Metabolomics Profiling Predicts Outcome of Tocilizumab in Rheumatoid Arthritis: an exploratory study

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Abstract

Introduction: To study metabolic signatures can be used to identify predictive biomarkers for a patient's therapeutic response.

Objectives: We hypothesized that the characterization of a patients' metabolic profile, utilizing one-dimensional nuclear magnetic resonance (^{1}H-NMR), may predict a response to tocilizumab in patients with rheumatoid arthritis (RA).

Methods: 40 active RA patients meeting the 2010 ACR/EULAR classification criteria initiating treatment with tocilizumab were recruited. Clinical outcomes were determined at baseline, and after six and twelve months of treatment. EULAR response criteria at 6 and 12 months to categorize patients as responders and non-responders. Blood was collected at baseline and after six months of tocilizumab therapy. ^{1}H-NMR was used to acquire a spectra of plasma samples.

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Consent to participate: Informed consent was obtained from all individual participants included in the study.

Availability of data and material: The datasets generated during the current study are available from the corresponding author on reasonable request.

Chenomx NMR suite 8.5 was used for metabolite identification and quantification. SPSS v.27 and MetaboAnalyst 4.0 were used for statistical and pathway analysis.

Results: Isobutyrate, 3-hydroxybutyrate, lysine, phenylalanine, sn-glycero-3-phosphocholine, tryptophan and tyrosine were significantly elevated in responders at the baseline. OPLS-DA at baseline partially discriminated between RA responders and non-responders. A multivariate diagnostic model showed that concentrations of 3-hydroxybutyrate and phenylalanine improved the ability to specifically predict responders classifying 77.1% of the patients correctly. At 6 months, levels of methylamine, sn-glycero-3-phosphocholine and tryptophan tended to still be low in non-responders.

Conclusion: The relationship between plasma metabolic profiles and the clinical response to tocilizumab suggests that ^{1}H-NMR may be a promising tool for RA therapy optimization. More studies are needed to determine if metabolic profiling can predict the response to biological therapies in RA patients.

Keywords

Metabolomic profiling; tocilizumab; therapeutic response; rheumatoid arthritis; NMR

1 INTRODUCTION

Our increasing understanding of the pathogenesis of rheumatoid arthritis (RA) has transformed the therapeutic options available for patients with this disease(McInnes & Schett, 2017). Therapy for RA was revolutionized two decades ago by the introduction of biologic agents, and specifically agents that target the inflammatory cytokine tumor necrosis factor (TNF)(Breedveld et al., 2006; Klareskog et al., 2004; Lipsky et al., 2000). Since the initial approval of etanercept, and shortly thereafter infliximab, three additional TNF-neutralizing antibodies (adalimumab, certolizumab pegol and golimumab) have been approved, as well as anti-T-cell (and/or dendritic cell) therapy, abatacept, the B-cell-depleting antibody, rituximab, and the IL-6 receptor antagonists, tocilizumab (TCZ) (Sebba et al., 2008) and sarilumab(Nam et al., 2017; Rein & Mueller, 2017).

Despite the obvious impact of biologic agents, studies reported that at least 20-40% of the patients were declared non-responders after starting a biologic therapy(Gavril et al., 2016; Greenberg et al., 2008). If remission is the goal, there is a clear lack of uniform success with current biologic agents. There could be several reasons for this, including the initiation of therapy too late in the course of a disease, an insufficient duration of the therapy, or an inappropriate biological therapy. If initiating RA therapy with the right biologic earlier in the course of the disease could help to reach the goal of remission, a greater effort should be made to develop tools used to employ 'personalized' medicine, which could be useful to accurately match a patient with the correct therapy.

IL-6 is found in serum and synovial fluid of patients with RA. IL-6 plays a critical role in the pathophysiology of RA *via* STAT3 pathway activation, including B-cell maturation and production of autoantibodies and Th17 differentiation (Yoshida & Tanaka, 2014). In addition, it contributes to joint destruction by inducing synovitis and osteoclast activation

(Yoshida & Tanaka, 2014). IL-6 also regulates glucose and lipid metabolism, IL-6 stimulates glycolysis, lipolysis in adipose tissue, and induces β oxidation via AMPK (Lehrskov & Christensen, 2019). Since only around 35% of the patients achieve remission after 6 months of TCZ, (Huang et al., 2019), biomarkers to identify patients that will respond to TCZ would be of interest.

Given the complexity and heterogeneous nature of RA, it is unlikely that a single cytokine or biomarker will provide a sufficient distinction between patients who will or will not respond to a given drug(Burska et al., 2014). Global biomarker signatures may represent more appropriate approaches for improving RA patient treatment protocols and outcomes. Metabolomics is the science of identifying and quantifying the biochemical state of the body by products of metabolism. The fundamental rationale in metabolomics is that perturbations caused by a disease in a biological system will lead to correlated changes in the concentration of certain metabolites. Since they can be detected in different biofluids, metabolomics is a useful approach for identifying biomarkers and metabolic alterations in patients(Armitage & Ciborowski, 2017; Suman et al., 2018). Metabolomics could also help to differentiate between responders and non-responders in different treatments(Barnes et al., 2016; Sweeney et al., 2016; van Wietmarschen et al., 2012; Young et al., 2013). One-dimensional nuclear magnetic resonance (^{1}H-NMR) allows the identification of polar metabolites in biofluids and tissue with less cost than mass spectrometry, and with very high reproducibility(Emwas, 2015).

The study of metabolic signatures could then be used to identify predictive biomarkers for patients' treatment responses. The aim of this study is to explore whether pre-treatment plasma metabolomic profiling predicts outcome of TCZ in patients with RA.

2 METHODS

2.1 Subjects

Forty patients (35 women and 5 men) meeting the 2010 ACR/EULAR classification criteria(Aletaha et al., 2010), that initiated treatment with TCZ were recruited from the Rheumatology Outpatient Clinic of the Sant Pau Hospital. The study was approved by the Institutional Board Review (number IIBSP-TOC-2016) and patients signed an informational consent form. All the patients had moderate or high disease activity according to the disease activity score using the erythrocyte sedimentation rate (DAS28-ESR). Demographic and clinical data including the number of swollen joints (SJC), tender joints (TJC), the Health Activity Questionnaire (HAQ), and the DAS-28-ESR, were collected at the baseline, and after six months and 12 months of TCZ treatment (8 mg/kg of TCZ every 4 weeks). Only one patient did not have the 12 months follow up visit. Non-fasting blood samples were collected (at the baseline and 6 months into the TCZ treatment) in 10 mL BD Vacutainer® CPT™ Cell Preparation Tubes with Sodium Heparin tubes for plasma separation. After 30 minutes of incubation at room temperature, the tubes were centrifuged for 20 min at 1800×g. The plasma was transferred into 1.7mL tubes, immediately frozen, and stored at −80 °C until they were analyzed for metabolite quantification. ESR, C-reactive protein (CRP), anti-cyclic citrullinated peptide antibodies (anti-CCP), rheumatoid factor (RF), and interleukin (IL)-6 (soluble and receptor) were also quantified.

2.2   Clinical Outcomes

Patients were classified in good responders. moderate-responders and non-responders, according to EULAR criteria(van Riel & Renskers, 2016). EULAR response criteria classify individual patients as non-, moderate, or good responders, depending on the extent of change and the level of disease activity reached. Since we only had 5 men in our cohort and all were responders, we decided to analyze them separately. Even though prior reports have not found sex to be a biomarker for response to TCZ(Narváez et al., 2016), the metabolic profile between men and women is different(Mittelstrass et al., 2011). Other than that, we did not have missing data.

2.3   Sample Preparation for Metabolic Extraction

The samples were thawed at room temperature for 30 minutes, and subsequently 150 μL of each sample was transferred to a deep well plate. The plates were spun at 2000xg at 4°C for 1 min (Eppendorf 5804 R centrifuge, A-2-DWP rotor). Methanol was taken directly from a −20°C freezer and 750 μL was added into each well while shaking using an Agilent Bravo 96-channel liquid handling robot. The plates were shaken at 850 rpm, 12°C for 30 min (Eppendorf Thermomixer Comfort), and then centrifuged at 2250 x g, 4°C for 60 min. After that, 600 μL of the supernatant was transferred to a new deepwell plate with the Agilent Bravo and dried overnight (Labconco CentriVap lyophilizer set to 20°C). Each dried pellet was washed with 50 μL of methanol-d4, shaken at 850 rpm, 12°C for 10 min before drying again for 1 hr at 20°C. To finish, 200 μL of buffer (37.5 mM sodium phosphate pD 6.95, 0.02% w/v sodium azide, 0.747 mM TSP-d4) was added and it was shaken at 850 rpm, 20°C for 1 h. 180 μL of each sample was transferred to a 3 mm SampleJet NMR tube with a Bruker SamplePro Tube L robot.

2.4   ^{1}H-NMR Acquisition, Processing, Metabolic Identification and Quantification

A 600 MHz Bruker Avance III spectrometer equipped with a cooled SampleJet and a 5 mm, room temperature BBI-probe was used for data acquisition. 1D ^{1}H spectra were acquired using the pulse sequence 'zgespe', encompassing water suppression through excitation, sculpting, and including a perfect echo sequence. The acquisition time was 2.04s, the relaxation delay was of 2s, the receiver gain 181 and 128 scans were collected into 64k points. A 0.3Hz exponential line broadening was applied before Fourier transformation and zero filling to 128k points. Spectra were phased, baseline-corrected, and referenced to TSP-d4, all performed in TopSpin3.5pl7 (Bruker BioSpin). Samples in the SampleJet carousel were kept at 6°C before and after insertion in the magnet. A daily quality assurance procedure was performed before sample data acquisition, involving temperature calibration checks, shim and water suppression quality, and consistent quantification using a reference sample, all according to the Bruker In Vitro Diagnostics for research (IVDr) SOP.

The metabolites detected in the 1D ^{1}H-NMR were identified and quantified using the software Chenomx NMR suite 8.5 professional (Chenomx Inc., Edmonton, Canada), which contains a library that matches compounds' peaks according to the chemical shift. Metabolite concentration was normalized to the TSP-d4. Forty-seven metabolites were quantified and 2-phenylpropionate was removed because it was detected in less than 50% of the patients. Metabolite concentrations were reported in micromoles (μM). Metabolomic

data was submitted at Metabolights (Haug et al., 2019) with the URL: www.ebi.ac.uk/metabolights/MTBLS3013,

2.5 Statistical Analysis

Metabolites were normalized by sum, log transformed and averaged, using MetaboAnalyst version 4.0, which is an open resource for metabolomics analysis(Chong et al., 2018; Chong et al., 2019). For predicting models that are based in algorithmic analysis, we used Partial Least Square Discriminant Analysis (PLS-DA), considered the gold standard for binary classification, to obtain the variable importance of projection and to determine the importance of individual metabolites in group discrimination(Mendez et al., 2019). We also used Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) to observe the difference between responders and non-responders. Pathway analysis was evaluated with the enrichment analysis tool available on MetaboAnalyst, using the set KEGG 2019, which contains 84 metabolite sets(Kanehisa et al., 2017).

Continuous variables were expressed as mean ± standard deviation (SD) and the categorical variables as a percentage. Differences between means for the continuous variables were assessed using paired and unpaired t-test analysis, differences between proportions using the chi-square test, and analysis of variance (ANOVA) Dunnett's post hoc tests for multiple group comparisons. Pearson's correlation coefficient was used to assess the association between variables. Linear regression was performed between each clinical characteristic/cytokine-metabolite pair, and age, diabetes and dyslipidemia status were controlled by including these factors as covariates in the model. We performed a binary logistic regression adjusted by age, anti-CCP, diabetes and dyslipidemia as dichotomic variables, interleukin 6 (its receptor and the ratio) as well as the metabolites with a $p<0.20$ in the binary analysis, to predict the relationship between the variables included and the response. We also performed a receiver operating characteristic (ROC) curve to evaluate the performance of the classification model using response as the variable of prediction. The value $p<0.05$ was considered statistically significant. The analysis was performed using SPSS v.27 (IBM Corp. Released 2019 v27.0. Armonk, NY: IBM Corp.).

3 RESULTS

3.1 Patient demographics and disease characteristics at baseline

Thirty-five women with active RA were recruited. Patient demographics, along with the disease characteristics and co-morbidities are summarized in Table 1. The average of age was 53 years old (± 10), and the average number of years since diagnosis was 11.8 years (±7.2). TJC and SJC were 9.26 (± 6.23) and 6.00 (± 4.08) respectively, with an average DAS28-ESR 5.77 (± 0.99). Prior to TCZ treatment, 34% of the patients were biological disease-modifying antirheumatic drug (bDMARD)- naïve. Patients had failed an average of 2 conventional synthetic DMARDs and an average of 1.33 bDMARDs. During TCZ treatment (8mg/kg), 31.4% of patients received TCZ as monotherapy, 42.9% received TCZ in combination with methotrexate (15.5 ± 8.36 mg/week), 5.7% with sulfasalazine (1.5 ± 0.7 mg/day), 22.9% with leflunomide (16.25 ± 5.17 mg/day) and, 68% with steroids (7.58

± 4.92 mg/day). Clinical parameters at baseline and 6 months are shown in supplementary table S1 and demonstrate the efficiency of TCZ.

3.2 Metabolic Profiling in responders vs non-responders at baseline

We first classified the 35 women in 2 groups according to their response to TCZ, determined by EULAR classification criteria. Patients with a >1.2 reduction of DAS28-ESR after 6 months of treatment and a DAS28-ESR 3.2 were considered responders (n=17). Patients with a DAS28-ESR reduction of 1.2 after 6 months of TCZ and a DAS28-ESR of > 3.2 were considered non-responders (14, moderate responders and 4 non-responders according to EULAR response, total n=18). No significant differences in clinical variables were observed at the baseline between groups, except for ESR which was higher in non-responders (39.67 ± 16.75 responders vs 61.75 ± 36.63 non-responders; p<0.002) (supplementary table S2).

A total of 46 polar metabolites were identified using ^{1}H-NMR spectroscopy and included in the analysis. We identified several amino acids, ketone bodies and several glycolytic intermediates among other metabolites. The ^{1}H-NMR spectra showed visible differences in the concentration of some metabolites between responders and non-responders at baseline (Supplementary Table S3 and Figure 1A).

After classifying RA patients according to EULAR response criteria, we performed a multivariate OPLS-DA with all the metabolites observing a partial discrimination between the groups. Principal components (1-5) explained 51.3% of the variance of the ^{1}H-NMR data. With a cutoff value of PLS-DA Variables Importance in Projection (VIP) > 1.0, we identified sarcosine, acetoacetate, 3-hydroxybutyrate, sn-glycero-3-phophocholine, glucose, acetone, carnitine, tryptophan, glutamine and o-phosphocholine as the key metabolites to differentiate between groups (Figure 1B, C, D). Heat map analysis clustered the groups according to the metabolic profile but was not able to fully classify them (Figure 1E).

Since our group previously found that the IL6/IL6R ratio was important to identify responders to TCZ(Diaz-Torne et al., 2018), we also evaluated if the addition of these parameters would better distinguish the groups (supplementary figure S1). Yet, IL-6, sIL-6R and the ratio did not achieve the cutoff value and did not improve the classification between responders and non-responders.

It is important to note that OPLS-DA did not show good discrimination when analyzing the subjects by age (young <55 years, old 55 years), DAS28-ESR (high activity vs moderate activity), RF (positive>25 IU/mL vs negative 25 IU/mL), ESR (High 30 mm/h vs normal < 30 mm/h), or CRP (negative <2.0 mg/L vs positive 2.0 mg/L) (Supplementary figure S2A and Supplemental material). However, based on the results of OPLS-DA, several metabolites discriminated between positive (>20 U/mL) and negative anti-CCP (20 U/mL) at baseline (Supplementary figure S2B and Supplemental material).

3.3 Significantly different enriched pathways and metabolites between responders and non-responders at baseline

We analyzed whether blood metabolites had a different metabolic fingerprint in both groups at baseline. We had some significant observations of different associations between metabolites at baseline in responders compared to non-responders (Figure 2A). In addition, when the metabolites were mapped to known metabolic pathways using MetaboAnalyst V.4.0, enriched pathways were significantly different in both groups (Figure 2B). In responders, sphingolipid and arginine/proline metabolism were the most enriched metabolic pathways, while glutathione metabolism and glycolysis/ gluconeogenesis pathways were the most important enrichment pathways in non-responders. These differences of metabolic enrichment pathways before treatment could condition the patient's response to TCZ.

To determine the significantly different metabolites between responders and non-responders, metabolites were analyzed by univariate analysis. Seven metabolites were significantly different between groups: 3-hydroxybutyrate (p= 0.01), isobutyrate (p=0.02), lysine (p= 0.05), sn-glycero-3-phosphocholine (p= 0.02), phenylalanine (p=0.02), tryptophan (p=0.01) and tyrosine (p= 0.03). Each of these displayed higher levels in responders than non-responders (Figure 3A and supplementary table S3). We did observe tendencies in the levels of these metabolites when analyzed between moderate responders and non-responders according to EULAR response (Figure 3B).

We also determined whether some of these metabolites were associated with clinical variables. Linear regression was performed between each clinical characteristic-metabolite pair; age, diabetes and dyslipidemia status were controlled by including these factors as covariates in the model. The regression coefficients for each clinical outcome-metabolite pair were used to form a clustered heatmap to lend insight into which clinical characteristics were correlated with which groups of metabolites. An important find was that ESR had a negative correlation with the levels of several amino acids (p<0.05). Some metabolites also correlated with swollen joints (p<0.05) (supplementary figure S3). We also performed linear regression between levels of IL-6, sIL-6R, and IL-6/IL-6R ratio and each metabolite pair adjusted as above (supplementary figure S4).

3.4 Multivariate analysis identifies 3-hydroxybutyrate and phenylalanine as predictors of response.

A binary logistic regression analysis, including the metabolites with p<0.20 in the univariate model after adjusting by age, anti-CCP positivity, diabetes, dyslipidemia, IL-6, sIL-6R, and IL-6/IL-6R ratio, showed that RA patients with elevated 3-hydroxybutyrate and phenylalanine at baseline had 1.5 times more chances of a response to treatment with TCZ, overall classifying 77.1% of the patients correctly. The classification model for response to TCZ at baseline showed a sensitivity of 94% and specificity of 66% with an AUC of 0.899 and a p<0.001 (Figure 4).

3.5 Metabolic profiling in men

5 men with active RA were recruited. Patient demographics, along with the disease characteristics and co-morbidities compared with woman are summarized in Supplemental

material. We did not observe differences in disease characteristics and comorbidities. Yet, the metabolic profile was different between men and women (Supplementary figure S5A, B). When we analyzed women and men together (n=40), we observed only slight differences in number of metabolites and metabolites predictors of response compared to only women (Supplementary figure S5C). The classification model for response to TCZ at baseline showed a sensitivity and specificity of 80% with an AUC of 0.886 and a p<0.001 (Supplementary figure S5D).

3.6   Metabolomic profile after treatment

Plasma collected after 6-month of TCZ treatment was also used for metabolomics analysis. Polar metabolite quantification at 6 months revealed several metabolites with increased concentration after treatment including) choline (p=0.008), creatine (p= 0.01), glutamine (0.006), glycine (p=0.04), lactate (p=0.006), leucine (p=0.05), lysine (p=0.006), proline (p=0.006), serine (0.002), threonine (p=0.04), and valine (0.02) (supplementary table S4). When comparing changes in metabolites before and after treatment in responders and non-responders, we observed that the concentration of metabolites did not change considerably in responders, while the concentrations of the metabolites increased until reaching responders' metabolite levels in non-responders, except for the acetone (Supplementary figure S6 and supplementary material).

We then classified the group of patients classified as non-responders at 6 months, in late responders (patient that did not respond at 6 months but did reach good response at 12 months according to the EULAR classification; n=8), and non-responders (patient that at 12 months were still non-responders or moderate responders; n=9). One patient was lost for the 12 months follow-up. Of interest we observed that patients who did not respond had levels of sG3PC, tryptophan and methylamine still downregulated, suggesting an alternative activated metabolic pathway that is not dependent of IL-6 (Figure 5 and supplementary material).

4   DISCUSSION

Predictive biomarkers for targeted therapies are an unmet clinical need since not all patients on these therapies will respond; around 50% of patients, for instance, will not achieve ACR50(Nishimoto et al., 2004). Global and targeted metabolomic studies using ^{1}H-NMR and/or mass spectrometry can uncover a set of metabolites associated with prognosis and response. In the present study, we successfully identified a set of polar metabolites in plasma that differentiate responders from non-responders to TCZ in RA patients using ^{1}H-NMR. These metabolites were higher in responders compared to non-responders. Of interest, some of these metabolites reached similar concentration after 6 months in both groups with the exception of SG3PC, tryptophan and methylamine, which were still downregulated in non-responders. Concentrations of circulating metabolites that belong to deregulated metabolic pathways can be either upregulated or downregulated. These upregulated or downregulated metabolites are not necessarily pathogenic, but a biomarker of an altered metabolic pathway. The fact that the late responders also normalize their concentration prior to their response, suggest that these metabolic pathways were IL-6 dependent in

these patients, but an alternative activated metabolic pathway that is not dependent of IL-6 was still dysregulated in non-responders. The metabolites that strongly identified TCZ responders were 3-hydroxybutyrate and phenylalanine.

Prior studies have described metabolic signatures that classify responders to several therapies(Kapoor et al., 2013; Priori et al., 2015; Sweeney et al., 2016; Takahashi et al., 2019; Teitsma et al., 2018). For instance, Priori et al observed higher levels of isoleucine, leucine, valine, alanine, glutamine, tyrosine, and glucose, as well as lower levels of 3-hydroxybutyrate(Priori et al., 2015) in etanercept responders. In our study, we observed a higher level of 3-hydroxybutyrate and isobutyrate in responders. 3-hydroxybutyrate is synthesized in the liver from acetyl-CoA and can be used as an energy source when blood glucose is low. IL-6 is crucial for hepatocyte homeostasis(Schmidt-Arras & Rose-John, 2016). Of note, an association between levels of IL-6 and ketosis was described(Jain et al., 2003), hence, levels of this metabolite could be reflecting IL-6 pathway activation in the liver. In addition, 3-hydroxybutyrate also has an anti-inflammatory role by blocking NOD-like receptor protein 3 (NLRP3) inflammasome(Møller, 2020; Newman & Verdin, 2017). Isobutyrate is a short chain fatty acid derived from the gut (Petrognani et al., 2020). Differences in levels of this metabolite could be associated with different microbiome between R and NR. Of note, prior studies have suggested an effect of variations within the human gut microbiome on bioavailability, clinical efficacy and toxicity of a wide array of drugs through direct and indirect mechanisms (Scher et al., 2020). Thus, levels of this metabolite can reflect the presence of microbiome more adequate for a TCZ response.

We recently identified 7 polar metabolites including phenylalanine and tyrosine that predicted response to rituximab(Sweeney et al., 2016). Yet, the levels of these metabolites were lower in responders instead of elevated. Interestingly, phenylalanine and tyrosine work closely with IL-6R which interacts with IL-6(Blanchetot et al., 2016). Phenylalanine levels were also found to vary after infusion of recombinant IL-6 and returned to control's levels when the infusion stopped suggesting a relationship between levels of IL-6 and phenylalanine(van Hall et al., 2008), and that the levels of IL-6 or ratio between IL-6/IL-6R might alter the concentration of phenylalanine. Tryptophan is another amino acid that has been associated with the IL-6 pathway, which is also elevated in responders to TCZ treatment, and it has antioxidant and anti-inflammatory properties through kynurenine, one of its metabolites. The 5-hydroxytryptophan, another product of tryptophan metabolism, reduces proinflammatory cytokines levels, such as IL-6 in animal models(Yang et al., 2015). In our study, the levels of several amino acids significantly changed after treatment, highlighting the important role of the IL-6 pathway in their metabolism.

Sphingolipid and arginine/proline metabolism were the most enriched metabolic pathways in responders, while glutathione metabolism and glycolysis/ gluconeogenesis pathways were the most important enrichment pathways in non-responders. Metabolic reprogramming, specifically the increment in glycolysis, has been implicated in immune and inflammation processes, as well as RA pathogenesis(Li et al., 2020; Mouton et al., 2020). Garcia-Carbonell showed that in RA fibroblast-like synoviocytes (FLS) compared to osteoarthritis FLS, the oxidative phosphorylation shifts to glycolysis, increasing the expression of transporters of glucose such as GLUT1(Garcia-Carbonell et al., 2016). Moreover, the

inhibition of glycolysis improves the clinical parameters of RA by decreasing activation of neutrophils and monocytes, as well as reducing the expression of cells associated with inflammation(Abboud et al., 2018). The fact that the glycolysis pathway was increased in non-responders could reflect higher levels of inflammatory cytokines which enhanced glycolysis. Even though, in our cohort, DAS28-ESR and CRP was not different between responders and non-responders, serum metabolites could identify more subtle changes than CRP or clinical outcomes.

In this study we were not able to identify lipids in our samples, but serine is critical for sphingolipid metabolism. Teitsma et al described sphingolipid metabolism also being a pathway that predicted response to TCZ(Teitsma et al., 2018) and suggested that sphinganine and sphingosine were also associated with IL-6 levels. Interestingly, several metabolites differentiate patients with and without anti-CCP antibodies. Methylamine, creatine, carnitine, and glycine were higher in patients who were anti-CCP positive. Of note, these metabolites merge into the urea cycle at different points likely impacting the levels of citrulline(Chandrasekharan et al., 2018; Dimitroulas et al., 2017). However, the significant increase of some metabolites in patients with anti-CCP positive was not associated with their response to TCZ. This observation is supported by Jonsson et al, who found no differences of anti-CCP and therapeutic response with methotrexate (Jonsson et al., 2018) or TCZ (Narváez et al., 2016). Finally, there is little data describing the correlation between metabolites and CRP in early rheumatoid arthritis. Young et al, showed in patients with early arthritis that choline, lactate, acetylglycine, urea, glucose, taurine, threonine, and fatty acids among other, correlated with CRP levels (Young et al., 2013). In our study, we only observed a negative correlation of CRP with isoleucine. Since our cohort have long-standing disease, these differences should be viewed with caution.

5   CONCLUSIONS

In conclusion, the relationship between plasma metabolic profiles and the clinical response to TCZ suggests that ^{1}H-NMR may be a promising tool for RA therapy optimization. Although the results are promising, our study has several limitations, not only the small sample size and the small male population in our cohort, but also the difficulty to control the number of variables involved in metabolite levels in plasma, including other co-morbidities and diet (Coras et al., 2020). More studies are needed to determine if metabolic profiling can predict response to biological therapies in RA patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding:

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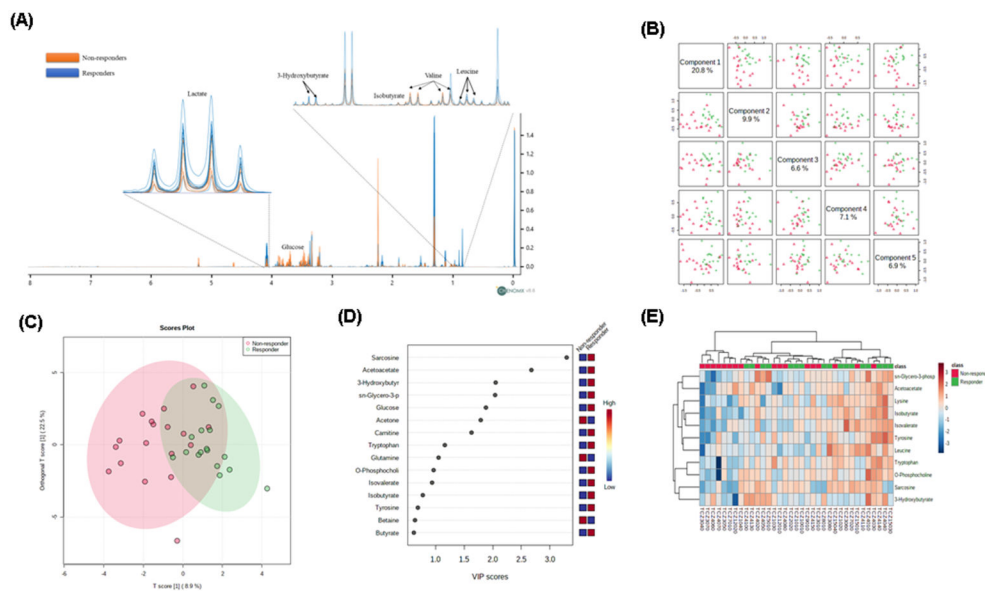
Fig 1.

Plasma metabolic profile of patients with RA discriminate responders and non-responders. **A)** ^{1}H-NMR of plasma from 12 patients (6 responders and 6 non-responders) with RA showing differences of metabolites intensities between responders and non-responders at baseline. The variation of polar metabolite concentrations indicates a characteristic metabolic profile in patients who achieve or not response after 6 months of treatment with TCZ. **B)** Pairwise score plots between the 5 principal components showing a total explained variance is 51.3%. **C)** Orthogonal PLS-DA between non-responders and responders at baseline. **D)** Important features identified by PLS-DA. **E)** Concentration of metabolites in format Heatmap based on Euclidean distances considering the 11 metabolites observed in figure 1D VIP>1, shows clustering between responders and non-responders according to metabolites concentration.
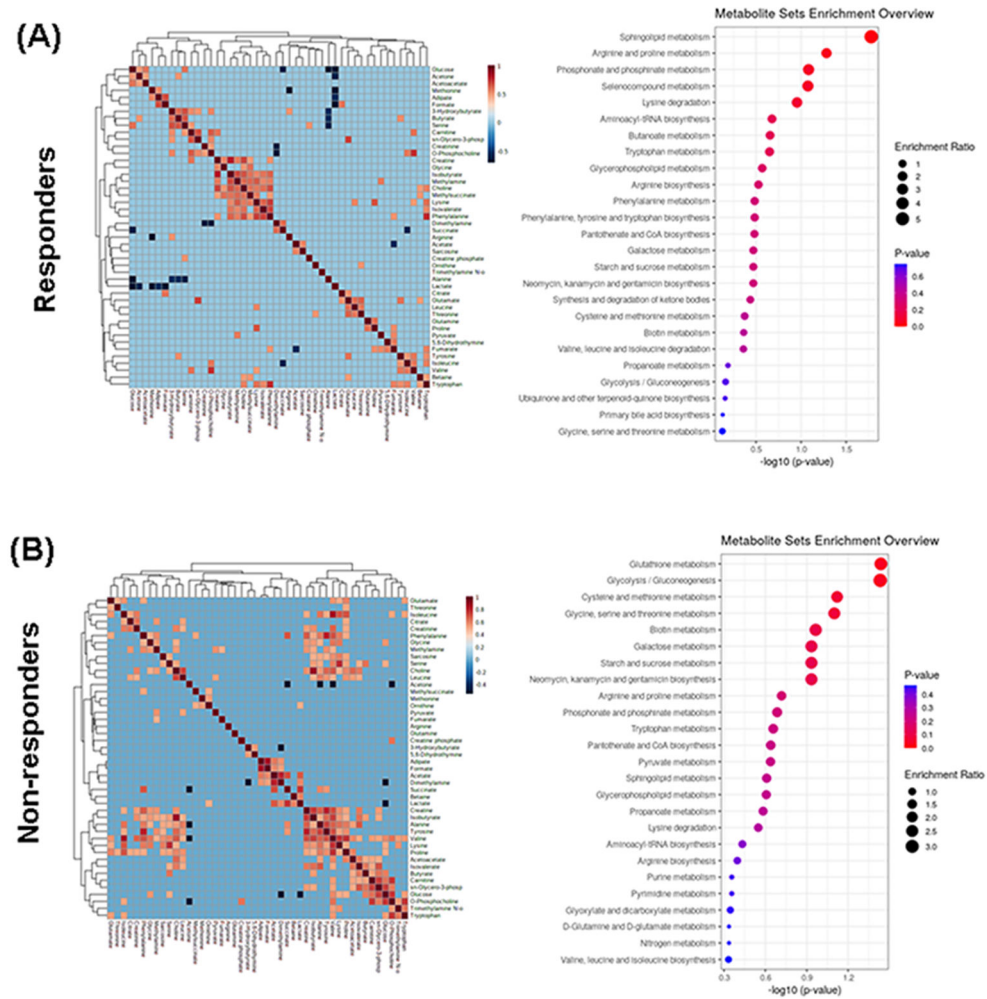
Fig 2.
Left. Heatmap and hierarchical cluster analysis indicating relationships between polar metabolites identified by ^{1}H-NMR in plasma from RA patients before treatment with TCZ. **A)** responders, **B)** non-responders. Pearson correlation with a Cutoff value of 0.5. The orange color indicates a positive correlation >0.5 and the dark blue indicates negative correlation <−0.5. **Right**. Summary of bar graphs for quantitative pathway enrichment analysis showing the significant changes on metabolic pathway involve before treatment with TCZ in **A)** responders and **B)** non-responders.

(A)

(B)
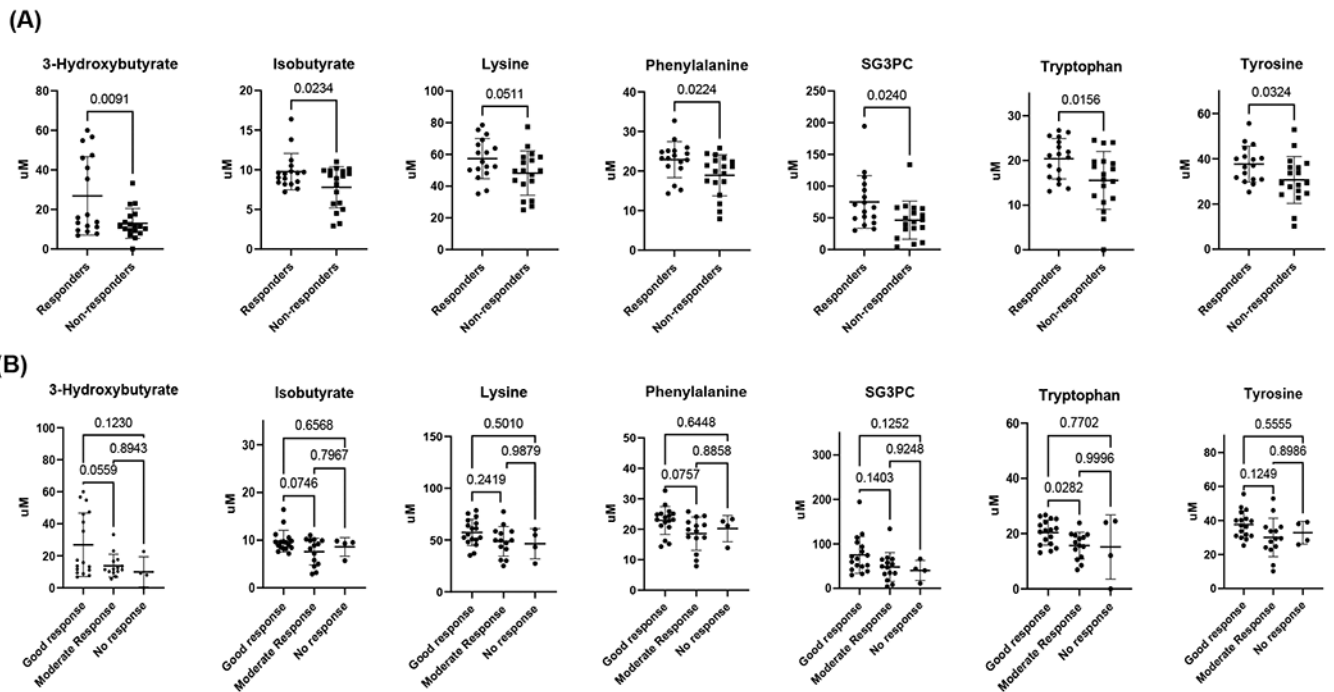
Fig 3.
A) Concentrations of specific polar metabolites at baseline that are different between TCZ responders and non-responders measured by ^{1}H-NMR before treatment with TCZ. These metabolites represent a group of potential biomarkers for predicting and assessing patient response to TCZ treatment. **B)** Concentrations of the same specific polar metabolites at baseline after dividing the non-responders between moderate-responder and non-responders at 6 months. SG3PC: Sn-glycero-3-phosphocholine. p values are shown in the graphics.

(A)
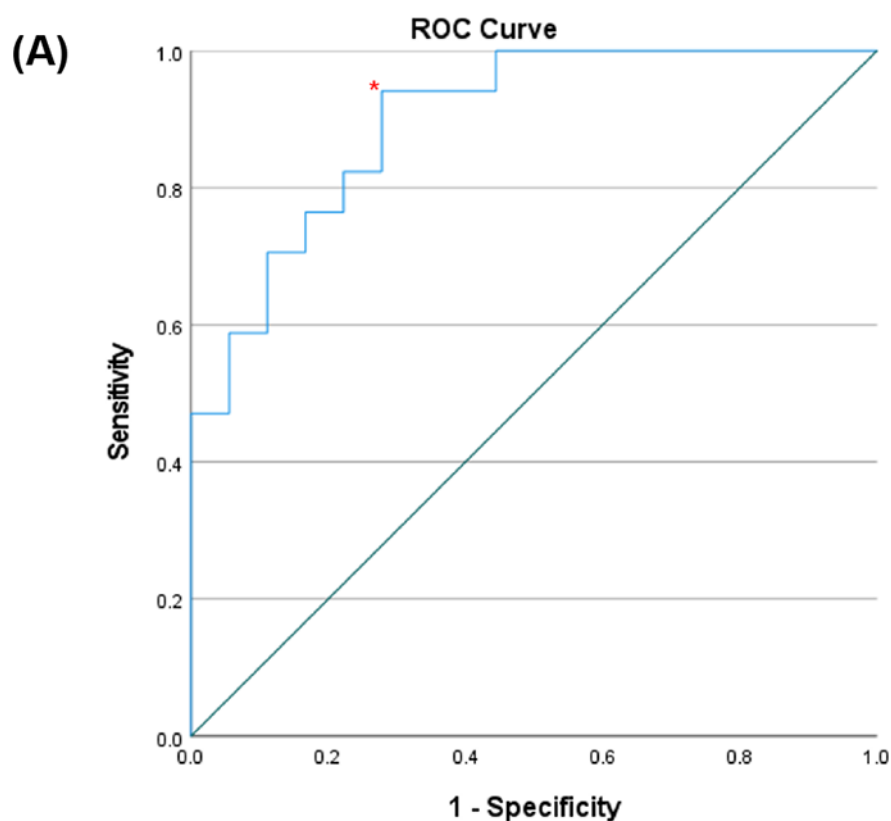
(B)

Variables	B	S.E.	Wald	Sig.	Exp (B)
3-Hydroxybutyrate uM	0.140	0.051	7.649	0.006	1.151
Phenylalanine uM	0.420	0.167	6.335	0.012	1.521

Fig 4.
A) Receiver operating characteristic (ROC) curve for the model probability for responders before treatment based on binary logistic regression. The area under the curve (AUC) was 0.899, the sensitivity of 94% and the specificity of 66% with a p<0.001. The patients with elevated phenylalanine and 3-hydroxybutyrate had 1.5 chances to respond to TCZ. **B)** Binary logistic regression analysis adjusted by age, anti-CCP positivity and interleukins. The variables considered for the logistic regression analysis were metabolites with p<0.20 in the bivariate model. The model correctly categorized 77.8% of non-responders and 76.5% of responders.
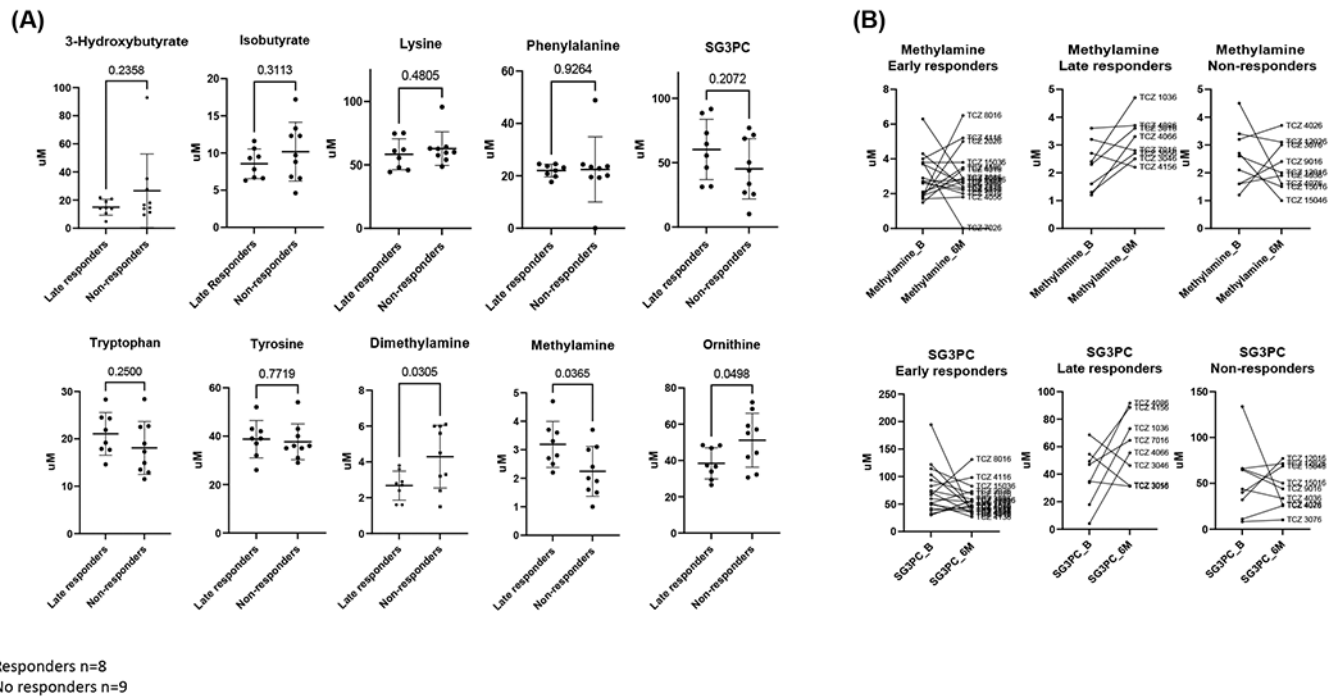
Fig 5.
A) Concentrations of specific polar metabolites at 6 months that are different between late TCZ responders and non-responders measured by ^{1}H-NMR. p values are shown in the graphics **B)** Metabolite trajectories of SG3PC and methylamine between baseline (B) and 6 months (6M) in early responders, late responders, and non-responders.

Table 1.

Characteristics of RA patients (women) included in the study.

Variable	Baseline
Age, years	53 ± 10
RF positive, n (%)	23 (65.7)
Anti-CCP positive %	23 (65.7)
Seropositive, n (%)	28 (80.0)
ESR	52.29 ± 31.44
CRP	1.98 ± 2.69
SJC	6.00 ± 4.08
TJC	9.26 ± 6.23
HAQ, score	1.41 ± 0.68
DAS28-ESR	5.77 ± 0.99
SDAI, score	29.53 ± 11.41
CDAI, score	27.54 ± 10.54
Tobacco, n (%)	9 (22.9)
Diabetes, n (%)	3 (8.6)
Hypertension, n (%)	8 (22.9)
Dyslipidemia	7 (20)

Continuous variables expressed in mean ± standard deviation; Categorical variables expressed in percentage. RF: rheumatoid factor; Anti-CCP: anti-cyclic citrullinated peptide; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; SJC: Swollen joint count; TJC: Tender joint count; HAQ: health assessment questionnaire; DAS-28: disease activity score in base of 28 joints; SDAI: simple disease activity index; CDAI: clinical disease activity index