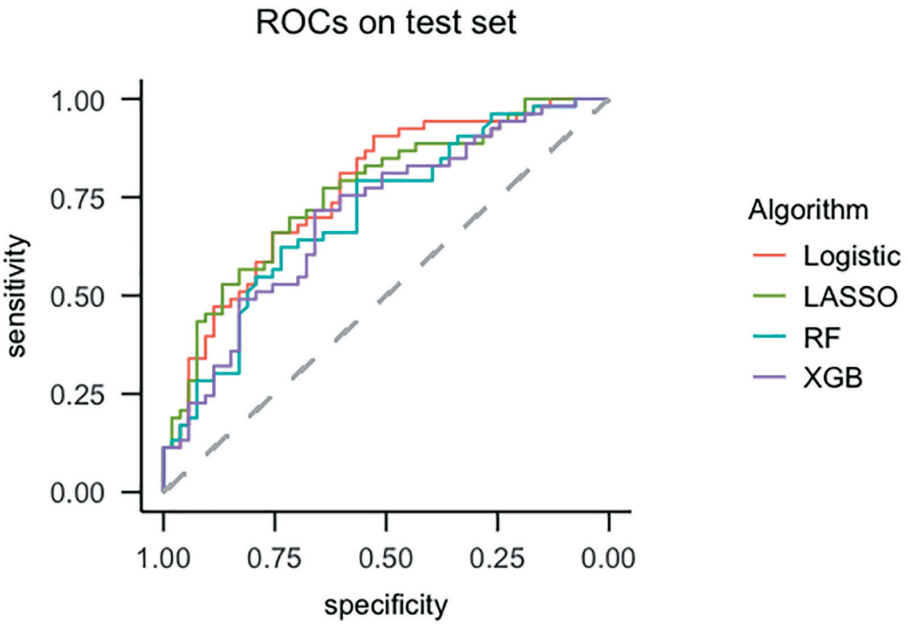


Figure 1. ROC curves of algorithms tested on test set (N=106). Abbreviations: RF=Random forest, Logistic = logistic regression, XGB=Extreme gradient boosting, LASSO = least absolute shrinkage and selection operator.

Sensitivity was significantly higher in logistic regression (p=0.02) and borderline significantly higher in LASSO (p=0.05) compared to random forest (Table 3). A sensitivity of 0.81 (logistic regression) indicates that 81% of all insufficient responders were correctly identified as such. The PPV, indicating percentage of predicted insufficient responders that were true insufficient responders, were comparable between algorithms.

Feature importance

Features' contributions to the model performances are presented in Figure 2. Features that were important for all algorithms were baseline DAS28 or DAS28 components (TJC28, ESR/CRP, HAQ). Depending on the algorithm also, current smoking, erythrocyte folate, ABCC3 genotype, BMI and the use of DMARDs/corticosteroids were important features in identification of insufficient responders. RF positivity, ACPA positivity and alcohol use were least important for the majority of the algorithms. Of all algorithms, LASSO performed most rigorous feature selection, selecting: DAS28, HAQ, TJC28, smoking, ESR, ABCC3 genotype, DMARD/corticosteroid use, CRP and gender. Although, gender and CRP were less important compared to the other selected features.

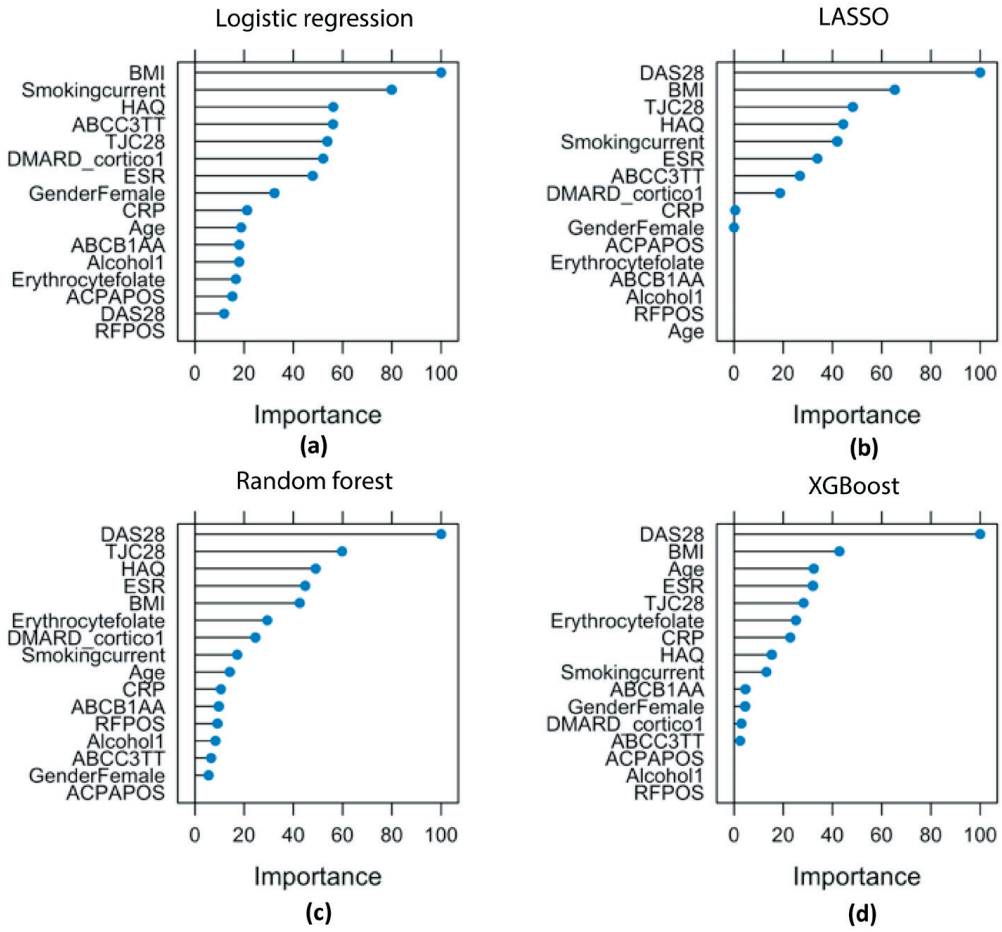


Figure 2. Feature importance plots of baseline features for (a) logistic regression, (b) LASSO, (c) random forest and (d) XGBoost in prediction of insufficient response at 3 months. Feature importance was determined based on regression coefficients (regression models) and Gini score (RF and XGBoost) of final models. Most important feature was set to 100 and the rest is relative to that feature. Abbreviations: DAS28= disease activity score 28, TJC28= tender joint count 28, HAQ= Health Assessment Questionnaire, ESR= erythrocyte sedimentation rate, BMI= body mass index, DMARD_cortico1=use of DMARDs or corticosteroids (0=no, 1=yes), Smoking (never/former versus current), CRP= c-reactive protein, ABCB1 AA vs AG/GG, ABCC3 TT vs TC/CC, RF= rheumatoid factor, Alcohol use (0=1 no, 1=yes), ACPA= anti-citrullinated protein (positive versus negative).

Feature selection

Feature selection was performed to boost model performances and retrieve more clinically applicable concise models. We started from the features selected by LASSO. Additionally, we excluded one out of two highly correlated features, e.g.: DAS28 and TJC28 (r=0.73) and CRP and ESR (r=0.61). TJC28 requires fewer clinical assessments compared to DAS28 and the outcome was based on DAS28-ESR, which is why TJC28 and ESR were chosen. Even though ABCC3 genotype was selected by LASSO, we excluded this feature because of its absence in the TCZ-monotherapy group and its minor contribution compared to the other features. Hence, features included after selection were: TJC28, HAQ, BMI, smoking,

ESR and the use of DMARDs/corticosteroid use. All models performed equally well with only 6 features (Table 4) compared to the complete set of features (=16 features; Table 1). The ROCs are presented in Supplemental Figure S1 and confusion matrices in Figure S2.

Table 4. Model performances on test set (N = 106) after feature selection.

	AUC (95%CI)	Sensitivity	Specificity	Accuracy	PPV	NPV
Logistic regression	0.78 (0.69 – 0.87)	0.83	0.60	0.72	0.68	0.78
LASSO	0.77 (0.68 – 0.86)	0.79	0.60	0.70	0.67	0.74
Random forest	0.76 (0.66 – 0.85)	0.79	0.62	0.71	0.68	0.75
XGBoost	0.77 (0.67 – 0.86)	0.79	0.62	0.71	0.68	0.75

Included features after feature selection were: TJC28, HAQ, BMI, smoking, ESR, DMARD/corticosteroid use.

Model assessment on TCZ-monotherapy arm

To assess whether the prediction model was specific for identification of insufficient responders to MTX (combination) therapy, the logistic regression model with 6 features was assessed on the TCZ-monotherapy arm of U-Act-Early. This group consisted of 101 patients of which 16 patients (16%) were insufficient responders at 3 months (DAS28 >3.2). Confusion matrices are presented in Supplemental Figure S3. Upon application of the model to the TCZ-monotherapy group, an AUC of 0.73 (95% CI: 0.60 – 0.86) was reached (Supplemental Figure S4) with a power of 86%, which was not significantly different from the AUC of 0.78 (95% CI: 0.69 – 0.87) with a power of 99% in the MTX combination therapy group (p=0.54). However, the sensitivity dropped significantly from 83% on the MTX combination therapy group to 69% on the TCZ-monotherapy group (p=0.03). Additionally, the model was better calibrated on the MTX (combination) therapy group than on the TCZ-monotherapy group in which the percentage of actual insufficient responders was largely overestimated (Figure 3). This was also confirmed in a logistic regression model assessing risk score, treatment group and their interaction in relation to insufficient response on the complete dataset. The interaction term was just insignificant (p=0.09).

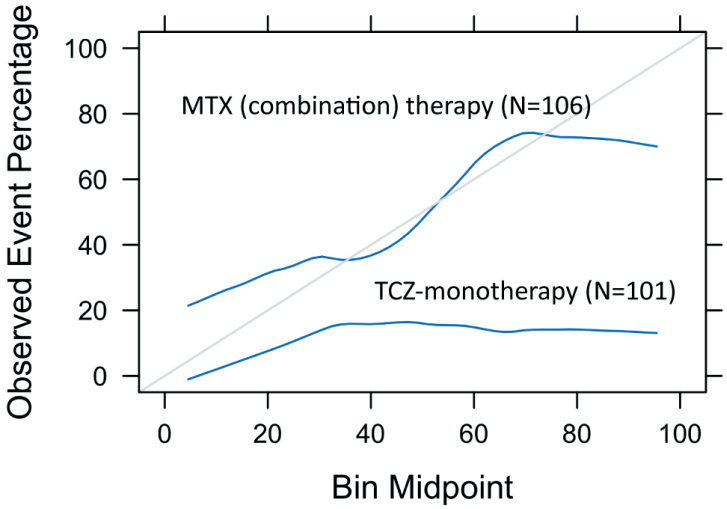


Figure 3. Calibration curves on test set of MTX (combination) therapy group and TCZ-monotherapy group. Logistic regression with 6 features (i.e., BMI, HAQ, smoking, ESR, TJC28, DMARD/corticosteroid use) was used to create calibration curves.

DISCUSSION

In this study, we showed that logistic regression performed equally well compared to machine-learning algorithms such as LASSO, random forest and XGBoost in prediction of insufficient response to MTX in RA patients on current dataset. This result is in accordance with a recent systematic review where no benefit was discovered for the use of machine-learning algorithms in clinical prediction models compared to logistic regression [26]. Nevertheless, the approach of data analysis used for machine learning could still be very useful. First, the machine-learning approach allows internal validation by splitting of the data into a train and a test set, thus reducing overfitting. Second, feature importance plots are an easy way to quickly inspect the importance of (combined) predictors on the outcome. Also, a larger number of features can be evaluated regardless of the number of cases. Furthermore, machine-learning algorithms such as XGboost or random forest may be superior if the relationship between features and the outcome is more complex (non-linear).

To enable comparisons between algorithm performances on the test set, we compared performance measures at the same cut-off on the ROC curve, for which any cut-off could have been chosen. In this study we have chosen the cut-off where most insufficient responders were correctly classified (highest sensitivity) and at least 60% of sufficient responders were correctly classified (specificity). However, the best threshold for the trade-off between sensitivity and specificity depends on the clinical goal, as previously discussed [2,7].

According to the feature plots, we made a selection and showed that all models could be reduced from 16 to 6 essential features for prediction of insufficient response. Features included were: TJC28/DAS28, HAQ, ESR/CRP, BMI, smoking and DMARD/corticosteroid use. Importantly, to select features according to feature importance plots, these plots should be carefully interpreted. Highly correlated features could make one feature seem irrelevant while that is not necessarily the case. An example is the low position of DAS28 in the logistic regression feature importance plot, which is due to its strong correlation with its component TJC28 (r=0.73). In this case TJC28 and baseline DAS28 were interchangeable, hence in clinical practice either one of the two correlated features could be used in the model. The same holds for ESR and CRP.

Our dataset contains a relatively high proportion (50%) of insufficient responders at 3 months, which can be explained by the design of the U-Act-Early trial. First of all, MTX dosage in U-Act-Early was slowly increased reaching a dosage of 25 mg/week only after 3 months, while this dosage was reached in tREACH within 3 weeks. This resulted in more insufficient responders from the U-Act-Early trial at 3 months. Moreover, all U-Act-Early patients received MTX-monotherapy, which in turn meant significantly more insufficient responders on MTX alone. This was accounted for in the model using the feature "DMARD/corticosteroids use".

The majority of baseline features selected by LASSO were clinical features (e.g. DAS28, HAQ, BMI, smoking) and were in accordance with features from previously validated prediction model on the same cohorts [7]. Also, same predictors were previously identified by others [5,6,27–29], although results on the direction of the effect of baseline DAS28 have been conflicting [5,6,30]. The exclusion of erythrocyte folate by LASSO was surprising, as this feature was required for the high AUC in our previous published model [7]. It seems that baseline ESR/CRP, not included in our previous model, could be used instead of erythrocyte folate to retain the high predictive power. ESR/CRP levels are easier to acquire compared to erythrocyte folate, hence inclusion of this predictor instead strongly facilitates model implementation. The fact that some features are interchangeable leads to multiple combinations of predictors with similar predictive power. This has the advantage that clinicians can choose to use a model based on feature availability in their own dataset. The model with 6 clinical features described in this study was therefore also uploaded in *Evidencio*: *https://www.evidencio.com/models/show/2415*).

Lastly, we showed that the final logistic regression model with 6 features performed better on the MTX (combination) therapy group than on the TCZ-monotherapy group, suggesting specific prediction of insufficient response to MTX (combination) therapy. Unfortunately, erythrocyte folate and ABCC3 genotypes, involved in the MTX metabolism [31], were not available in the TCZ-monotherapy group; hence their contribution to specific prediction to MTX combination therapy could not be assessed. Baseline CRP/ESR and TJC28 are more generic predictors for response, shown to be associated with TCZ response in RA patients (CRP/ESR) and with etanercept response (TJC28) in Juvenile Idiopathic

Arthritis (JIA) patients [32,33]. Nevertheless, even with these generic predictors, the sensitivity dropped significantly from 83% on the MTX (combination) therapy group to 69% in the TCZ-monotherapy group. Additionally, the calibration curves showed that predicted and observed risks fairly match in the MTX combination therapy group, while predicted risks largely overestimate the actual number of insufficient responders in the TCZ-monotherapy group (Figure 3).

Strengths of this study are that algorithms were tested head-to-head in the same group enabling direct comparisons of algorithm performances. Additionally, the final model was assessed on an independent therapy group starting TCZ without previous DMARD use suggesting that the model specifically identified insufficient responders to MTX (combination) therapy. Limitations were the relatively small number of patients included. It is noteworthy that the number of cases in the TCZ monotherapy was limited (N=16), however the ROC curve for this group still had a power of 86%. Also we may have missed new features that could potentially improve prediction to MTX (combination) therapy (e.g. global DNA methylation [34]) because we were limited to data availability in all included cohorts. Contrarily, clinical features currently included in the model are often readily available and commonly assessed, which eases implementation of the model into clinical practice.

In conclusion, logistic regression and machine-learning algorithms were on par in the prediction of insufficient response to MTX (combination) therapy. The model could be reduced to 6 features and was specific for prediction of insufficient response in a MTX (combination) therapy group.

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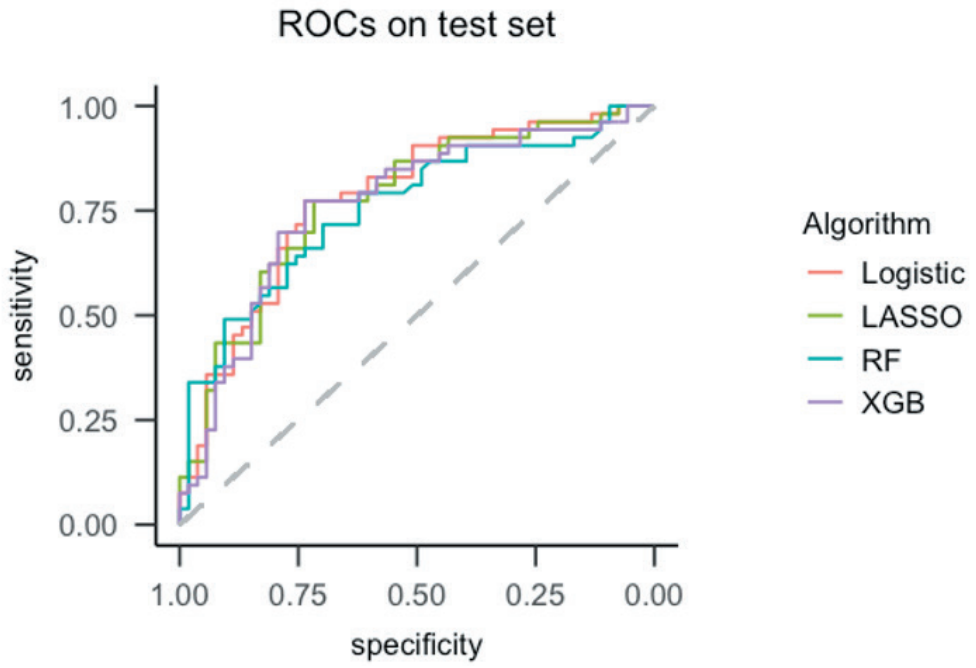
SUPPLEMENTARY FILES

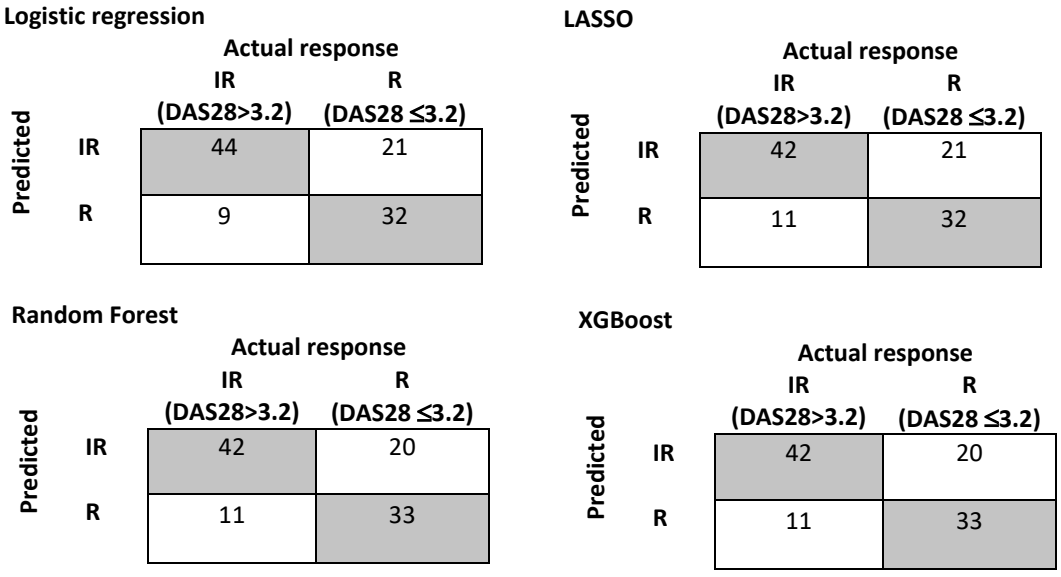
Table S1. Algorithm performances on the train set (N = 249).

Algorithm	AUC
Logistic regression	0.72
LASSO regression	0.73
Random Forest	0.71
XGBoost	0.73

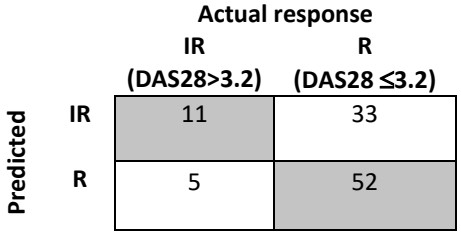
Algorithms performances are the result of 10-fold cross validation on the train set. 50% were insufficient responders. Insufficient response was determined at 3 months (DAS28>3.2).



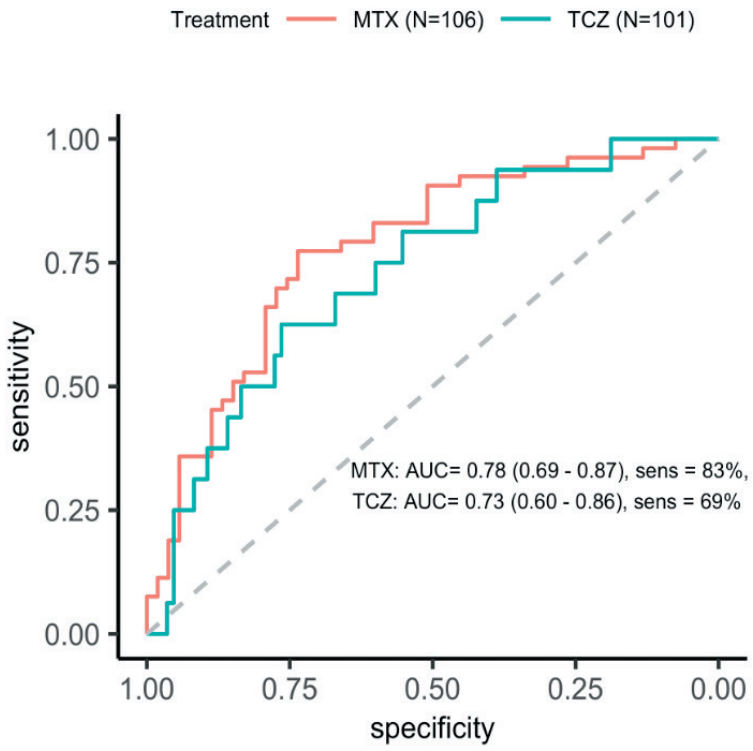
Supplementary Figure S1. ROC curve of models tested on test set (N=106) after feature selection. Features included in the model were: TJC28, HAQ, BMI, smoking, ESR, DMARD/cortico use Logistic= logistic regression, LASSO= LASSO regression, RF= random forest, XGB= XGBoost.



Supplementary Figure S2. Confusion matrices on test set (N=106) after feature selection. Features included in the model were: TJC28, HAQ, BMI, smoking, ESR, DMARD/cortico use. Thresholds were chosen where sensitivity was highest and specificity was at least 0.60. Grey-scale cells are correctly identified patients. IR=insufficient responder (DAS28 >3.2), R=sufficient responder (DAS28 ≤3.2)



Supplementary Figure S3. Confusion matrix of logistic regression on TCZ monotherapy group. IR=insufficient responder (DAS28 >3.2), R=sufficient responder (DAS28 ≤3.2).

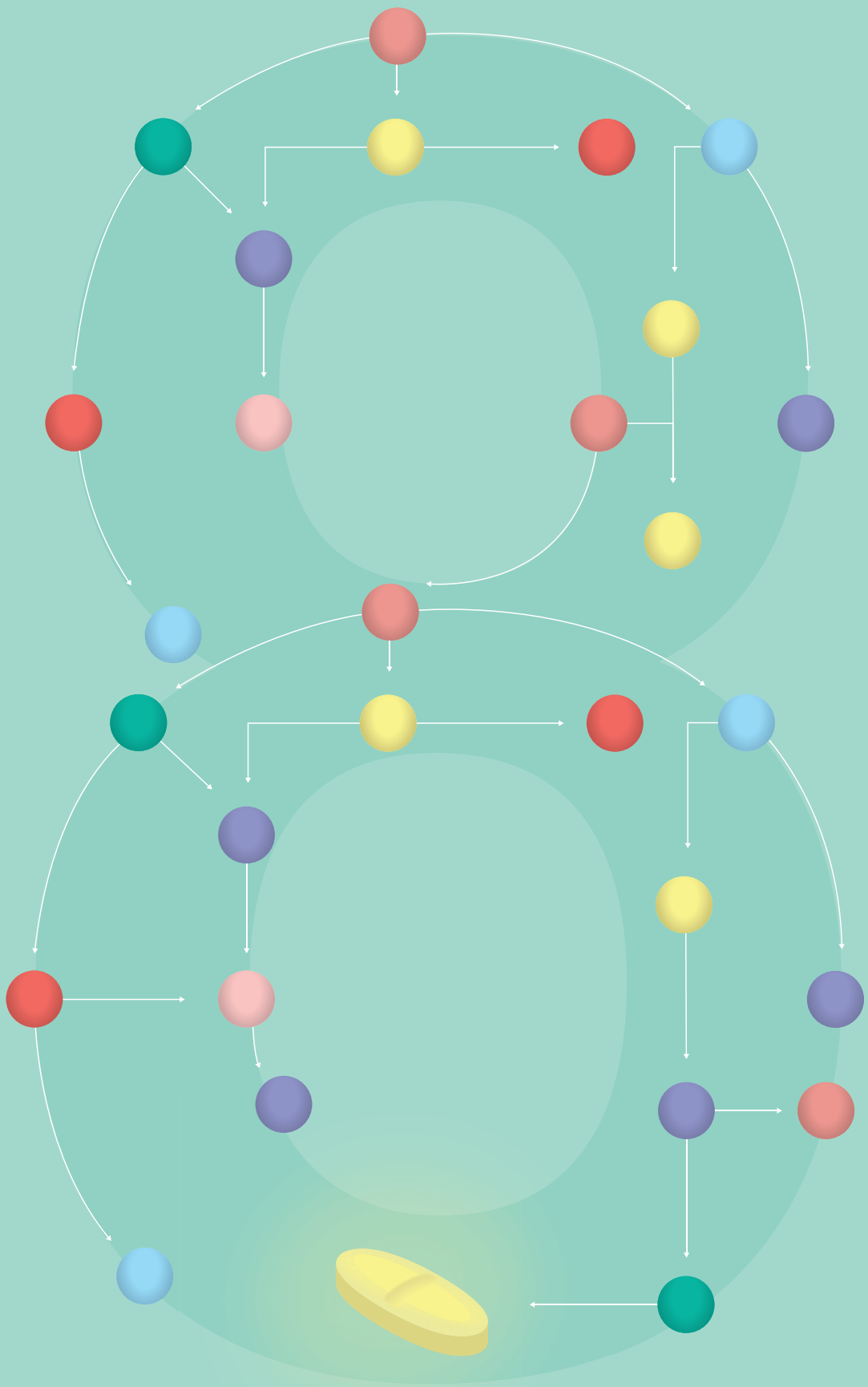


Supplementary Figure S4. ROC curves of performance of final logistic regression model on test set MTX combination therapy and on TCZ monotherapy group. Features included in the model: TJC28, HAQ, BMI, smoking, ESR, DMARD/corticosteroid use. Sensitivity in both groups was determined at its highest point for which the specificity was at least 0.60.

Table S2. Logistic regression on complete dataset (MTX combination + TCZ monotherapy).

	Estimate	St. error	P-value
Intercept	-3.15	0.73	<1.73 x 10-5
Treatment (MTX combi)	0.86	0.82	0.29
Riskfactor	0.04	0.02	0.02
Treatment x riskfactor#	0.03	0.02	0.09

Interaction term between predictors. Predictors included in calculation risk factor: BMI, HAQ, smoking, ESR, TJC28, DMARD/cortico use.



Chapter 8

Discussion
and future perspectives

DISCUSSION AND FUTURE PERSPECTIVES

The main objective of this thesis was to develop a prediction model to identify early rheumatoid arthritis (RA) patients prior to treatment that will insufficiently respond to MTX therapy. Secondary aims were: 1) to examine new potential epigenetic and metabolic biomarkers for insufficient response, 2) to externally validate a previously developed prediction model, 3) to assess if prediction could be improved using machine-learning algorithms compared to classical multivariable logistic regression. The main findings to these aims are presented in Figure 1. In order to identify potential epigenetic biomarkers, we first assessed whether stored blood samples could be used for epigenetic analyses.

STABILITY EPIGENETIC MARKERS UPON STORAGE

From a longitudinal study in 90 individuals we observed that global DNA methylation and DNA hydroxymethylation are stable over at least 18 months when stored at -80°C. At -20°C a slight decrease (-6.1%) in DNA methylation stability was observed in stored DNA samples. Global DNA hydroxymethylation on the other hand was more stable in DNA samples compared to stored blood samples after 6 months, independent of storage temperature. Freezing and thawing of blood or DNA samples did not influence the stability of global DNA methylation or DNA hydroxymethylation up to at least 3 cycles, which is in accordance with a results of a study by Li *et al.* [1]. Even though, stored samples can be used for association studies, fresh samples are still preferred over stored samples as these deliver higher DNA yield [2,3]. However, if fresh samples are not available, we recommend the usage of DNA samples stored at -80°C for global epigenetic association studies.

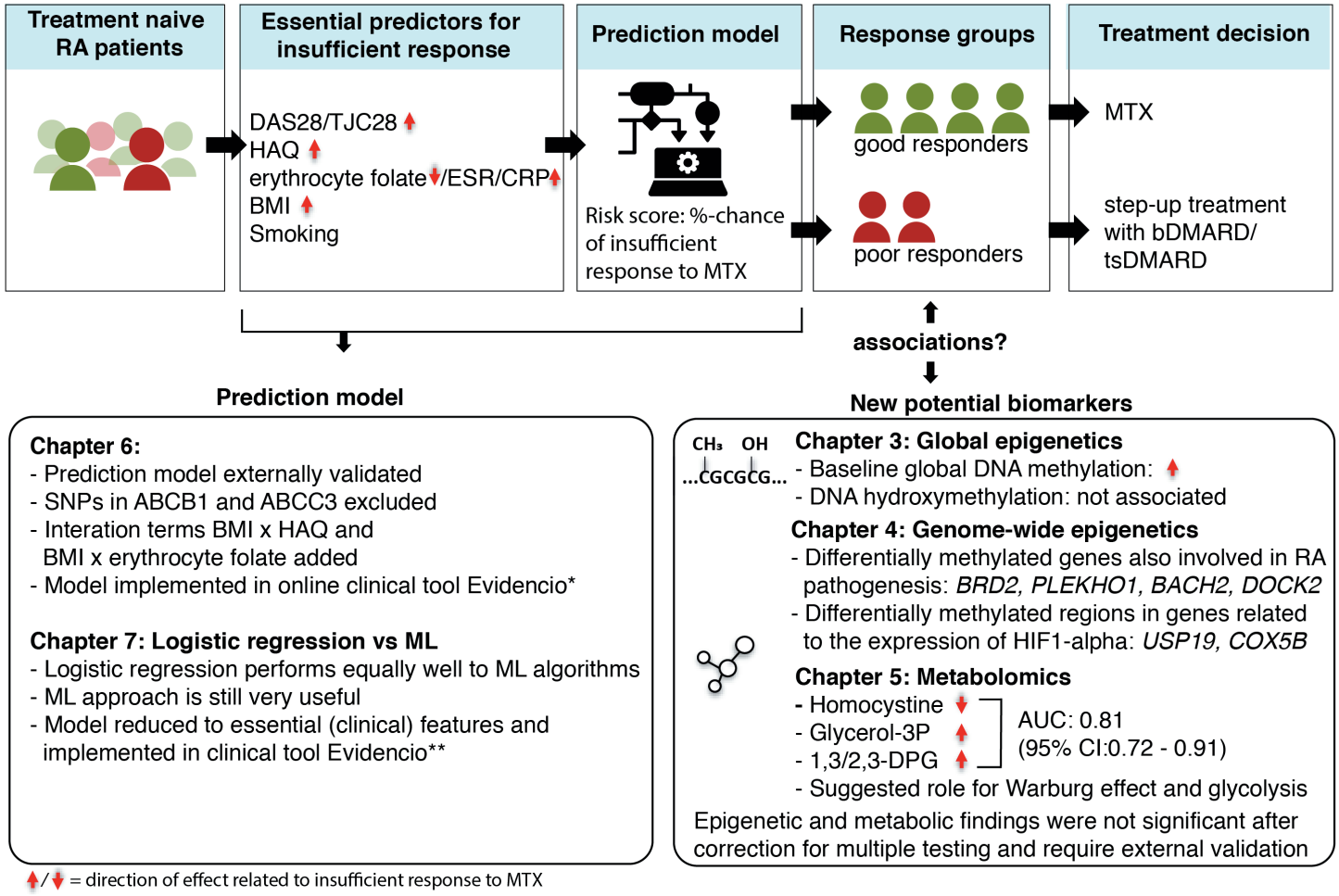


Figure 1. Schematic overview of main findings discussed in this thesis. Abbreviations: DAS28= disease activity score 28, TJC28= tender joint count 28, ESR= erythrocyte sedimentation rate, CRP= C-reactive protein, BMI= body mass index, MTX= methotrexate, b/tsDMARD= biologic/targeted synthetic disease modifying anti-rheumatic drug, ABCB1/C3= ATP-binding cassette transporter family member B1/C3, ML= machine learning, HAQ= health assessment questionnaire, glycerol-3P= glycerol-3-phosphate, 1,3-DPG/2,3-DPG= 1,3/2,3-diphosphoglyceric acid

*https://www.evidencio.com/models/show/2191 **https://www.evidencio.com/models/show/2415

DETERMINANTS OF INSUFFICIENT RESPONSE TO MTX

Potential epigenetic and metabolic biomarkers for response

In this thesis, we observed baseline differences in global DNA methylation **(chapter 3)**, baseline DNA methylation at genomic positions **(chapter 4)** as well as in baseline metabolite concentrations **(chapter 5)** between good and poor responders (Figure 1). We showed that higher global DNA methylation was significantly associated with insufficient response to MTX after correction for multiple testing, which was validated with a second technique.

Overall, observed effect sizes were medium to small. In the EWAS study, the largest standardized effect size of top hits in the EWAS study could be translated to 0.8 change in SD ΔDAS28 per one SD difference in baseline DNA methylation (*BACH2* gene) **(chapter 4)**, which is similar to a cohen's d between good and poor responders of 0.64. The largest difference in baseline metabolite levels between good and poor responders was <2 log2 fold change, which can be translated to a cohen's d of 0.76 **(chapter 5)**. In the metabolomics and EWAS studies, numerous tests were conducted in parallel on the same group of patients and strict correction for multiple testing may have hampered the finding of significant hits. Overall, considering the small to medium effect sizes observed and the fact that RA is a complex multidisciplinary disease, we consider it unlikely that one of these biomarkers can be used as single predictor for treatment since RA is a complex multidisciplinary disease [4,5].

In **chapter 5** we showed that a combination of 3 metabolites (decreased homocystine, increased glycerol-3-phosphate and increased 1,3-diphosphoglyceric acid/2,3-diphosphogelyceric acid [1,3/2,3-DPG]) together constructed a receiver operating characteristic (ROC) curve with high area under the curve (AUC) of 0.81 (95% confidence interval: 0.72-0.91). Even though these results are promising, results were obtained from a relatively small group with enhanced contrast (41 poor vs 41 good responders), hence external validation is required. When validated, their contribution to the full prediction model could be examined.

Global DNA methylation results investigated in this thesis were already confirmed and internally validated by a second technique. In fact, results of **chapter 3** suggest an additive role for baseline global DNA methylation to the previous published prediction model [6], since the standardized effect size of global DNA methylation (β= 0.15) was similar to that of erythrocyte folate (β= -0.17) and BMI (β= 0.14) in the same model **(chapter 3)** and baseline erythrocyte folate levels and global DNA methylation only weakly correlated (R=0.34, p=0.00061; **chapter 3**); hence, baseline global DNA methylation like erythrocyte folate is an independent predictor for insufficient response to MTX. Both low erythrocyte folate and high BMI were previously identified as predictors for insufficient response to MTX in RA patients [6] and were validated as predictors in **chapter 6**. Whether baseline global DNA methylation can actually improve the predictive power of the model should

be determined by investigating whether adding this predictor to the validated prediction model [7] significantly and independently improves its diagnostic accuracy.

Since epigenetic markers are reversible, treatment could hypothetically also induce epigenetic changes. Provided that this happens during the first few weeks from treatment, epigenetic changes could potentially be used to predict response at a later time point. MTX inhibits methionine-adenosine transferase (MAT) and dihydrofolate reductase (DHFR) in 1-carbon [8–10], which indirectly results in the inhibition of the methyl donor S-adenosyl methionine (SAM). For this reason, MTX was expected to decrease global DNA methylation during MTX treatment. However, we did not observe any changes in global DNA methylation over the first 3 months of treatment and a minor increase in DNA hydroxymethylation was not related to response **(chapter 3).** In contrast to this hypothesis, others observed an increase in global DNA methylation during the first months of MTX treatment in T lymphocytes and monocytes [11–13]. The fact that we did not observe any differences may be explained by differences in cell types assessed, or the fact that patients in our study also received folic acid during MTX treatment. Patients taking folate were excluded in the study of Kim *et al.*, while the folate status in the other two studies was not reported [12,13]. Since 2013, RA patients are recommended to take folic acid during MTX therapy in order to reduce adverse events [14]. Because MTX is an anti-folate, folic acid acts on the same pathway as MTX. Thus, folic acid could hypothetically relieve MTX's inhibition of methyl group donation. The relationship between MTX, folic acid and their effect on global DNA methylation could be further investigated in *in vitro* studies. Until this relationship is better understood, results over time should be interpreted with care.

In contrast to DNA methylation, we do not have any indications that global DNA hydroxymethylation is a potential predictor of response to MTX in RA. No baseline differences were observed in relation to response, nor at – and over 3 months in whole blood leukocytes **(chapter 3)**. Others observed increased expression of baseline ten-eleven translocation (TET) enzymes in T lymphocytes and monocytes of RA patients compared to healthy controls [12]. Nonetheless, in that same study also no baseline differences in global DNA hydroxymethylation were observed. TET enzymes facilitate oxidation of DNA methylation into DNA hydroxymethylation, which is the first step in the demethylation process. TET mediated demethylation of promoter regions is important for gene expression and differentiation. For example, differentiation of naïve CD4 cells into regulatory T cells as well as stable expression of T regulatory require TET mediated demethylation of enhancer regions in *Foxp3* [15–17].

In this thesis, differentially methylated positions (DMP) assessed in the EWAS study **(chapter 4)** were not significant after correction for multiple testing. Nevertheless, some CpGs in or near identified genes (*BRD2, PLEKHO1, BACH2, DOCK2*) could be interesting targets for future studies as these have been associated with IL-6 production, leukocyte differentiation and regulation, which are all important events in the pathogenesis and

development of RA [18–24]. So far, only two other EWAS studies have been performed in relation to MTX response. In T-lymphocytes of RA patients, 2 baseline DMPs were identified in relation to MTX response at 6 months using the 450 k array after extensive filtering of probes to reduce multiple comparisons [25]. These DMPs were situated in the *ADAMTSL2* gene, involved in TGF-β regulation [26] and the *BTN3A2* gene, related to adaptive immune responses[27,28]. Even though, we were not able to replicate these results in PBMCs **(chapter 4)**, decreased ADAMTSL2 expression was observed in fibroblast-like synoviocytes (FLS) after MTX treatment [29]. Besides baseline differences, early epigenetic changes upon MTX treatment were assessed in a 450 k array study on whole blood leukocytes of RA patients [30]. Here, the investigators identified 6 DMPs at 4 weeks of MTX treatment that correlated with DAS28 and/or changes in DAS28 components SJC28 and CRP over 6 months [30]. In the same study, no significant differences were observed at baseline.

Potential links between epigenetic and metabolic findings

Even more interesting than these single DMPs are some potential links between epigenetic and metabolic findings that require further investigation. First of all, two underlying metabolic processes identified in relation to MTX treatment response in **chapter 5** were glycolysis and the Warburg effect. Under normal circumstances, ATP production depends on oxygen and is primarily produced through the mitochondrial oxidative phosphorylation (OXPHOS) pathway. Under hypoxic circumstances, ATP is produced through the less efficient glycolysis pathway resulting in lactate production, which does not require oxygen. However, in cancer cells a switch from the OXPHOS pathway to glycolysis was observed despite the presence of oxygen (aerobic glycolysis). This phenomenon is called the "Warburg effect" [31–33]. The last years, a substantial body of literature already described upregulation of glycolysis in relation to RA development [34–37]. Also, cell specific metabolic signatures and metabolic changes in relation to immune cell responses were investigated in the field of 'immunometabolism' [34,38–41]. The fact that different immune cells comprise different metabolic signatures can be used to specifically target pro-inflammatory cells and thereby restore the balance between pro- and anti-inflammatory cells. Targeting the 'immunometabolism' is therefore currently a widely discussed new treatment option in RA [38,39,42]. Hypothetically, these altered metabolic processes could also influence current treatment responses.

In this thesis, we observed some potential interesting links between epigenetic differences and the Warburg effect/glycolysis that were both related to MTX response. First of all, the Warburg effect is regulated through transcription factor HIF1- α. In **chapter 4,** we identified a potential differentially methylation region (DMR) comprising 6 CpGs in the ubiquitin carboxyl-terminal hydrolase 19 (*USP19*) gene, which encodes an enzyme that rescues HIF1- α degradation [43]. HIF1- α in turn binds and suppresses the proximal promoter of cytochrome c oxidase subunit 5B (COX5B), encoding an enzyme in the mitochondrial electron transport chain (OXPHOS), which was also identified as potential

DMR comprising 2 CpGs in **chapter 4** and favours glycolysis. Moreover, Cronstein and colleagues described that MTX indirectly inhibits NF-kB in T-cells via the induction of long intergenic non-coding RNA p21 (lncRNA-p21) [10]. LncRNA-p21 in turn upregulates HIF1- α under hypoxic circumstances [10]. Moreover, a targeted DNA methylation study in regulatory T cells showed increased DNMT expression accompanied by elevated DNA methylation in an upstream enhancer of the FoxP3 region upon MTX treatment. This resulted in increased expression FoxP3 and CTLA-4 and restored the suppressive function of T regulatory cells [44,45], yet CTLA-4 expression can also decrease glycolysis in T-cells [46]. Finally, a study in fibroblast-like synoviocytes (FLS) showed that methotrexate could significantly reduce hexokinase-2 (HK2) expression which is important for glycolysis [29,47]. These studies suggest that immunometabolism in RA could be accompanied by epigenetic changes and together may result in cellular phenotypes that are associated to insufficient response to MTX treatment. Such metabolic and epigenetic interplay has also been earlier described in cancer [48,49] and requires further investigation in RA as well as in relation to therapy response.

Hitherto, the field of epigenetic biomarkers in relation to RA response is still at the beginning. Meanwhile, the field is still evolving; new platforms are already available that can distinguish DNA methylation from DNA hydroxymethylation at genomic positions [50]. This may reveal new insights on differentially expressed genes as DNA methylation and hydroxymethylation could have distinct regulatory roles [51]. A study in osteoarthritic (OA) chondrocytes already showed multiple differentially DNA hydroxymethylated positions in key OA genes that could be distinguished from healthy chondrocytes [52]. Also, the increased TET enzyme expression levels that were observed at baseline in RA PBMCs compared to healthy controls [12] could be related to increased hydroxymethylation at specific genomic positions which was not assessed. Generally speaking, more epigenetic studies are required to better understand underlying differences related to treatment response in RA. To do so, material and cell type considerations are required, as described below.

Tissue considerations for epigenetic studies

The use of whole blood for DNA methylation association studies and specifically in RA requires some considerations. First of all, whole blood is a cell mixture consisting of granulocytes (i.e. neutrophils, eosinophiles, basophiles and mast cells) and PBMCs (i.e. lymphocytes, monocytes and macrophages). Due to the different cellular functions, different cell types comprise different DNA methylation signatures [53,54], hence adjustment for cell type composition in association studies is highly important. Since cell type composition measurements are not always available, a widely used measure described by Houseman et al. could be used to predict cell types from DNA methylation signatures [55]. Importantly, this method uses a reference-based algorithm which is based on sorted cell type methylation patterns of only six healthy male donors[54]; hence, this method is not

perfect. For future studies, a recently developed package: tensor composition analysis (TCA), which is able to reveal cell-type specific associations from bulk methylation data may be a better alternative [56]. Secondly, RA is an infiltrative disease where leukocytes (including: granulocytes, CD4+, CD4- T-lymphocytes, B-lymphocytes) migrate from peripheral blood towards the synovium due to increased expression of adhesion molecules and chemokines [57–59]. In the synovium, these cells produce proinflammatory cytokines resulting in a constant state of inflammation affecting the joints [57]. Sorted cells from biopsies of the site of action (e.g. infiltrated T lymphocytes or synovial fibroblasts) may therefore be better representatives of the disease pathogenesis compared to PBMCs and may therefore be better predictors for response to MTX. In fact, even similar cell types from different locations in the body could have different methylome signatures, which stresses the importance of the place of biopsy taken [60]. On the contrary, also baseline differential methylated positions in peripheral blood isolated T – lymphocytes have been related to response to MTX [25] and peripheral blood naïve T – lymphocytes and fibroblast like synoviocytes in RA were observed to share hypermethylated sites [61]. This suggests that peripheral blood lymphocytes could still be useful although results from sorted cells are probably more specific. On-going single cell analyses therefore may give more insight in the role of specific cellular subsets and underlying processes [62,63].

In addition, DNA methylation and hydroxymethylation are only a subset of epigenetic modifications. Other modifications that could influence gene regulation are for instance histone modifications [64] and microRNAs which were predictive for insufficient response to MTX [65–69], etanercept [70] and Infliximab [71]. Epigenetic modifications can in turn affect pre-mRNA splicing [72,73], resulting in alternative splicing variants. For instance a partial retention of intron 8 (8PR)/wild-type in the FPGS, leading to abrogated FPGS enzyme required for MTX-polyglutamation formation was associated with MTX resistance in acute lymphoblastic leukemia (ALL) and non-response to MTX in RA patients [74,75]. Also for these epigenetic markers, above-mentioned suggestions should be taken into consideration.

Integration of -omic analyses

In this thesis, we primarily discussed epigenetic and metabolic predictors and how these could be intertwined in pathways in relation to treatment response. However, ideally, not only epigenetic and metabolomic data are combined, but instead data of multiple –omic fields (genomics, epigenomics, transcriptomics, proteomics and metabolomics) should all be integrated in multi -omics analyses, providing a full picture of interactions between underlying processes and networks in relation to MTX response. This has already shown promising results in the COMBINE study in relation to anti-TNF response [76] and in the prediction of adalimumab and etanercept response using integration of DNA methylation and RNA sequencing results [77]. At the same time, larger cohorts should be formed. Either in the form of meta-analyses: combining patient results, or by the inclusion of many more patients in centralized consortia. An example of such a consortium is the "Maximising

Therapeutic Utility for Rheumatoid Arthritis (MATURA)" consortium in the UK that consists of national collaborations to examine biomarkers for drug response in RA patients [78].

PREDICTION MODELS FOR INSUFFICIENT RESPONSE

Validation and integration of prediction models

In the end, results from integrated analyses could lead to better understanding and maybe lead to specific markers that can be used as biomarkers or used in prediction models. In this thesis, a previously developed prediction model was validated in an external cohort **(chapter 6)** and logistic regression could equally well predict insufficient response to MTX compared to machine-learning algorithms. From **chapter 6 and 7,** we can conclude that clinical parameters (e.g. DAS28, TJC28) were strongest predictors for insufficient response to MTX in RA, followed by BMI, HAQ and smoking. ABCB1 genotype previously identified did not contribute to the predictive power of the model in the current thesis when validated in a larger cohort **(chapter 6)**. The contribution of ABCB3 genotype on the other hand was debatable **(chapter 6),** and the LASSO algorithm did select this feature for prediction in **chapter 7**. However, the model performed equally well when ABCB3 genotype was replaced for ESR/CRP concentrations, which are easier to assess in a clinical setting.

To date, results from targeted SNP analyses have been inconsistent and results of GWAS studies have been disappointing in the prediction of insufficient response to MTX in RA patients [79–81]. Most evidence for a relation between SNPs and treatment efficacy has been observed for SNPs in ATIC, DHFR, FPGS, MTHFR, MTR, SLC19A1/RFC1 and TYMS genes [79]. However, in the largest GWAS study so far (N=1424 RA patients), no SNPs reached genome-wide significance in relation to changes in DAS28 over 6 months nor in relation to changes in single DAS28 components (SJC28, TJC28, CRP) [82]. Also, in a study on the MATURA consortium, the authors compared 11 methods on the ability to predict treatment response in RA patients using genome-wide SNP data. Again, SNPs only contributed very little to the predictive power of the model compared to a model with clinical variables alone [83]. Experts in the field agree that SNPs are probably not going to be single predictors for response [79,83], yet they could potentially be combined with other predictors for instance by using polygenic risk scores [84]. Especially, models that assumed a more complex underlying genetic architecture and models created on large (simulated) sample sizes achieved slightly better [83], although other predictors seem much stronger as was also observed in this thesis (**chapter 6** and **7**). So far, in this thesis we have validated two prediction models for insufficient response that can be tested in a clinical setting. Both models (with and without laboratory predictors) were uploaded in Evidencio [85]. A prediction model is always a trade-off between sensitivity and specificity and the choice for best cut-off value depends on the clinical goal. In this thesis, we aimed to identify as many insufficient responders as possible (sensitivity= 81%), with a reasonable high specificity

(at least > 60%) **(chapter 7)**. However, another cut-off value could be chosen with higher specificity, allowing good responders to safely start MTX therapy while some insufficient responders will be missed. This would still be a win in comparison to the current "trial and error" treatment strategy in which every patient starts MTX for at least 3 – 6 months until they appear to be insufficient responders. These patients could potentially benefit from immediate step-up treatment with for instance abatacept [86] or JAK-inhibitors [87–89]. Whether or not to step-up treatment prior to MTX initiation should always be a shared-decision between clinician and patient, weighing the risk of potential adverse events and potential benefit of step-up treatment [90–93].

Such a shared-decision between clinician and patient could also improve patient adherence to treatment [94]. Lack of treatment adherence complicates the search for biomarkers to treatment response in RA [95], since patients that do not take their medication are falsely reported as non-responders. Important factors for non-adherence are disease duration and higher disease activity [95,96]. On the contrary, good counselling by the clinician and patient education could improve adherence [97] as well as low disease activity, which stresses the need for quick control of the disease activity [98]. Predictive scores for insufficient response according to a prediction model could support shared-decision making between clinician and patient as described in **chapter 6.** A low score for prediction of insufficient response could maybe help clinicians to prescribe MTX with more certainty and win patient's trust in treatment and in that way improve adherence. Similarly, patients that are predicted good responders may feel more urges to adhere to their medication to reach the predicted goal.

IMPLICATIONS AND FUTURE STUDIES

From this thesis we can conclude that it is unlikely that single predictors are strong enough to predict MTX treatment response in such a complex disease as RA. It would probably be better to capture the whole "pathogenic signature" combining –omic results to get a good understanding of underlying processes that could predict someone's response to treatment. Even though, logistic regression performed equally well in prediction of insufficient response compared to machine-learning algorithms in case of our clinical prediction model, machine-learning algorithms are still preferred when integrating larger datasets and more complex (non-linear) relationships and could therefore be used for the integration of -omic datasets [99–101] or system biology, which includes dynamic interactions between genes, proteins and metabolites to gain a better understanding of an organism [102]. Moreover, integration of data from electronic health records with –omic data using machine learning algorithms (translational bioinformatics) could facilitate the translation to clinical implementation [103,104]. Finally, while the search for more accurate biomarkers continues we have developed two prediction models for insufficient response to MTX in RA of which the first one was internally [6] and externally

validated [7]. The second model was enhanced using machine-learning methods, where we used internal cross-validation and the model was tested on an unseen data set. In spite of the fact that both prediction models are ready to be tested in a clinical setting, the latter only contains clinical variables and is therefore easiest to execute. For instance in a directive approach, where a control group (standard of care) could be compared to a prediction model guided group that either start MTX (standard of care) for predicted responders or MTX + bDMARD for predicted insufficient responders [105].

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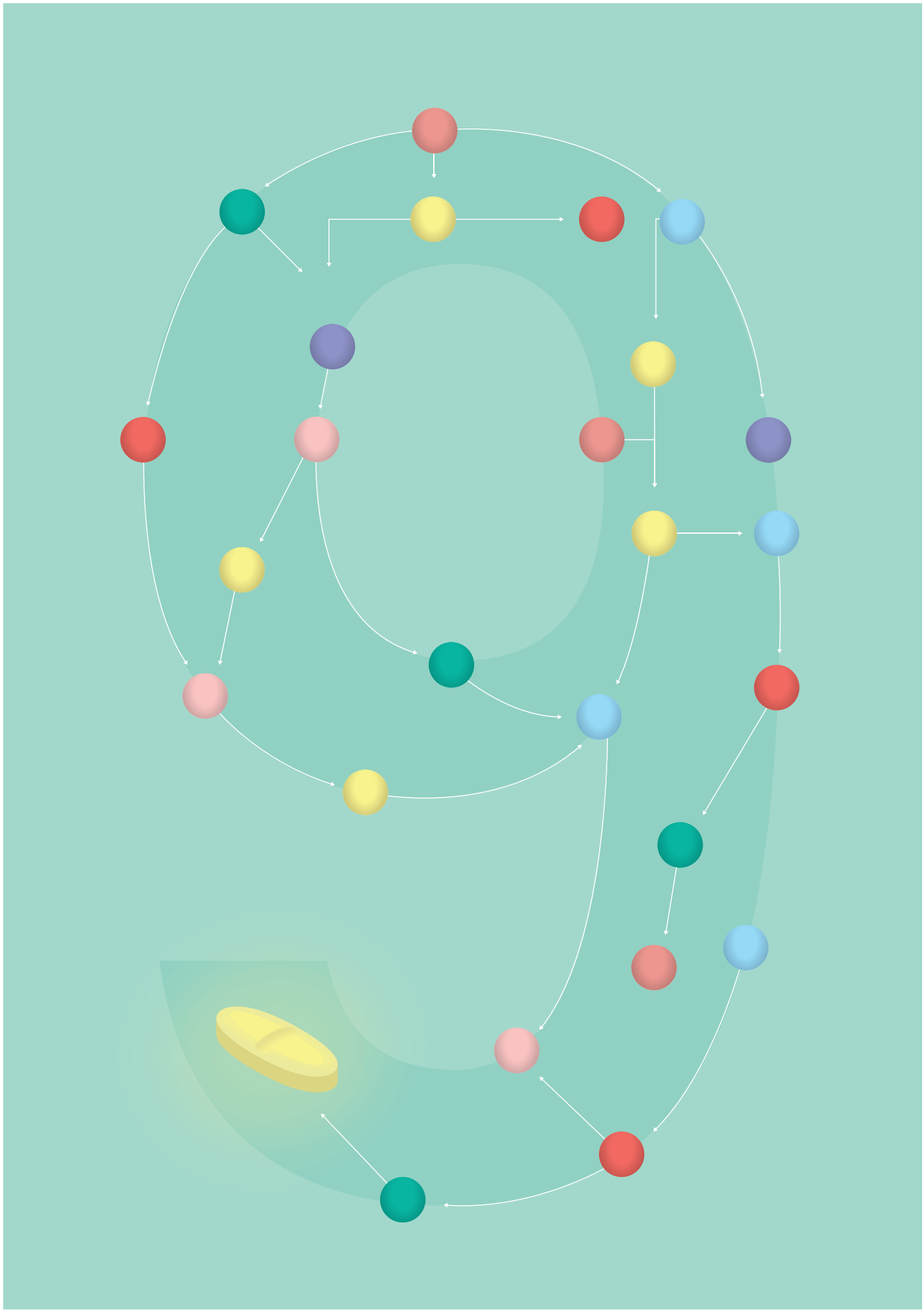


Chapter 9

Summary & samenvatting

ENGLISH SUMMARY

Methotrexate (MTX) is first-line therapy in Rheumatoid Arthritis (RA). Despite its relatively high response rates and safety, up to 40% of RA patients do not benefit from MTX and require step-up treatment with biologic or targeted synthetic disease modifying anti rheumatic drugs (b/ts DMARDs). Up to now, treatment response can be determined the soonest at 3 to 6 months from treatment initiation. Since early control of the disease activity results in better long-term outcomes, insufficient responders would benefit from sufficient treatment from the start. To identify insufficient responders prior to MTX treatment initiation there is a need for determinants of insufficient response. This would allow the use of personalized medicine in which MTX is only prescribed in case patients have a high chance of successful response and if not they could step-up treatment directly from the start. In the first part of this thesis we assessed epigenetic and metabolic determinants for insufficient response to MTX. In the second part, we validated a previously developed prediction model for insufficient response to MTX in RA and we assessed the use of machine-learning algorithms compared to conventional multivariable logistic regression in such clinical prediction models.

Part I

Regarding a role for DNA methylation as biomarker in response to MTX, in **chapter 2** we first investigated whether stored samples could be used for epigenetic association studies. We showed in a longitudinal study (n=90) that global DNA methylation and hydroxymethylation where stable when assessed in blood or DNA samples stored up to 18 months at -80°C. DNA methylation in DNA samples stored at -20°C showed a slight decrease after 18 months, while the stability of DNA hydroxymethylation was independent of temperature and more stable in DNA samples compared to blood. Freezing and thawing up to 3 cycles of blood and DNA samples stored at -80°C did not affect the stability. Hence, DNA samples stored at -80°C can be used best for global DNA (hydroxy)methylation association studies.

The association between global DNA (hydroxy)methylation and MTX response in leukocytes of 294 RA patients was assessed in **chapter 3**. We observed that higher baseline global DNA methylation was significantly associated with insufficient response to MTX. This finding was confirmed with a second technique. Global DNA hydroxymethylation on the other hand was not associated with MTX response at baseline or at- and over the first three months of treatment. Since MTX inhibits 1-carbon metabolism that provides methyl groups for DNA methylation, MTX was expected to reduce DNA methylation over time, which we did not observe. In fact, others found the opposite result. This can be explained by the fact that whole blood was assessed, which is a composite of different cell types, that comprise different methylomes or by the prescription of folic acid 24 hours after MTX therapy to reduce adverse events, which acts on the same pathway and could interfere

with MTX's actions on DNA methylation. However, the relationship between erythrocyte folate, MTX and their effect on global DNA methylation is not completely understood and should be further investigated in *in vitro* studies. Baseline global DNA methylation was only weakly correlated with erythrocyte folate at baseline and therefore acted as independent predictor for insufficient response to MTX. In future studies, baseline global DNA methylation can be further assessed as predictor for insufficient response in the full prediction model.

The assessment of global DNA methylation was taken a step further in an epigenome-wide association study (EWAS), where we investigated differences in DNA methylation at genomic positions (DMP) or regions (DMR) in 69 RA patients in relation to response to MTX (**chapter 4**). As there were no large differences observed in DNA methylation in relation to response and many tests (>850,000) were performed in relation to the limited number of patients, no genome-wide significant hits were observed. Still, some DMPs identified in or near genes related to the pathogenesis of RA (i.e. *BRD2, PLEKHO1, BACH2, DOCK2*) could be interesting to further investigate in future studies. Also, DMRs in genes related to the expression of HIF1-alpha (i.e. *USP19, COX5B*) are interesting as this is an important regulator of glycolysis and the Warburg effect, two metabolic processes that have been upregulated in RA.

In **chapter 5** we aimed to identify metabolic determinants that could discriminate 41 insufficient responders to MTX from 41 good responders to MTX in an untargeted metabolomics study. Single metabolites that could best discriminate these two response groups were: homocystine, glycerol-3-phosphate and 1,3/2,3-biphosphoglyceric acid. However, these findings should first be confirmed in a larger patient group as the group size was limited and findings were not significant after correction for multiple testing. Underlying metabolic events identified in relation to MTX response were glycolysis and the Warburg effect.

Taken together the results of the EWAS and metabolomics study, these suggest that underlying epigenetic and metabolic pathways/events could play a role in MTX response rather than single determinants. Integration of multiple -omic studies is required to gain more insight into underlying processes that may explain the difference in response to MTX in RA. A better understanding of underlying events could contribute to a more specific search for new biomarkers that can be added to a prediction model for insufficient treatment response.

Part II

In the second part of this thesis, we externally validated a previously developed prediction model for insufficient response **(chapter 6).** The model included baseline DAS28, health assessment questionnaire (HAQ), erythrocyte folate, BMI, smoking and two single nucleotide polymorphisms (SNPs) in MTX transporter genes *ABCB1* and *ABCC3*. The two SNPs did not sufficiently contribute to the predictive power of the model and were

excluded to facilitate clinical implementation of the model. Interaction terms between HAQ and erythrocyte folate and HAQ and BMI improved the predictive power of the model and were therefore included. From the final model a receiver operating characteristic (ROC) curve was constructed with a high area under the curve (AUC) of 0.75 and 95% confidence interval (CI) of 0.69 – 0.81. Subsequently, the model was uploaded in the online tool *Evidencio* for clinical use and to facilitate the possibility for other researchers to externally validate the model on their own dataset.

In **chapter 7** we compared the use of multivariable logistic regression to machine-learning algorithms in the prediction of insufficient response to MTX in RA. We showed that logistic regression equally performed to advanced machine-learning algorithms. Importantly, the dataset consisted of straightforward linear relationships. In case of higher dimensional data and non-linear interactions the use of machine-learning algorithms is recommended. Apart from the algorithms, the approach that is used in machine learning including: 1) the intention to *predict* results 2) the use of internal cross-validation and 3) feature reduction, lead to more validated, generalizable prediction models and enhance clinical implementation. The final model in **chapter 7** performed better on the MTX (combination) therapy group (for which it was developed) than on a RA patient group starting toculizumab (TCZ) monotherapy, suggesting that the model predicts response specifically to MTX (combination) therapy. The following baseline clinical predictors were included in the final model: DAS28/tender joint count 28 (TJC28), HAQ, erythrocyte sedimentation rate (ESR)/C-reactive protein (CRP), BMI and smoking. The AUC of the ROC constructed from the final model was 0.78 (95% CI = 0.69-0.87), and this model was also uploaded in *Evidencio*.

This thesis contributed to the search of new biomarkers and the prediction of insufficient response to MTX to move towards personalized medicine in RA. We identified high baseline global DNA methylation as potential determinant of insufficient response to MTX. Other single epigenetic and metabolic determinants that were identified in this thesis first require external validation. Results of integrated –omic studies could give more insight in underlying events related to MTX insufficient response, such as glycolysis and the Warburg effect identified as suggestive underlying events in this thesis. These could lead to a more specific direction of where to find potential new determinants for insufficient response. Furthermore, previously developed prediction model for insufficient response to MTX in RA was externally validated. This model together with a new clinical model that was optimized using a machine-learning approach were integrated in an online tool *Evidencio* and can be tested in a clinical study for shared-decision making between clinician and patient to enable personalized medicine.

NEDERLANDSE SAMENVATTING

Methotrexaat (MTX) is de eerstelijnsbehandeling in rheumatoïde artritis (RA) patiënten. Ondanks de hoge veiligheid van MTX en het gegeven dat het bij de meeste patiënten goed aanslaat, heeft tot 40% van de patiënten geen baat bij het gebruik van MTX en is additionele behandeling nodig met biologische of synthetische medicijnen (biological/ targeted synthetic disease-modifying antirheumatic drugs: b/tsDMARD's). Op dit moment kan de werking van het MTX niet eerder dan 3 tot 6 maanden na de start van de behandeling bepaald worden. Aangezien vroege controle van de ziekteactiviteit leidt tot betere lange termijn uitkomsten, zouden onvoldoende responders van MTX er baat bij hebben om direct vanaf het begin een intensievere behandeling te starten. Om voorafgaand aan de MTX behandeling te bepalen wie er onvoldoende gaat reageren, moeten eerst determinanten/voorspellers van onvoldoende response geïdentificeerd worden. Aan de hand van deze determinanten is gepersonaliseerde geneeskunde mogelijk waarin MTX alleen wordt voorgeschreven aan patiënten met een hoge kans op een succesvolle respons. Aan patiënten met een lage kans op succesvolle respons op MTX kan de behandeling direct vanaf het begin verzwaard worden met additionele therapie. In het eerste deel van dit proefschrift hebben we epigenetische en metabole determinanten onderzocht voor onvoldoende respons op MTX. In het tweede deel hebben we een eerder ontwikkeld predictiemodel gevalideerd voor onvoldoende respons op MTX bij RA en hebben we het gebruik van machine-learning algoritmen vergeleken met conventionele multivariabele logistische regressie in dergelijke klinische predictiemodellen.

Deel I

Met betrekking tot een rol voor DNA methylering als biomarker voor respons op MTX, hebben we in **hoofdstuk 2** onderzocht of DNA (hydroxy)methylering stabiel is wanneer opgeslagen bij -20°C en -80°C, zodat het gebruikt kan worden voor epigenetische associatie studies. We hebben in een longitudinale studie (n=90) laten zien dat globale methylering en hydroxymethylering stabiel zijn in bloed en DNA monsters opgeslagen voor 18 maanden bij -80°C. DNA methylering bepaald in DNA monsters opgeslagen bij -20°C lieten na 12 maanden een lichte afname zien, terwijl de stabiliteit van globale DNA hydroxymethylering onafhankelijk was van de opslag temperatuur maar stabieler is in opgeslagen DNA monsters vergeleken met bloed monsters. Het herhaaldelijk vriezen en dooien van bloed en DNA monsters opgeslagen bij -80°C had geen invloed op de stabiliteit van globale DNA (hydroxy)methylering tot ten minste 3 cycli. Derhalve concluderen wij dat voor epigenetische studies het best DNA opgeslagen kan worden bij -80°C.

In **hoofdstuk 3** is onderzocht of globale DNA (hydroxy) methylering voor start van methotrexaat therapie geassocieerd is met de respons. Hogere baseline globale DNA methylering was significant geassocieerd met onvoldoende MTX respons. Deze bevinding werd bevestigd met een tweede techniek. Globale DNA hydroxymethylering

was daarentegen niet geassocieerd met MTX respons. Aangezien MTX het 1-koolstofmetabolisme remt, dat methylgroepen levert voor DNA methylering, werd verwacht dat DNA methylering zou afnemen na behandeling met MTX. In onze studie zagen we daarentegen geen significante veranderingen in DNA methylering tijdens de behandeling met MTX. Deze resultaten kunnen verklaard worden door het feit dat wij naar volbloed gekeken hebben dat uit verschillende soorten bloedcellen bestaat, die elk verschillende methyleringsprofielen bevatten. Ook foliumzuur wat 24 uur na MTX therapie wordt voorgeschreven om bijwerkingen te verminderen in onze patiënten zou de methylering beïnvloed kunnen hebben. De relatie tussen foliumzuur, MTX en hun gezamelijke effect op globale DNA methylering is echter niet volledig duidelijk en zou verder onderzocht kunnen worden in *in vitro* studies. Baseline globale DNA methylering was slechts zwak gecorreleerd met erythrocyt folaat en fungeerde daarom als onafhankeijke voorspeller voor onvoldoende respons op MTX. Baseline globale DNA methylering zal in toekomstige studies verder onderzocht kunnen worden als voorspeller voor onvoldoende respons in het volledige predictiemodel.

Globale DNA methylering werd nader onderzocht in een epigenoom-brede associatiestudie (EWAS). In deze EWAS hebben we gekeken naar differentieel gemethyleerde posities (DMP) en regio's (DMR) in het genoom van 69 RA patiënten in relatie tot MTX respons **(chapter 4).** We vonden geen grote verschillen in DNA methylering tussen responders en non-responders op MTX. Vanwege het grote aantal tests (> 850.000) dat werd uitgevoerd in verhouding tot het beperkte aantal patiënten, werden geen significante hits waargenomen na correctie voor meervoudig testen. Toch kunnen sommige DMP's interessant zijn om nader te onderzoeken in toekomstige studies, bijvoorbeeld DMPs die in of nabij genen liggen die eerder gerelateerd zijn aan de pathogenese van RA (*BRD2, PLEKHO1, BACH2, DOCK2*). Ook zijn DMR's in genen gerelateerd aan de expressie van HIF1-alfa (*USP19, COX5B*) interessant omdat dit een belangrijke regulator is van glycolyse en het Warburg-effect, twee metabolische processen die verhoogd zijn in RA patiënten.

In **hoofdstuk 5** hebben we een metabolomics-studie uitgevoerd om metabole determinantent te identificeren die onvoldoende responders op MTX (N=41) van goede responders (N=41) zouden kunnen onderscheiden. Metabolieten die deze twee responsgroepen het beste konden onderscheiden waren: homocystine, glycerol-3-fosfaat en 1,3/2,3-difosfoglycerinezuur. Deze bevindingen zullen echter eerst in een grotere patiëntengroep bevestigd kunnen worden, aangezien de groepsgrootte beperkt was en de bevindingen niet significant waren na correctie voor het uitvoeren van meervoudige testen. Onderliggende metabole routes die werden geïdentificeerd in verband met MTX-respons waren glycolyse en het Warburg-effect. De resultaten van de EWAS- en metabolomics-studie samengenomen, suggereren dat onderliggende epigenetische en metabole routes een rol zouden kunnen spelen bij MTXrespons in plaats van op zichzelf staande determinanten. Integratie van meerdere -omic studies is vereist om meer inzicht

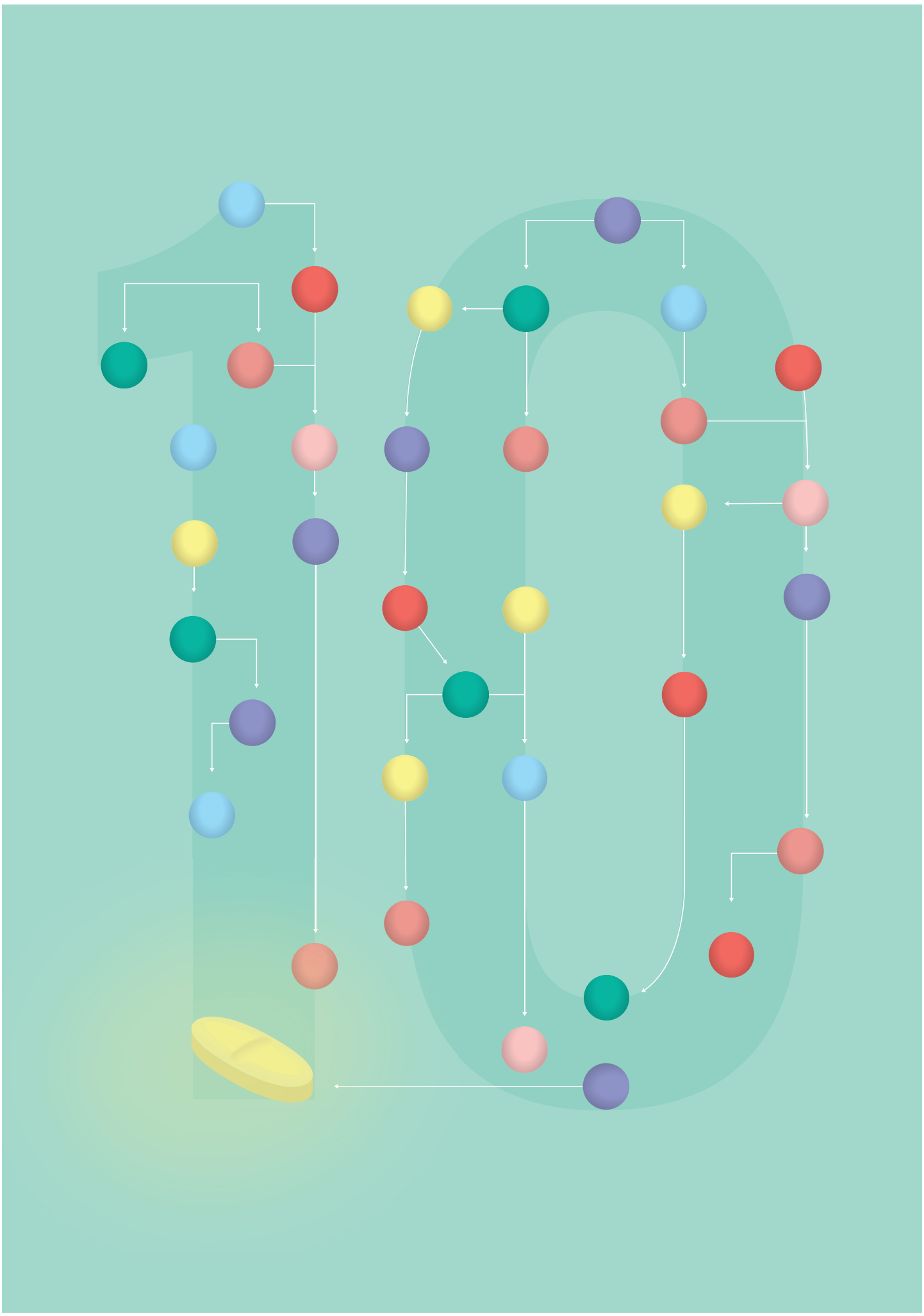
te krijgen in onderliggende processen die het verschil in respons op MTX bij RA kunnen verklaren. Een beter begrip van onderliggende gebeurtenissen zou kunnen bijdragen aan een specifiekere zoektocht naar nieuwe biomarkers die kunnen worden toegevoegd aan een predictiemodel voor onvoldoende respons op MTX behandeling.

Deel II

In het tweede gedeelte van dit proefschrift hebben we eerst een eerder ontwikkeld predictiemodel voor onvoldoende respons op MTX behandeling extern gevalideerd **(hoofdstuk 6)**. Het predictiemodel bevat de volgende variabelen: baseline DAS28, een gezondheidsbeoordelingsvragenlijst (HAQ), erytrocytfolaat, BMI, roken en twee enkele nucleotide polymorfismen (SNP's) in MTX-transportgenen *ABCB1* en *ABCC3*. De twee SNP's droegen onvoldoende bij aan de voorspellende kracht van het model en werden uit het predictiemodel gehaald om de klinische implementatie van het model te bevorderen. Interactietermen tusen HAQ en erytrocytfolaat en HAQ en BMI verbeterden de voorspellende kracht van het model en werden daarom juist in het model opgenomen. Op basis van het uiteindelijke model werd een Receiver Operating Characteristic (ROC) curve geconstrueerd met een groot oppervlakte onder de curve ('area under the curve'; AUC) van 0,75 en een 95% betrouwbaarheidsinterval van 0,69 - 0,81. Vervolgens werd het model geüpload in de online tool *Evidencio* voor klinisch gebruik en om het voor andere onderzoekers mogelijk te maken om het model extern te valideren op hun eigen dataset. Vervolgens hebben we in **hoofdstuk 7** het voorspellend vermogen van multivariabele logistische regressie met machine-learning algoritmen vergeleken bij het voorspellen van onvoldoende respons op MTX bij RA. We hebben aangetoond dat logistische regressie even goed de response op MTX voorspelt als geavanceerde machine-learning algoritmen. Belangrijk is dat de dataset bestond uit eenvoudige lineaire relaties. In het geval van hoger dimensionale gegevens en niet-lineaire interacties wordt het gebruik van machine-learning algoritmen aanbevolen. Over het algemeen leidt machine learning tot beter gevalideerde en generaliseerbaardere predictiemodellen wat bevorderend is voor klinische implementatie. Dit komt met name doordat machine-learning modellen gemaakt worden met als doel om een uitkomst te *voorspellen* en doordat er gebruik gemaakt wordt van interne validatie. Het best presterende model in **hoofdstuk 7** presteerde beter op de MTX (combinatie) therapiegroep (waarvoor het werd ontwikkeld) dan op een RA-patiëntengroep die startte met toculizumab (TCZ) monotherapie, wat suggereert dat het model specifiek de respons op MTX (combinatie) therapie voorspelt. De volgende klinische baseline voorspellers werden opgenomen in het uiteindelijke model: DAS28/aantal pijnlijke gewrichten 28 (tender joint count 28: TJC28), HAQ, erythrocytbezinkingssnelheid (BSE) / C-reactief proteïne (CRP), BMI en roken. De AUC van de ROC geconstrueerd op basis van het uiteindelijke model was 0,78 (95% BI = 0,69-0,87), en dit model werd ook geüpload in *Evidencio*.

Dit proefschrift heeft bijgedragen aan de zoektocht naar nieuwe biomarkers en het

voorspellen van onvoldoende respons op MTX en is een stap richting gepersonaliseerde geneeskunde in RA. We identificeerden baseline globale DNA-methylering als mogelijke determinant voor onvoldoende respons op MTX. Andere afzonderlijke epigenetische en metabole determinanten die in dit proefschrift werden geïdentificeerd, vereisen eerst externe validatie. Resultaten van geïntegreerde –omic studies zouden meer inzicht kunnen geven in onderliggende gebeurtenissen gerelateerd aan onvoldoende respons op MTX, zoals glycolyse en het Warburg-effect welke werden geïdentificeerd als suggestieve onderliggende gebeurtenissen in dit proefschrift. Deze zouden in een specifiekere richting kunnen wijzen waar potentiële nieuwe determinanten voor onvoldoende respons kunnen worden gevonden. Daarnaast, werd het eerder ontwikkelde predictiemodel voor onvoldoende respons op MTX bij RA extern gevalideerd. Dit predictiemodel en een nieuw klinisch model dat werd geoptimaliseerd met behulp van een machine-learning benadering, werden beiden geïntegreerd in een online tool *Evidencio*. Deze kunnen worden getest in een klinische studie voor gedeelde besluitvorming tussen arts en patiënt om gepersonaliseerde behandeling mogelijk te maken.



Chapter 10

Appendices

Curriculum vitae
PhD portfolio
List of publications
Dankwoord

CURRICULUM VITAE

Helen Gosselt was born 9th of June 1991 in Utrecht. She grew up in Breukelen together with her brother Maarten and sister Isabel. In 2009 she graduated from the secondary school RSG Broklede where she had attended bilingual VWO. Hereafter, she started Biomedical Sciences at the Vrije Universiteit in Amsterdam. During her bachelor studies she became interested in oncology research. After graduation she travelled through Asia whereafter she started her Masters in Oncology at the Vrije Universeit. During her Master she got the opportunity to write a literature study at the NKI and performed a research internship at the Hubrecht Institute in Utrecht in the group of Jop Kind. After travelling for another 2 months in Central America she started her PhD in the department of Clinical Chemistry at the Erasmus MC and Amsterdam UMC under supervision of dr. Sandra G. Heil, prof.dr. Robert de Jonge and prof.dr. Johanna M.W. Hazes, resulting in this thesis. During her PhD project she supervised five students performing their research internships in the Erasmus MC or VUmc.

ERASMUS UNIVERSITY ROTTERDAM

PHD PORTFOLIO

Helen Gosselt

Description	Organizer	EC
Required courses		
Scientific Integrity (2017)		0.30
CC02 Biostatistical Methods I: Basic Principles (2017)		5.70
Biomedical English Writing (2018)		2.00
Other courses		
Basic Course on 'R' (2016)	Molmed	1.80
Ensembl Gene Browsing workshop (2017)	Molmed	0.60
CPO-course: Patient Oriented Research (2017)		0.30
Epigenetics Bootcamp (2017)	Columbia University-New York	1.00
CE08 Repeated Measurements (2018)		1.70
SNP Course: SNPs and Human Diseases (2018)	Molmed	2.00
Data science for healthcare (2018)	Amsterdam Center for	2.00
Career support workshop (2020)	Business Analytics VU	0.15
Conferences		
7th Clinical Epigenetics Meeting 2017 (2017)		1.00
Molmed Day 2017 (2017)	Molmed	0.30
EULAR 2017 (2017)	EULAR	1.00
38th European Workshop for Rheumatology Research (2018)		1.00
Molmed Day 2018 (2018)	Molmed	0.30
EULAR 2018 (2018)	EULAR	1.00
PEMED 2018 - Personalized and Precision Medicine International Conference (2018)		1.00
Molmed Day 2019 (2019)	Molmed	0.30
NVKC - voorjaarscongres - pitch (2019)	NVKC	1.00
PACE Consortium Meeting (2019)	The Generation R Study Group	1.00
2019 ACR/ARP Annual Meeting (2019)	American College of Rheumatology	1.00
NVKC najaarscongres – pitch (2020)	NVKC	1.00
Teaching activities		
Supervision student (HBO), 5 months full time (2017)		2.00
Supervision student (HBO), 6 months parttime (2018)		1.00
Supervision Master student (VUmc), 4 months part time (2018)		1.00
Supervision Master student (VUmc), 6 months part time (2019)		1.00
Supervision Master student (VUmc), 2 months part time (2019)		0.50
Total EC		**32.95**

List of publications

In this thesis

Gosselt, H. R., van Zelst, B. D., de Rotte, M. C., Hazes, J. M., de Jonge, R., & Heil, S. G. (2019). Higher baseline global leukocyte DNA methylation is associated with MTX non-response in early RA patients. *Arthritis research & therapy*, *21*(1), 157.

Gosselt, H. R., Griffioen, P. H., van Zelst, B. D., Oosterom, N., de Jonge, R., & Heil, S. G. (2020). Global DNA (hydroxy) methylation is stable over time under several storage conditions and temperatures. *Epigenetics*, 1-9.

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Gosselt HR, Muller IB, Jansen G, van Weeghel M, Vaz FM, Hazes JMW, Heil SG, de Jonge R. Identification of Metabolic Biomarkers in Relation to Methotrexate Response in Early Rheumatoid Arthritis. *Journal of Personalized Medicine*. 2020; 10(4):271. *https://doi.org/10.3390/jpm10040271x*

Gosselt, H. R., Verhoeven, M. M., de Rotte, M. C., Pluijm, S. M., Muller, I. B., Jansen, G., ... & Hazes, J. M. (2020). Validation of a Prognostic Multivariable Prediction Model for Insufficient Clinical Response to Methotrexate in Early Rheumatoid Arthritis and Its Clinical Application in Evidencio. *Rheumatology and Therapy*, 1-14.

Gosselt HR, Verhoeven MMA, Bulatović-Ćalasan M, Welsing PM, de Rotte MCFJ, Hazes JMW, Lafeber FPJG, Hoogendoorn M, de Jonge R. Complex Machine-Learning Algorithms and Multivariable Logistic Regression on Par in the Prediction of Insufficient Clinical Response to Methotrexate in Rheumatoid Arthritis. *Journal of Personalized Medicine*. 2021; 11(1):44. *https://doi.org/10.3390/jpm11010044*

Other publications

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Kevin Stroek, Allerdien Visser, **Helen R. Gosselt**, Catharina P.B. van der Ploeg, Nitash Zwaveling-Soonawala, Annemieke C. Heijboer, Annet M. Bosch, A.s. Paul van Trotsenburg, Anita Boelen, Mark Hoogendoorn, Robert de Jonge. Newborn screening for congenital hypothyroidism: man versus machine. *Under revision*

Dankwoord

"Time flies, but not memories." - Ika Natassa

De jaren zijn voorbij gevlogen en ik heb het ontzettend naar mijn zin gehad. Helaas komt aan alle leuke dingen een eind, maar in het geval van mijn proefschrift heb ik daar vrede mee. Ik heb het gelukkig niet alleen hoeven doen en ik wil daarom ook iedereen die heeft bijgedragen aan dit proefschrift of op een andere manier betrokken is geweest en steun heeft geleverd in de komende pagina's bedanken.

Allereerst wil ik alle *patiënten* bedanken die hebben deelgenomen aan de studies die beschreven staan in dit proefschrift en *alle anderen* die betrokken waren bij het opzetten van de studies. Zonder jullie materiaal en inzet was dit proefschrift er niet geweest.

Dan mijn promotieteam. Allereerst, Prof. Dr. R. de Jonge, beste *Robert*, ik wil je heel erg bedanken voor je positieve instelling en enthousiasme. Je zag alleen maar mogelijkheden en gaf mij veel vrijheid om te doen wat ik leuk vond. Naast de inhoudelijke discussies waar ik veel van heb geleerd, was er ook altijd ruimte voor een grapje. Bedankt voor de kansen die je mij hebt geboden om continu iets nieuws te leren en knopen door te hakken wanneer nodig.

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