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PDF issue: 2025-05-25

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(Citation)
Rheumatology,58(12):2153-2161

(Issue Date)
2019-12

(Resource Type)
journal article

(Version)
Accepted Manuscript

(Rights)
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(URL)
https://hdl.handle.net/20.500.14094/90006680

Biomarkers identified by serum metabolomic analysis to predict biological treatment response in rheumatoid arthritis patients

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Abstract

Objectives. Biological treatment has recently revolutionized the management of RA. Despite this success, approximately 30-40% of the patients undergoing biological treatment respond insufficiently. The aim of this study was to identify several specific reliable metabolites for predicting the response of RA patients to TNF-α inhibitors (TNFi) and abatacept (ABT), using capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS).

Methods. We collected serum from RA patients with moderate or high disease activity prior to biological treatment, and obtained the serum metabolomic profiles of these samples using CE-TOFMS. The patients' response was determined 12 weeks after starting biological treatment, according to the EULAR response criteria. We compared the metabolites between the response and non-response patient groups and analysed their discriminative ability.

Results. Among 43 total patients, 14 of 26 patients in the TNFi group and 6 of 17 patients in the ABT group responded to the biological treatment. Of the metabolites separated by CE-TOFMS, 196 were identified as known substances. Using an orthogonal partial least-squares discriminant analysis, we identified 5 metabolites as potential predictors of TNFi responders and 3 as predictors of ABT responders. Receiver operating characteristic analyses for multiple biomarkers revealed an area under the curve (AUC) of 0.941, with a sensitivity of 85.7% and specificity of 100% for TNFi, and an AUC of 0.985, with a sensitivity of 100% and specificity of 90.9% for ABT.

Conclusion. By metabolomic analysis, we identified serum biomarkers that have a high ability to predict the response of RA patients to TNFi or ABT treatment.

Key words: biomarker, biological treatment, TNF-α inhibitors, abatacept, rheumatoid arthritis, metabolomics, CE-TOFMS

Rheumatology Key Messages

・Serum metabolomic analysis was useful for identifying biomarkers for predicting the response to biological treatment.

・Betonicine, glycerol 3-phosphate, N-acetylalanine, hexanoic acid, and taurine were associated with the response to TNFi.

・Citric acid, quinic acid, and 3-aminobutyric acid levels were associated with the response to ABT.

Introduction

RA is a chronic systemic autoimmune disease characterized by immune-cell infiltration and proliferation in the synovium, leading to progressive joint destruction. Although the etiology of RA is not fully understood, recent evidence indicates that fibroblast-like synoviocytes (FLS) play an important role in initiating and driving RA [1]. The intimal lining of the synovium displays remarkable changes in RA, with a marked increase in cellularity. Hypercellularity is due to an increase in both cell types, namely macrophage-like cells and FLS [2]. The macrophage-like cells display a highly activated phenotype and produce pro-inflammatory cytokines, chemokines and growth factors. These products can activate FLS and induce FLS to produce mediators, especially IL-6, prostanoids and matrix metalloproteinases (MMPs). This process establishes a paracrine/ autocrine network that can perpetuate synovitis and contribute to destruction of the cartilage and bone tissues [2, 3].

Recently, biological agents, predominantly antibodies such as TNF-α inhibitors (TNFi) and abatacept (ABT), which target specific inflammatory pathways, have revolutionized the management of RA. ABT is a fusion protein of cytotoxic T lymphocyte-associated Antigen 4 (CTLA-4) and the Fc portion of IgG1. It selectively modulates the interaction of CD80/ CD86 on antigen-presenting cells with CD28 on T cell that normally senses the co-stimulatory signal required for the full activation of T cell. This results in decreased T cell

activation and eventually decreased joint inflammation [4]. Despite the success of biological treatment, a substantial proportion of the patients (approximately 30–40%) responds insufficiently [5]. At the initiation of biological treatment, it is currently impossible to distinguish future responders from non-responders; therefore, the treatment is tested by trial and error. This approach is inefficient, because the clinical response to biological treatment can only be assessed after at least three months of treatment. Within this time-frame, non-responders might develop joint damage or experience toxic side effects. Furthermore, inefficient treatment increases healthcare costs due to intensive monitoring, increased complications, higher morbidity, and medication. Thus, the ability to identify responders and non-responders before initiating biological treatment, so the most optimal agent can be selected for each patient, is desirable. Toward this goal, many approaches have been explored, mostly involving the evaluation of clinical parameters, proteins, or mRNA biomarker profiles, but none has been successful enough to be implemented in clinical practice [6].

The relatively new field of metabolomics, which is the comprehensive study of low-molecular-weight metabolites, has been steadily advancing. Metabolomics is a rapidly developing approach in biomarker research, in which a large number of small-molecule metabolites is measured in biological fluids, tissues, and cells. Since metabolism closely influences an organism's phenotype, the characteristics of a disease are thought to more closely reflect alterations in the levels of metabolites than changes in gene or protein expression [7]; thus, metabolomics may have an advantage over genomics and proteomics in identifying biomarkers. Indeed, studies have revealed that metabolomic profiles associated with a disease or therapeutic response to treatment can show measurable differences from baseline or controls [8, 9]. Thus, metabolomics provides a novel perspective in the search for new disease biomarkers and drug targets.

Though metabolites can be endogenous, including lipids, small peptides, amino acids and carbohydrates or exogenous, such as drugs and food additives, lipids, amino acids and

carbohydrates are the most abundant metabolites in plasma [10]. Alteration of lipid metabolism leads to changes in cellular functions such as cell growth and inflammation. Carbohydrates have important roles in metabolism and signalling. Increases in glucose uptake and glycolysis have been associated with increased cellular proliferation by generating ATP and providing substrates for the synthesis of proteins, nucleic acids and lipids. Amino acids are nutrients and substrates for macromolecular synthesis. For example, glutamine, which is highly abundant in blood, provides nitrogen and carbon for the *de novo* synthesis of amino acids and synthesis of nucleotides through the hexosamine pathway during cellular proliferation. Thus, serum metabolites reflect what is happening at the cellular level.

Metabolomics aims to investigate the overall metabolomic activity, and thus takes into account the genetic and environmental background, the effects of which are integrated into the results [7]. The extent of metabolic changes and types of metabolites seen may therefore be good markers for RA. Metabolomic approaches in RA have already contributed to the understanding of RA and its subtypes, as well as the effect of drug treatment [11]. However, studies using metabolomic profiling to predict patients' response to biological treatment are limited. Notably, to our knowledge, metabolomics has not been applied to predict the clinical response to ABT. In addition, only three previous studies have used metabolomics to predict the clinical response to TNFi, two of which used nuclear magnetic resonance (NMR) spectroscopy and one used liquid chromatography coupled to mass spectrometry (LC-MS) [12, 13, 14]. Although these studies showed that metabolomics was effective for predicting the response to TNFi using all the detected metabolites or using a combination of clinical parameters and some metabolites, it is expensive to measure all the metabolites, including the cost of equipment. Therefore, the ability to predict a patient's response to biological treatment using a limited number of reliable metabolites is desirable.

Capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS) is a novel and promising method that separates and detects ionic compounds based on the different

migration rates of charged metabolites [15]. Compared to NMR and LC-MS, CE-TOFMS requires only a small injection volume and has better resolution. Major advantages of CE-TOFMS include its extremely high resolution, versatility, and ability to simultaneously quantify virtually all the charged low-molecular-weight compounds in a sample [16]. Although the efficacy of CE-TOFMS has been demonstrated in various human clinical studies [17, 18], there is no report applying it to RA.

The aim of this study was to identify several specific reliable metabolites to predict the response of RA patients to TNFi and ABT using CE-TOFMS, a more sophisticated technique than those used in previous TNFi metabolomics studies. In addition, this is the first report to identify serum biomarkers to predict the clinical response to ABT using metabolomic analysis.

Methods.

Patients

Patients with a diagnosis of RA according to the ACR criteria [19] and designated to start TNFi, such as etanercept, golimumab, infliximab, certolizumab pegol, and adalimumab, or ABT were prospectively enrolled from Kobe University Hospital. Allocation of biological treatment was made by discretion of attending physicians. Eligible patients had a DAS in 28 joints defined as a CRP (DAS28-CRP) greater than 2.7 despite prior treatment with disease-modifying antirheumatic drugs (DMARDs) or biological agents, and were about to start or switch to biological treatment [20]. ‘Starting biological treatment’ was defined as a start of a biological agent in patients not receiving any biological agents. ‘Switching to biological treatment’ was defined as a change to another biological agent in patients who had been receiving a biological agent, due to treatment failure or side effects. When switching to biological treatment, we started a new biologic agent after the period of administration interval of the previous biological agent. The disease activity was assessed by DAS28-CRP

before and 12 weeks after starting the biological treatment regimen. According to the EULAR-CRP response criteria [21], we classified the RA patients as responders (good response) and non-responders (moderate or no response) 12 weeks after starting the biological treatment regimen. This study was approved by the Ethics Committee of Kobe University Hospital and complied with the principles of the Declaration of Helsinki. All patients provided written informed consent.

Peripheral blood sample collection

We collected fasting serum samples of the RA patients prior to starting or switching to biological treatment. The samples were allowed to stand at room temperature for 1 h, and were then centrifuged at 2000*g* for 10 min at room temperature to obtain the sera. The supernatant was stored in aliquots at −80 °C until further use. All samples used for the present study were allowed to undergo no more than two freeze/thaw cycles.

Metabolite extraction

The metabolite extraction and metabolomic analysis were conducted at Human Metabolome Technologies (HMT), Tsuruoka, Yamagata, Japan. Briefly, 50 µl of serum was added to 450 µl of methanol containing internal standards (solution ID: H3304-1002, HMT) at 0 °C to inactivate native enzymes. This solution was then thoroughly mixed with 500 µl of chloroform and 200 µl of Milli-Q water and centrifuged at 2300*g* at 4 °C for 5 min. The 400-µl upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cut-off filter to remove proteins. The filtrate was then centrifugally concentrated and re-suspended in 25 µl of Milli-Q water for metabolomic analysis at HMT.

Metabolomic analysis

The metabolomic analysis was conducted using the HMT Advanced Scan package via CE-TOFMS, as described previously [22, 23]. Briefly, the CE-TOFMS analysis was carried out using an Agilent CE capillary electrophoresis system equipped with an Agilent 6210 TOFMS, Agilent 1100 isocratic high performance liquid chromatography pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI–MS sprayer kit (Agilent Technologies, Waldbronn, Germany). The systems were controlled by Agilent G2201AA ChemStation software version B.03.01 for CE and connected by a fused silica capillary (50 μm i.d. × 80 cm total length) with a commercial electrophoresis buffer (H3301-1001 and H3302-1021 for cation and anion analyses, respectively; HMT) as the electrolyte. The spectrometer was scanned from *m/z* 50 to 1000 [22]. The peaks were extracted using MasterHands automatic integration software (Keio University, Tsuruoka, Yamagata, Japan), and peak information including the *m/z*, peak area, and migration time was obtained [24]. Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded, and the remaining peaks were annotated according to the HMT metabolite database based on their *m/z* values and migration times. The areas of the annotated peaks were then normalized based on the internal standard levels and sample amounts, to obtain the relative level of each metabolite.

Statistical analysis

Metabolite levels were used to develop a model for predicting a patient's response to TNFi or ABT 12 weeks after starting the treatment regimen. An overview of the analyses is provided in Fig. 1 and is presented in detail below.

First, the normalized peak areas of identified metabolites were filtered, log-transformed, and scaled for further analyses. Pareto scaling was used to reduce the selection of features with the highest variance. Second, orthogonal partial least squares discriminant analyses (OPLS-DA) were used to identify metabolomic signatures that could predict the response to

TNFi or ABT. Third, to identify the metabolites that contributed to the differentiation between responders and non-responders to biological treatment, predictive variable importance in projection (VIP) and S-plot were used [25, 26]. Metabolites with a VIP > 2 and an absolute value of modelled correlation from the OPLS-DA > 0.5 in the S-plot were selected. Although the average of the squared VIP scores equals 1 and "greater than one rule" is generally used as a criterion for variable selection, this is not a statistically justified limit and therefore we used threshold of 2 for VIP to identify the metabolites that more contributed to the group separation. In addition, we used threshold of 0.5 for an absolute value of modelled correlation because correlation of 0.5 to 1.0 is generally interpreted as "large" [27]. Fourth, prediction models for detecting responders to biological treatment using multiple selected biomarkers were constructed with OPLS-DA. The validity of the models was assessed by principal component analyses (PCA) using selected multiple biomarkers. Receiver operating characteristic (ROC) analyses were then carried out to evaluate the usefulness of the prediction model, and 95% confidence intervals (CI) were calculated by bootstrapping (N = 20,000). Data were presented as the median (the 1st to 3rd quartile) unless otherwise noted. The chi-squared test and Wilcoxon rank sum test were used to compare differences in clinical data between responders and non-responders to TNFi or ABT with $P < 0.05$ as the level of significance. All the statistical analyses were conducted by R version 3.2.3 (R Development Core Team, Vienna, Austria). We used "ropols" package for OPLS-DA, "pROC" for ROC analyses.

Results.

Patients with RA

A total of 43 patients with RA were enrolled, and their characteristics are shown in Table 1, 2. Before starting TNFi treatment, all patients had moderate or high RA disease activity, as reflected by a median DAS28-CRP of 4.05 (the 1st to 3rd quartile: 3.71 - 4.58). At 12 weeks

after starting the biological treatment regimen, 14 (53.8%) of the 26 patients treated with TNFi were identified as responders. The DAS28-CRP was significantly decreased to 1.92 (1.25 - 2.32) in the responders, but not to 3.44 (2.97 - 5.19) in the non-responders.

Before starting ABT treatment, all patients had moderate or high RA disease activity, as reflected by a DAS28-CRP of 3.77 (3.45 - 4.50). At 12 weeks after starting the biological treatment regimen, 6 (35.3%) of the 17 patients treated with ABT were identified as responders. The DAS28-CRP was significantly decreased to 2.23 (2.07 - 2.31) in the responders, but not to 3.73 (2.61 - 3.98) in the non-responders.

Distinct clustering of metabolites for responders versus non-responders to biological treatment

In this study, we used CE-TOFMS for comprehensive RA serum metabolomic profiling. A total of 564 metabolites were obtained, 196 of which were identified as known substances. Using the 196 known metabolites, OPLS-DA revealed a good separation between the serum spectra of responders versus non-responders to TNFi or ABT (Fig. 2), indicating that some metabolites contributed to a differentiation between responders and non-responders to biological treatment.

Metabolites contributing to the differentiation between responders and non-responders to biological treatment

We next sought to identify the metabolites that contributed to the differentiation between responders and non-responders to biological treatment, by VIP and S-plot. Five metabolites were selected as key metabolites for classifying responders versus non-responders in the TNFi group, and three metabolites were selected in the ABT group (Table 3). The mean

concentration of betonicine was elevated, while glycerol 3-phosphate, N-acetylalanine, hexanoic acid, and taurine were decreased in the responders to TNFi. Citric acid and quinic acid were elevated, while 3-aminobutyric acid was decreased in the responders to ABT. These metabolites displayed individual area under the ROC curve (AUC) values of 0.679 to 0.800 in response to TNFi and of 0.765 to 0.864 to ABT.

Effectiveness of using multiple biomarkers for detecting responders to biological treatment

We next used glycerol 3-phosphate, betonicine, N-acetylalanine, hexanoic acid, and taurine as multiple biomarkers to detect responders to TNFi, and 3-aminobutyric acid, citric acid, and quinic acid to detect responders to ABT. OPLS-DA using these multiple biomarkers revealed a good separation between the serum spectra of responders versus non-responders to TNFi or ABT (Supplementary Fig. 1), whereas PCA support the validity of these models (Supplementary Fig. 2) [28]. The ROC analyses for detecting responders to TNFi using these multiple biomarkers revealed an AUC of 0.941 (95% CI: 0.822 to 1.000), with a sensitivity of 85.7% (95% CI: 70.0 to 100) and specificity of 100% (95% CI: 80.0 to 100), whereas the ROC analyses for ABT revealed an AUC of 0.985 (95% CI: 0.914 to 1.000), with a sensitivity of 100% (95% CI: 83.3 to 100) and specificity of 90.9% (95% CI: 78.6 to 100), suggesting that these multiple biomarkers are useful for detecting responders to biological treatment (Fig. 3). When the cut-off values were set to obtain 100% of sensitivity, consequent median specificities with 95% CI were 33.3% (95% CI: 8.3 to 66.7) for glycerol 3-phosphate, 0.0% (95% CI: 0.0 to 0.0) for betonicine, 33.3% (95% CI: 8.3 to 83.3) for N-acetylalanine, 8.3% (95% CI: 0.0 to 75.0) for hexanoic acid, 33.3% (95% CI: 8.3 to 66.7) for taurine, and 50.0% (95% CI: 16.7 to 100.0) for multiple biomarkers in TNFi treatment. When similar approach was applied to ABT treatment, consequent median specificities were 81.8% (95% CI: 54.6 to 100.0) for 3-aminobutyric acid, 45.5% (95% CI: 9.1 to 100.0) for citric acid,

54.6% (95% CI: 27.3 to 90.9) for quinic acid, and 100.0% (72.7 to 100.0) for multiple biomarkers.

Discussion

In this study, we sought to apply baseline metabolomic profiling using CE-TOFMS to identify potential biomarkers for predicting RA-patient responders to TNFi or ABT treatment. This is the first report to use CE-TOFMS for measuring serum metabolites of patients with RA. We identified five metabolites, betonicine, glycerol 3-phosphate, N-acetylalanine, hexanoic acid, and taurine, as multiple biomarkers for a patient response 12 weeks after starting TNFi treatment and three metabolites, citric acid, quinic acid, and 3-aminobutyric acid, for ABT treatment, with high sensitivity and high specificity, suggesting that these multiple biomarkers are useful for detecting responders to biological treatment. In addition, this is the first report to identify serum metabolomic biomarkers to predict the response of RA patients to ABT.

To identify the metabolites that contributed to the differentiation between responders and non-responders to biological treatment, we used VIP and S-plot. Metabolites with a VIP > 2 and absolute value of modelled correlation from the OPLS-DA > 0.5 in the S-plot were selected. Based on these evaluations, five metabolites for the response to TNFi and three metabolites for the response to ABT were selected as metabolite biomarker candidates. These metabolites individually displayed low AUC values in response to TNFi or ABT. These results indicated that the single metabolite biomarkers were not practical for screening, and that the use of multiple biomarkers is likely to be better for distinguishing responders and non-responders with high sensitivity and specificity, even though previous metabolomic approaches focusing on single biomarkers have been widely applied.

Several reports show a relationship between taurine and inflammatory disease including RA. Taurine is positively correlated with CRP or ESR in RA [29] and is associated with

oxidative stress [30], suggesting that this metabolite is associated with an inflammatory phenotype. Our results showed that taurine levels were low in responders to TNFi, and were consistent with previous studies supporting taurine as a candidate biomarker for RA. Interestingly, although a higher taurine level was associated with the response to MTX treatment in a study by Wang et al. [31], we found the opposite result for a response to TNFi treatment, suggesting that taurine may be a candidate biomarker for choosing MTX or TNFi for RA treatment.

Our findings showed that the citrate levels were high in responders to ABT treatment. Van Linthoudt et al. showed that the citrate concentrations are significantly lower in the synovial fluid of RA patients than in that of osteoarthritis patients [32], and Kapoor et al. showed that the urine concentration of citrate in RA patients is increased after infliximab treatment [12]. Collectively, citrate appears to be associated with a low inflammatory status in RA.

Our results also showed that the quinic acid levels were high in responders to ABT treatment. Quinic acid is found in plants, particularly in cranberries. Jung et al. showed that quinic acid has inhibitory effects on inflammation activation and oxidative stress in macrophages [33], and Jang et al. showed that it inhibits MAP kinase and NF-κB signalling pathways in vascular smooth muscle cells [34]. However, the role of quinic acid in metabolism has not been clearly elucidated, and requires further investigation.

Glycerol 3-phosphate is an intermediate metabolite of the glycolysis system and is one of the constituents of glycerophospholipids. Low levels of glycerol 3-phosphate indicate that glycerophospholipid synthesis is activated. However, the association of glycerol 3-phosphate with the TNFi treatment response is unknown and requires further investigation.

Betonicine, N-acetylalanine and 3-aminobutyric acid are amino acids. Hexonic acid, commonly referred to as caproic acid, is known as a catalyst for saturated fatty acid biosynthesis. The biological functions of these metabolites are largely unknown. Here we report that high levels of betonicine and low levels of N- acetylalanine and hexonic acid were

associated with a response 12 weeks after starting TNFi treatment, and that low levels of 3-aminobutyric acid were associated with a response to ABT treatment. However, the biological functions of these metabolites and their association with the pathology of RA require further investigation. Thus, while some of the metabolites we identified as associated with the response to biological treatment were previously described as RA-associated metabolites, the relationships of the other metabolites in RA were not previously reported.

Although there is no report on predicting the clinical response to ABT using metabolomics, three previous studies used metabolomics to predict the clinical response to TNFi in RA patients. Citrate, which was found to predict a response to ABT in our study, was found to that to TNFi in one previous study that used urine samples [12], suggesting that citrate may be involved in the pathology of RA. On the other hand, the other metabolites that we identified as predictors were different from the metabolites identified in the two previous reports that used serum samples from RA patients. Several differences in the study design compared to our study might explain these apparent discrepancies, such as the different analytical platform (technique and targeted panels) and statistical methods applied.

Several limitations of this study should be acknowledged. First, this study did not validate the predictive power of these metabolites using other cohorts. Second, this study only presents a preliminary result, as only a limited number of cases were available at the time we carried out our experiment. To address these points, we are now conducting a validation study in which we are measuring the level of these metabolites using other cohorts. Third, we collected blood samples mostly from patients with established RA who were already receiving DMARDs or biological treatment and were not at an early stage of RA; thus, our study did not include patients that had never been treated for RA and the discrepancies or biases might be occurred by concomitant drugs. On the other hand, this study may more accurately reflect the situation experienced in real clinical practice. Forth, because we started a new biologic agent after the period of administration interval of the previous biological

agent when switching to biological treatment, the effect of the previous biological DMARD may partly influence baseline DAS28-CRP, response, and eventual predictive accuracy both in the TNFi and ABT group.

To the best of our knowledge, this is the first report to use CE-TOFMS as a metabolomic technique for measuring the serum metabolites of patients with RA. Furthermore, this is also the first report to use metabolomic analysis to identify serum metabolomic biomarkers to predict the response to ABT. In this study we identified five metabolites in TNFi and three metabolites in ABT that correlated with a response to treatment for RA, with high sensitivity and high specificity. When the prediction rule is further validated, non-responders can be identified using these biomarkers and offered more suitable treatment, thereby preventing joint damage and potentially toxic side-effects, and decreasing healthcare costs.

References

1 Pap T, Muller-Ladner U, Gay RE, Gay S. Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. Arthritis Res. 2000;2:361-7.

2 Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev. 2010;233:233-55.

3 Bottini N, Firestein GS. Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. Nat Rev Rheumatol. 2013;9:24-33.

4 Linsley PS, Wallace PM, Johnson J *et al*. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. Science. 1992;257(5071):792–795.

5 Kahlenberg JM and Fox DA. Advances in the Medical Treatment of Rheumatoid Arthritis. Hand Clin. 2011;27:11–20.

6 Cuppen BVJ, Welsing PMJ, Sprengers JJ *et al*. Personalized biological treatment for rheumatoid arthritis: a systematic review with a focus on clinical applicability. Rheumatology. 2016;55:826–839.

7 Brindle JT, Antti H, Holmes E *et al*. Rapid and noninvasive diagnosis of the presence and severity

of coronary heart disease using 1H-NMR-based metabonomics. Nat Med 2002;8:1439–44.

8 Guma M, Tiziani S, Firestein GS. Metabolomics in rheumatic diseases: desperately seeking biomarkers. Nat Rev Rheumatol. 2016;12:269–281.

9 Yoshida M, Hatano N, Nishiumi S *et al*. Diagnosis of gastroenterological diseases by metabolome analysis using gas chromatographymass spectrometry. J Gastroenterol. 2012;47:9–20.

10 Quehenberger O. and Dennis EA. The human plasma lipidome. N. Eng. J. Med. 2011;365:1812-1823.11 Priori R, Scrivo R, Brandt J *et al*. Metabolomics in rheumatic diseases: The potential of an emerging methodology for improved patient diagnosis, prognosis, and treatment efficacy. Autoimmun Rev. 2013;12:1022–30.

12 Kapoor SR, Filer A, Fitzpatrick MA *et al*. Metabolic profiling predicts response to anti-tumor necrosis factor α therapy in patients with rheumatoid arthritis. Arthritis Rheum. 2013;65:1448–1456.

13 Priori R, Casadei L, Valerio M, Scrivo R, Valesini G, Manetti C. 1H-NMR-Based Metabolomic Study for Identifying Serum Profiles Associated with the Response to Etanercept in Patients with Rheumatoid Arthritis. PLoS One. 2015;10:e0138537.

14 Cuppen BVJ, Fu J, van Wietmarschen HA *et al*. Exploring the Inflammatory Metabolomic Profile to Predict Response to TNF-α Inhibitors in Rheumatoid Arthritis. PLoS One. 2016;11:e0163087.

15 Suzuki Y, Fujimori T, Kanno K, Sasaki A, Ohashi Y, Makino A. Metabolome analysis of photosynthesis and the related primary metabolites in the leaves of transgenic rice plants with increased or decreased Rubisco content. Plant Cell Environ. 2012;35:1369–79.16 Soga T, Ohashi Y, Ueno Y, Naraoka H, Tomita M, Nishioka T. Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. J Proteome Res. 2003;2:488–94.

17 Soga T, Sugimoto M, Honma M *et al*. Serum metabolomics reveals gamma-glutamyl dipeptides as biomarkers for discrimination among different forms of liver disease. J Hepatol. 2011;55:896–905.

18 Hirayama A, Kami K, Sugimoto M *et al*. Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. Cancer Res. 2009;69:4918–25.

19 Arnett FC, Edworthy SM, Bloch DA *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.

20 Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PLCM. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.

21 van Gestel AM, Prevoo MLL, van't Hof MA, van Rijswijk MH, van de Putte LB, van Riel PLCM. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis. Arthritis Rheum 1996;39:34–40.

22 Ohashi Y, Hirayama A, Ishikawa T *et al.* Depiction of metabolome changes in histidine-starved Escherichia coli by CETOFMS. Mol BioSyst 2008;4:135–47.

23 Ooga T, Sato H, Nagashima A *et al*. Metabolomic anatomy of an animal model revealing homeostatic imbalances in dyslipidaemia. Mol BioSyst 2011;7:1217–23.

24 Sugimoto M, Wong DT, Hirayama A, Soga T, Tomita N. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. Metabolomics 2010;6:78–95.

25 Galindo PB, Eriksson L, Trygg J. Variable influence on projection (vip) for orthogonal projections to latent structures (opls). Journal of Chemometrics 2014;28:623-32.

26 Wiklund S, Johansson E, Sjostrom L *et al*. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. Anal Chem 2008;80:115-22.

27 Cohen Jacob . Statistical Power Analysis for the Behavioral Sciences, 1988.

28 Worley B. and Powers R. PCA as a practical indicator of OPLS-DA model reliability. Curr Metabolomics. 2016;4(2):97-103.

29 Young SP, Kapoor SR, Viant MR *et al*. The impact of inflammation on metabolomic profiles in patients with arthritis. Arthritis Rheumatol 2013;65:2015-23.

30 Schirra HJ, Anderson CG, Wilson WJ *et al*. Altered metabolism of growth hormone receptor mutant mice: a combined NMR metabolomics and microarray study. Plos One 2008;3:e2764.

31 Wang Z, Chen Z, Yang S *et al*. ^{1}H NMR-based metabolomic analysis for identifying serum biomarkers to evaluate methotrexate treatment in patients with early rheumatoid arthritis. Exp Ther Med 2012;4:165-71.

32 Van Linthoudt D, Salani I, Zender R, Locatelli P, Ott H, Schumacher HR Jr. Citrate in synovial fluid and its relation to inflammation and crystal presence. J Rheumatol 1996;23:502-5.

33 Jung S, Song SW, Lee S *et al*. Metabolic phenotyping of human arherosclerotic plaques: Metabolic alterations and their biological relevance in plaque-containing aorta. Artherosclerosis 2018;269:21-8.

34 Jang SA, Park DW, Kwon JE *et al*. Quinic acid inhibits vascular inflammation in TNF-α-stimulated vascular smooth muscle cells. Biomed Pharmacother 2017;96:563-71.

Acknowledgements

S.T. designed the study, performed the experiment, analysed the data, and drafted the manuscript. A.M., J.S., and A.O. made substantial contributions to the study concept and design, the analysis and interpretation of data, drafting the article, and revising the manuscript. All authors read and approved the final manuscript.

Funding: This study was partly supported by the Human Metabolome Technologies Research Grant for Young Leaders in Metabolomics 2014 from Human Metabolome Technologies Inc. (to S. Takahashi).

Disclosure statement: The authors have declared no conflicts of interest.

TABLE 1 Baseline characteristics of RA patients, by TNFi treatment group

Unless where indicated otherwise, continuous variables are described as median (the 1st to 3rd quartile) while categorical variables are shown as number (percentage). [a]According to the EULAR-CRP response criteria, RA patients were classified as responders (good response) and non-responders (moderate or no response) 12 weeks after starting biological treatment. [b]Age are shown as mean ± standard deviation. [c]HAQ-DI data were available for 8 respondersto TNFi and 7 non-responders.

TNFi: TNF-α inhibitors. DAS28: DAS in 28 joints. HAQ-DI: HAQ disability index for RA. csDMARDs: conventional synthetic DMARDs.

TABLE 2 Baseline characteristics of RA patients, by ABT treatment group

Unless where indicated otherwise, continuous variables are described as median (the 1st to 3rd quartile) while categorical variables are shown as number (percentage). [a]According to the EULAR-CRP response criteria, RA patients were classified as responders (good response) and non-responders (moderate or no response) 12 weeks after starting biological treatment. [b]Age are shown as mean ± standard deviation. [c]HAQ-DI data were available for 5

respondersto ABT and 8 non-responders.

ABT: abatacept. DAS28: DAS in 28 joints. HAQ-DI: HAQ disability index for RA. csDMARDs: conventional synthetic DMARDs.

TABLE 3 Metabolites contributing to the differentiation between response and non-response to biological treatment assessed using CE-TOFMS

[a]Extent of contribution to the differentiation between responders and non-responders to biological treatment. [b]↑ indicates an up-regulation of the metabolite in serum samples from patients who had a good response to biological treatment. ↓ indicates a down-regulation. [c]Variable importance of the projection (VIP) scores were calculated using OPLS-DA. CE-TOFMS: capillary electrophoresis-time-of-flight mass spectrometry. OPLS-DA: orthogonal partial least-squares discriminant analysis. TNFi: TNF-α inhibitors. ABT: abatacept.

Table 1 for revision. Baseline DAS28-CRP and response proportion by previous biological DMARD in the TNFi group

	Patient with receiving previous biological DMARD (n=9)	Patient without receiving previous biological DMARD (n=17)	*P*-value
Baseline DAS28-CRP, median (1st to 3rd quartile)	4.06 (3.42－4.99)	4.04 (3.71 – 4.65)	0.94
Response proportion, no (%)	5 (55.6)	9 (52.9)	0.90

Table 2 for revision. Baseline DAS28-CRP and response proportion by previous biological DMARD in the ABT group

	Patient with receiving previous biological DMARD (n=5)	Patient without receiving previous biological DMARD (n=12)	*P*-value
Baseline DAS28-CRP, median (1st to 3rd quartile)	3.77 (3.51 – 5.12)	3.79(3.29 – 4.40)	0.49
Response proportion, no (%)	2(40.0)	4 (33.3)	0.79

	TNFi response			ABT response		
Rank [a]	Metabolite [b]		VIP score[c]	Metabolite		VIP score
1	Glycerol 3-phosphate	↓	2.175	3-Aminobutyric acid	↓	2.594
2	Betonicine	↑	2.164	Citric acid	↑	2.455
3	N-Acetylalanine	↓	2.148	Quinic acid	↑	2.271
4	Hexanoic acid	↓	2.097			
5	Taurine	↓	2.071			

TABLE 3 Metabolites contributing to the differentiation between response and

non-response to biological treatment assessed using CE-TOFMS

Figure Legends

Fig. 1 Flowchart for predicting response to biological treatment

Fasting serum samples of RA patients with a DAS28-CRP > 2.7 were collected prior to starting or switching to biological treatment (n=43). (A) CE-TOFMS was used for comprehensive RA serum metabolomic profiling. (B) RA patients were classified as responders (good response) and non-responders (moderate or no response) according to the EULAR-CRP response criteria 12 weeks after starting biological treatment. (C) Metabolites were compared between the responders and non-responders within the TNFi and ABT treatment groups. The discriminative ability of metabolites, extracted as multiple biomarkers, was analysed. DAS28-CRP: DAS in 28 joints using the CRP. CE-TOFMS: capillary electrophoresis-time-of-flight mass spectrometry.

Fig. 2 Differentiation between good responders and non-responders to biological treatment based on CE-TOFMS metabolomic fingerprinting

Score plot from OPLS-DA separating responders and non-responders to TNFi (A) and ABT (B) using metabolites identified from CE-TOFMS. Fasting serum samples of RA patients prior to starting or switching to biological

treatment were used. The patients' response was determined 12 weeks after starting biological treatment, based on the EULAR-CRP response criteria. Circles represent responders, and crosses represent non-responders. OPLS-DA: orthogonal partial least-squares discriminant analysis. TNFi: TNF-α inhibitors. ABT: abatacept. CE-TOFMS: capillary electrophoresis-time-of-flight mass spectrometry.

Fig. 3 ROC curves for detecting responders to biological treatment with multiple biomarkers using bootstrapping (N = 20,000)

ROC curves for the 5 baseline biomarkers (glycerol 3-phosphate, betonicine, N-acetylalanine, hexanoic acid, and taurine) to detect responders to TNFi (A) and for the 3 baseline biomarkers (3-Aminobutyric acid, citric acid, and quinic acid) to detect responders to ABT (B), selected by a predictive VIP > 2 and absolute value of modelled correlation from the OPLS-DA > 0.5 in the S-plot. ROC: Receiver operating characteristic. TNFi: TNF-α inhibitors. ABT: abatacept. VIP: variable importance in projection. OPLS-DA: orthogonal partial least-squares discriminant analysis.

RA patients with DAS28-CRP > 2.7 (N=43)

Serum samples before biological treatment

A. Metabolome analysis

CE-TOFMS

Metabolome data

B. Clinical assessment

Clinical evaluation 12 weeks after treatment

TNFi	ABT
14 responders	6 responders
12 non responders	11 non responders

Clinical data

Combine metabolome data with clinical data

C. Prediction of response to biological treatment

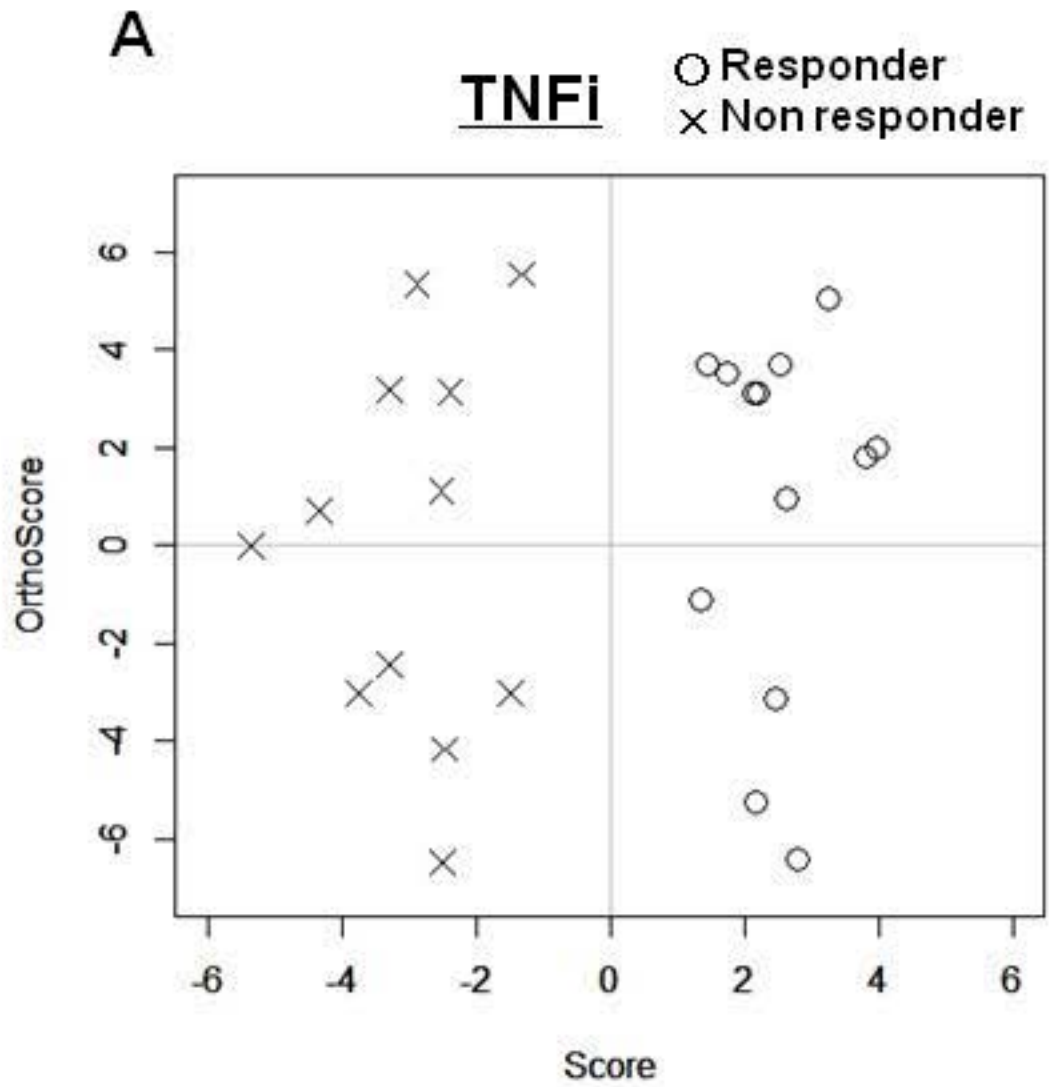
Identify metabolomic signatures for predicting the response to biological treatment using OPLS-DA

Metabolites selected with VIP>2 and |p(corr)|>0.5)

Metabolites extracted as multiple biomarkers

ROC analysis using bootstrapping (N=20000)

A


TNFi
Responder
Non responder
OrthoScore
Score
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-2
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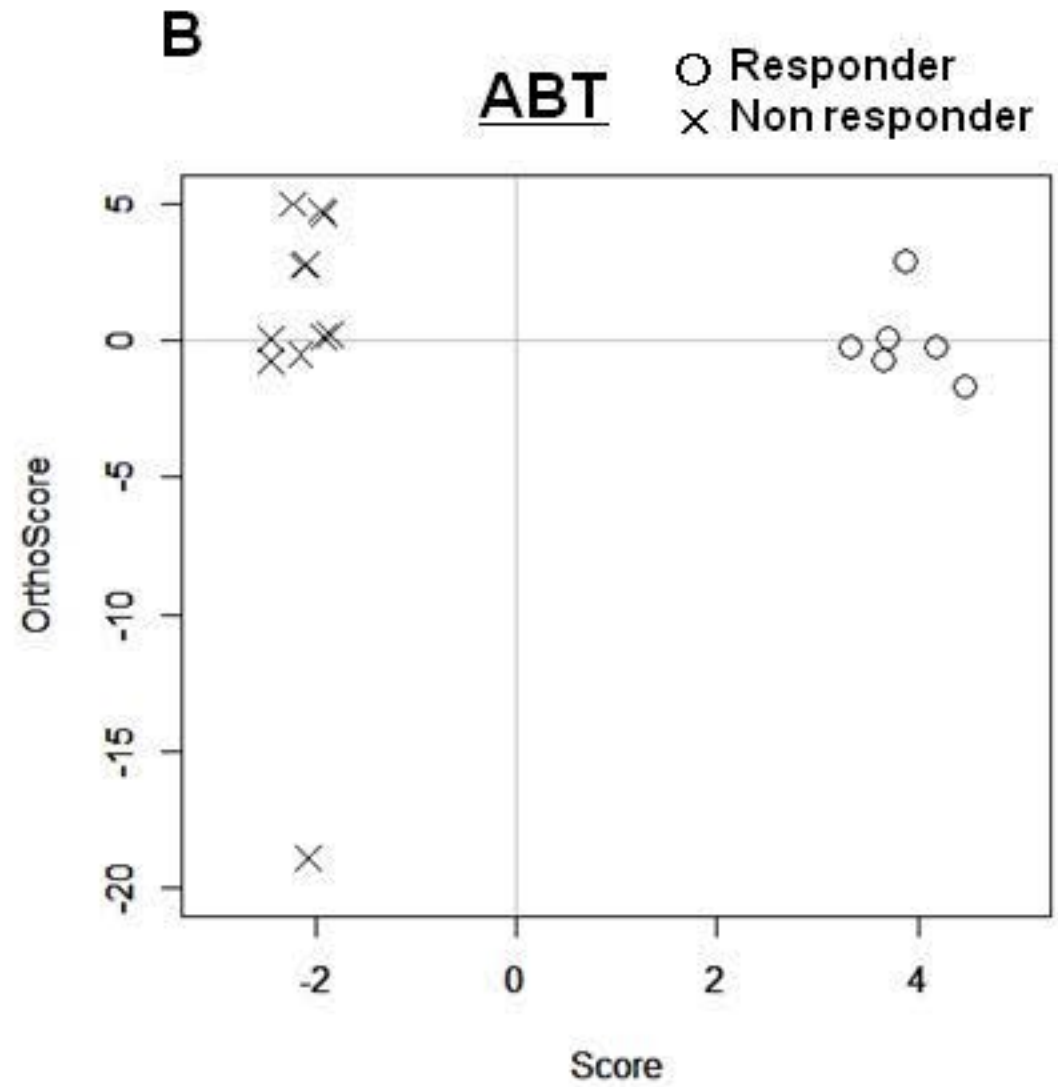
-6
-6
-4
-2
0
2
4
6

B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Non responder
OrthoScore
Score
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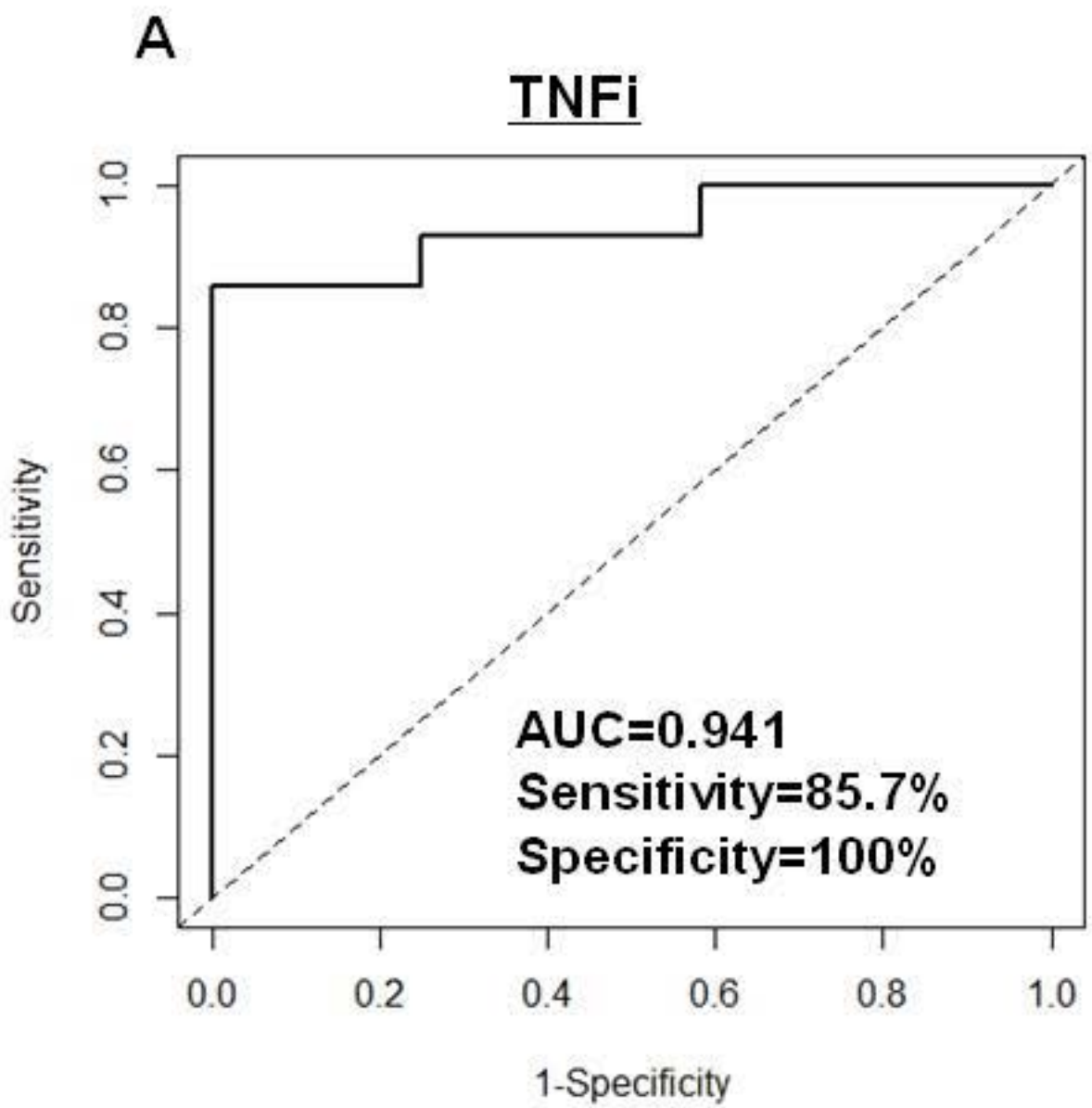
4


A
TNFi
Sensitivity
1-Specificity
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0.4
0.6
0.8
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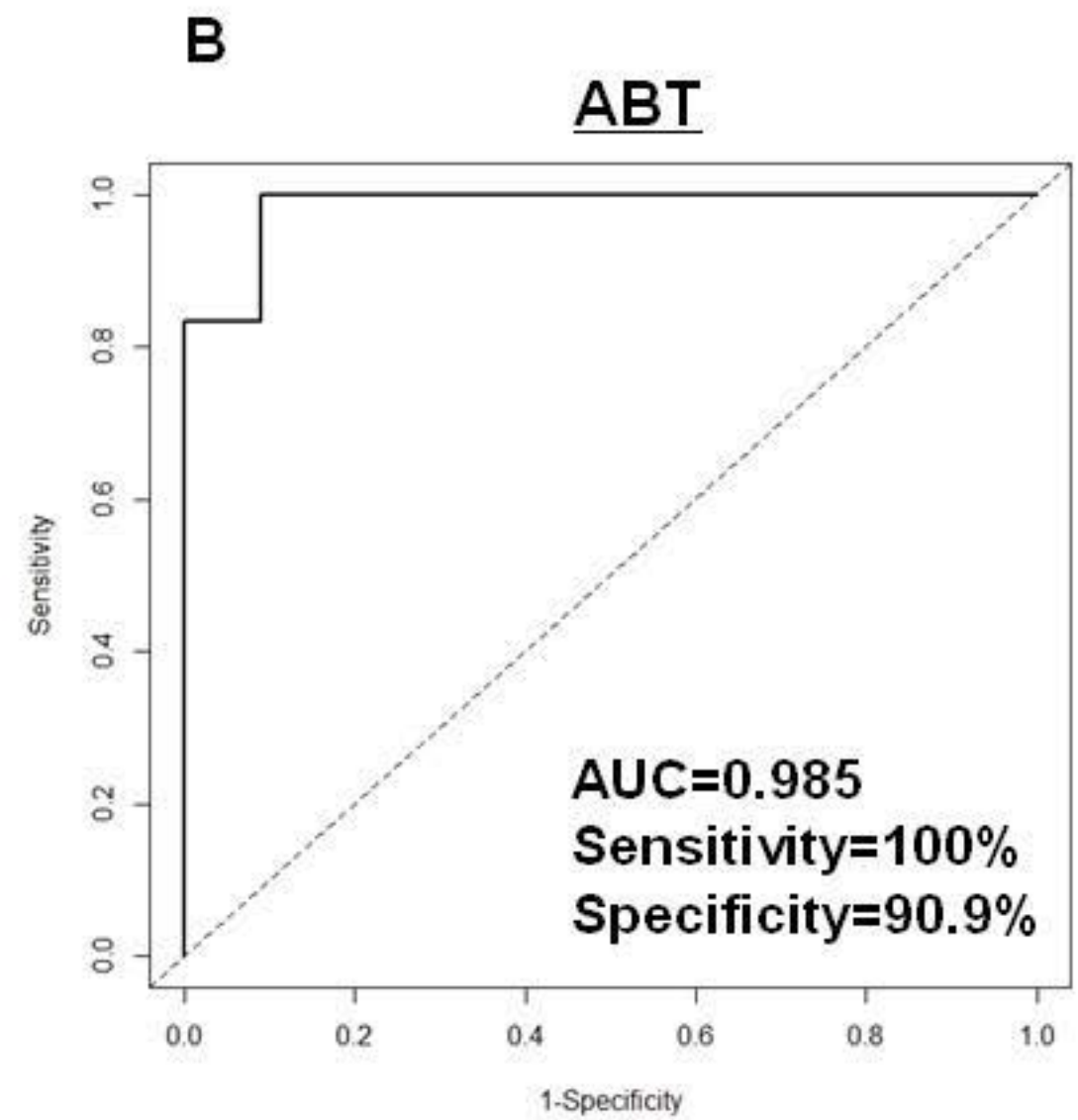
AUC=0.941
Sensitivity=85.7%
Specificity=100%


B
ABT
Sensitivity
1-Specificity
0.0
0.2
0.4
0.6
0.8
1.0
AUC=0.985
Sensitivity=100%
Specificity=90.9%

Supplementary Fig. 1

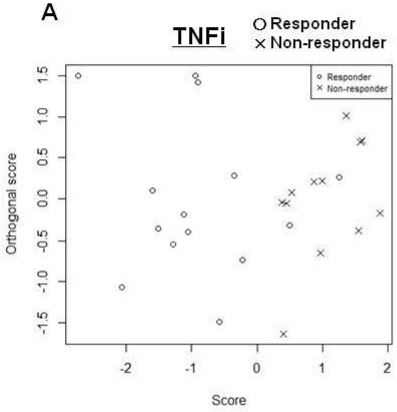
Differentiation between good responders and non-responders to biological treatment based on multiple biomarkers

Score plot from OPLS-DA separating responders and non-responders to TNFi (A) and ABT (B) using multiple biomarkers. Glycerol 3-phosphate, betonicine, N-acetylalanine, hexanoic acid, and taurine were used as multiple biomarkers to detect responders to TNFi, whereas 3-aminobutyric acid, citric acid, and quinic acid were used to detect responders to ABT. Fasting serum samples of RA patients prior to starting or switching to biological treatment were used. The patients' response was determined 12 weeks after starting biological treatment, based on the EULAR-CRP response criteria. Circles represent responders, and crosses represent non-responders. OPLS-DA: orthogonal partial least-squares discriminant analysis. TNFi: TNF-α inhibitors. ABT: abatacept.

Supplementary Fig. 2

Validity between good responders and non-responders to biological treatment based on multiple biomarkers

Score plot from PCA separating responders and non-responders to TNFi (A) and ABT (B) using multiple biomarkers. Glycerol 3-phosphate, betonicine, N-acetylalanine, hexanoic acid, and taurine were used as multiple biomarkers to detect responders to TNFi, whereas 3-aminobutyric acid, citric acid, and quinic acid were used to detect responders to ABT. Fasting serum samples of RA patients prior to starting or switching to biological treatment were used. The patients' response was determined 12 weeks after starting biological treatment, based on the EULAR-CRP response criteria. Circles represent responders, and crosses represent non-responders. PCA: principal component analyses. TNFi: TNF-α inhibitors. ABT: abatacept.


A
TNFi
Responder
Non-responder
Orthogonal score
Score
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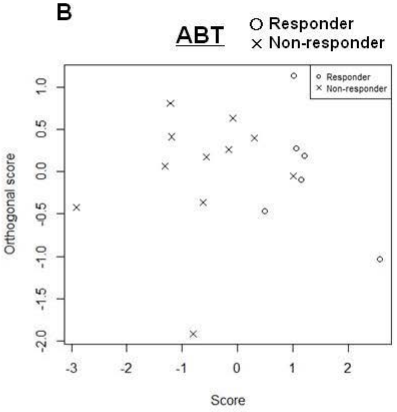
0.5
0.0
-0.5
-1.0
-1.5
-2
-1
0
1
2


B
ABT
Responder
Non-responder
Orthogonal score
Score
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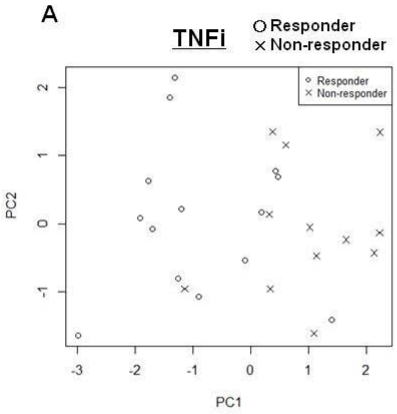
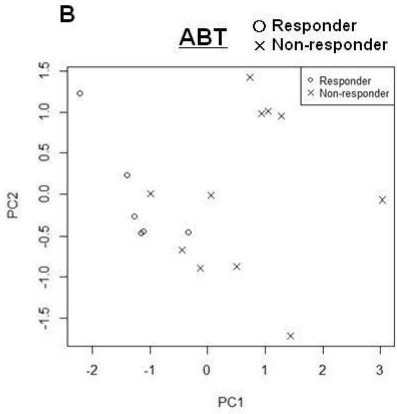
-0.5
-1.0
-1.5
-2.0
-3
-2
-1
0
1
2


A
TNFi
Responder
Non-responder
Responder
Non-responder
PC2
PC1
-3
-2
-1
0
1
2


B
ABT
Responder
Non-responder
Responder
Non-responder
PC2
PC1
-2
-1
0
1
2
3
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-1.0
-0.5
0.0
0.5
1.0
1.5