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SERMENT D'HIPPOCRATE

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RESUME

Objectifs : Analyser les changements métabolomiques 4 semaines après l'initiation de l'adalimumab, un anti-TNF chez les patients atteints de spondylarthrite axiale (SpA) et de polyarthrite rhumatoïde (PR) et étudier la relation avec la réponse clinique à 12 et 26 semaines.

Méthodes : Nous avons effectué des analyses en post-hoc du sérum de patients atteints de SpA de l'étude COMARIS (NCT01895764) et de patients atteints de PR de l'étude AFORA (NCT01382160) par chromatographie liquide couplée à la spectrométrie de masse à haute résolution (LC/HRMS) à l'inclusion (S0) et 4 semaines (S4) après l'initiation du traitement par adalimumab. Les réponses cliniques ont été évaluées à 12 et 26 semaines. Les variations des concentrations de métabolites entre S0 et S4 ont été comparées entre les répondeurs et les non-répondeurs en analyse univariée, multivariée non supervisée en composantes principales (PCA) puis multivariée supervisée par des analyses discriminantes partielles des moindres carrés (PLS-DA), à l'aide du logiciel METABOANALYST. Les variables d'intérêt, définies comme celles ayant un score VIP (Variable Influence on Projection) supérieur ou égal à 2 ont été sélectionnées. Enfin, nous avons étudié les différentes voies métaboliques dans lesquelles ces variables d'intérêt étaient impliquées.

Résultats : Soixante-quatorze patients ont été inclus dans la cohorte COMARIS, dont 43 répondeurs et 31 non-répondeurs. Soixante-trois patients ont été inclus dans la cohorte AFORA, dont 52 répondeurs et 11 non-répondeurs. En analyse univariée, la leucine, l'hypoxanthine et la n-acétyl-l-alanine étaient abaissées chez les répondeurs atteints de SpA. La carnosine, le cortisol et la purine étaient abaissés chez les répondeurs atteints de PR, tandis que la l-méthionine et la 5-oxo-l-proline étaient augmentées chez ces sujets. Nous n'avons pas été en mesure de différencier les patients de chaque cohorte en fonction de leur réponse en utilisant la PCA et la PLS-DA. Cependant, chez les patients atteints de SpA, cinq métabolites avec un score VIP >2 de l'analyse PLS-DA, y compris le PAF-C16, un facteur d'activation plaquettaire, et le LysoPC (18:0), un composant des membranes cellulaires, semblaient pertinents. Diverses voies métaboliques ont été identifiées dans les deux cohortes : métabolisme des glycérophospholipides ; métabolisme de l'arginine et de la proline ; métabolisme de l'alanine, de l'aspartate et du glutamate ; biosynthèse de la phénylalanine, de la tyrosine et du tryptophane; métabolisme de la cystéine et de la méthionine; biosynthèse du pantothénate et CoA, et biosynthèse de l'aminoacyl-ARNt.

Conclusion : Chez les patients atteints de PR et de SpA, l'adalimumab induit des changements précoces dans le métabolome impliquant des voies telles que le métabolisme des acides aminés essentiels et non essentiels et le stress oxydatif. Ce résultat pourrait guider les investigations futures afin de trouver des marqueurs prédictifs de la réponse thérapeutique aux anti-TNF dans les rhumatismes inflammatoires chroniques.

Mots-clés : métabolomique ; spondylarthrite axiale ; polyarthrite rhumatoïde ; adalimumab ; prédiction de la réponse ; LC/HRMS ; acides aminés ; stress oxydatif.

ABSTRACT

Objectives: To analyse metabolomic changes 4 weeks after initiation of adalimumab, a TNF inhibitor in patients with axial spondyloarthritis (SpA) and Rheumatoid Arthritis (RA) and to study the relationship with clinical response at 12 and 26 weeks.

Methods: We performed post-hoc analyses of serum from patients with SpA from the COMARIS study (NCT01895764) and from patients with RA from the AFORA study (NCT01382160) by liquid chromatography coupled to high-resolution mass spectrometry (LC/HRMS) at inclusion (W0) and 4 weeks (W4) after initiation of adalimumab therapy. Clinical responses were assessed at 12 and 26 weeks. The variations in metabolites concentrations between W0 and W4 were compared between responders and non-responders in univariate, multivariate unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA), using METABOANALYST software. The variables of interest, defined as those with a VIP (Variable Influence on Projection) score greater than or equal to 2 were selected. Finally, we studied the different metabolic pathways in which these variables of interest were involved.

Results: Seventy-four patients were included in the COMARIS cohort, including 43 responders and 31 non-responders. Sixty-three patients were included in the AFORA cohort, including 52 responders and 11 non-responders. In univariate analysis, leucine, hypoxanthine, and n-acetyl-l-alanine were lowered in SpA responders. Carnosine, cortisol, and purine were lowered in RA responders, whereas l-methionine and 5-oxo-l-proline were increased in these subjects. We were unable to differentiate the patients from each cohort according to their response using both PCA and PLS-DA. However, in SpA patients, five metabolites with a VIP-score >2 from the PLS-DA analysis including PAF-C16, a platelet activating factor, and LysoPC(18:0), a component of cell membranes, appeared relevant. Various metabolic pathways were identified in both studies: glycerophospholipid metabolism; arginine and proline metabolism; metabolism of alanine, aspartate, and glutamate; biosynthesis of phenylalanine, tyrosine, and tryptophan; cysteine and methionine metabolism; biosynthesis of pantothenate and CoA, and biosynthesis of aminoacyl-tRNA.

Conclusion: In patients with RA and SpA, adalimumab induced early changes in the metabolome involving pathways such as essential and non-essential amino acid metabolism and oxidative stress. This finding may guide future investigations to find predictive markers of therapeutic response to TNF inhibitors in chronic inflammatory rheumatic diseases.

Keywords: metabolomics; spondylarthritis; rheumatoid arthritis; adalimumab; response prediction; LC/HRMS; amino acids; oxidative stress.

LISTE DES ABBREVIATIONS

ACR	American College of Rheumatology
AFORA	Serum Concentration of Adalimumab as a Predictive Factor of clinical Outcomes in Rheumatoid Arthritis
ARNt	Acide Ribonucléique de transfert
ASDAS	Ankylosing Spondylitis Disease Activity Score
ATRA	all-trans-retinoic acid
bDMARDS	biologic Disease Modifying Anti-Rheumatic Drug
CoA	Coenzyme A
COMARIS	Effect of the Combination of Methotrexate and Adalimumab on Reduction of Immunization in Ankylosing Spondylitis
CRP	C-reactive protein
csDMARDS	conventional synthetic Disease Modifying Anti-Rheumatic Drug
DAS28	Disease Activity Score on 28 joints
ESR	Erythrocyte Sedimentation Rate
EULAR	European Alliance of Associations for Rheumatology
HLA-B27	Human Leukocyte Antigen B27
HUGO	Hôpitaux Universitaires du Grand Ouest
IPP	Interphalangeal proximal
LC/HRMS	Liquid chromatography coupled to high-resolution mass spectrometry
LPC (18:0)	Lysophosphatidylcholine 18:0
MCP	Metacarpophalangeal
MRI	Magnetic Resonance Imaging
PAF	Platelet Activating Factor
PCA	Principal Component Analysis
PLS-DA	Partial Least Squares Discriminant Analysis
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
S0, S4, S12, S26	Semaine 0, semaine 4, semaine 12, semaine 26
SpA	Spondyloarthritis/Spondylarthrite axiale
TNF	Tumor Necrosis Factor
tRNA	transfert Ribonucleic Acid
tsDMARDS	targeted synthetic Disease Modifying Anti-Rheumatic Drugs
VIP-score	Variable Influence on Projection score
VS	Vitesse de sédimentation
W0, W4, W12, W26	Week 0, week 4, week 12, week 26

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Résumé général du travail de thèse

1. Introduction

1.1. Spondyloarthrite et Polyarthrite rhumatoïde

Le terme « spondyloarthrite » désigne un groupe de maladies inflammatoires chroniques caractérisées par des points communs sur le plan clinique et génétique. Ces pathologies peuvent toucher aussi bien le squelette axial que les articulations périphériques. La prévalence de l'atteinte axiale (Spondylarthrite Ankylosante) dans la population mondiale varie entre 0,32% et 1,4% avec des particularités régionales et un gradient Nord-Sud **(1)**.

Cliniquement, les patients atteints de spondylarthrite axiale présentent des douleurs de rythme inflammatoire du rachis ainsi que des fesses, parfois à bascule avec une importante raideur matinale. Parmi les manifestations périphériques les plus fréquentes, nous pouvons citer les arthrites et les enthésites retrouvées chez 30 à 50% des patients atteints de spondylarthrite axiale au diagnostic ou ayant présenté ces symptômes dans les années précédant le diagnostic. Enfin, des symptômes extra-articulaires tels que l'uvéite antérieure – atteinte extra-articulaire la plus fréquente – peuvent venir compléter le tableau clinique **(1)**.

Sur le plan pathogénique, de multiples mécanismes sont à noter tels qu'une prédisposition génétique faisant intervenir certains antigènes du complexe majeur d'histocompatibilité, le plus connu étant le gène HLA-B27 **(2)**. Des mécanismes immunitaires sont également à mentionner, notamment ceux faisant intervenir des cytokines pro-inflammatoires telles que le Tumor Necrosis Factor (TNF) et les interleukines 23 et 17, dont les concentrations sont élevées dans le sérum des patients atteints de spondylarthrite **(3)**. Cependant, l'ensemble de la physiopathologie n'est pas totalement connu à ce jour.

La polyarthrite rhumatoïde, quant à elle, touche environ 0,5 à 1% de la population mondiale avec des variations régionales **(4)**. En France, elle est estimée à 0,3% avec là encore des particularités régionales **(5)**. Sur le plan clinique, elle se présente comme une atteinte symétrique des petites et moyennes articulations avec des douleurs de rythme inflammatoire et une importante raideur matinale. Sa physiopathologie n'est pas non plus complètement connue mais elle serait la conséquence d'une interaction entre des facteurs environnementaux tels que le tabac **(6,7)**, le changement dans le microbiote buccal et intestinal **(8)** et un terrain génétique prédisposé. La combinaison de ces éléments entrainerait un état inflammatoire au niveau des membranes synoviales faisant intervenir à nouveau le TNF **(9)**.

En ce qui concerne l'activité de ces pathologies, celle-ci peut être évaluée par des scores tels que l'ASDAS (Ankylosing Spondylitis Disease Activity Score) développé en 2009 pour la spondyloarthrite **(10,11)** et le DAS28 (Disease Activity Score 28), développé dans les années 90 pour la polyarthrite rhumatoïde **(12)** qui sont tous les deux des scores composites tenant compte de l'auto-évaluation de la maladie par le patient associée à celle du clinicien et d'un marqueur biologique de l'inflammation, la Protéine C-réactive (CRP) ou la vitesse de sédimentation (VS). La spondylarthrite est considérée comme inactive pour un score ASDAS inférieur à 1,3 ; modérée pour un score compris entre 1,3 et 2,1 ; active pour un score compris entre 2,1 et 3,5 et étant très active pour un score au-delà de 3,5 **(13)**. La polyarthrite rhumatoïde est considérée comme étant en rémission pour un score DAS28 inférieur à 2,6 ; d'activité faible pour un score compris entre 2,6 et 3,2 ; d'activité modérée pour un score compris entre 3,2 et 5,1 et enfin d'activité élevée pour un score supérieur à 5,1 **(14,15)**. Ainsi, il est possible de définir la réponse thérapeutique de ces deux pathologies en fonction de la variation de leur score d'activité entre l'introduction du traitement et la réévaluation par le clinicien.

1.2. Thérapeutiques actuellement disponibles

La prise en charge de la spondylarthrite axiale et de la polyarthrite rhumatoïde repose sur l'utilisation de traitements symptomatiques tels que les anti-inflammatoires non stéroïdiens, la corticothérapie orale et les traitements de fond désignés aujourd'hui par le terme csDMARDS pour « conventional synthetic Disease Modifying Anti-Rheumatic Drug » (Méthotrexate, Leflunomide, Sulfasalazine notamment) en première intention. Depuis plusieurs années, les traitements de fond se sont étoffés d'alternatives que sont des molécules ciblant des voies d'activation cellulaire ou des marqueurs de l'inflammation appelés bDMARDS pour « biologic Disease Modifying Anti-Rheumatic Drug » ou tsDMARDS pour « targeted synthetic Disease Modifying Anti-Rheumatic Drugs » **(16)**. Parmi celles-ci, on distingue :

- bDMARDS :
    - Les anti-TNF : adalimumab ; certolizumab ; etanercept ; golimumab ; infliximab
    - Les anti-interleukine 6 : tocilizumab ; sarilumab
    - Le modulateur de la costimulation des lymphocytes T : abatacept
    - Le rituximab
- tsDMARDS : baricitinib ; tofacitinib ; upadacitinib

Ces molécules peuvent être et sont de plus en plus utilisées en association ou en monothérapie chez les sujets ayant une réponse incomplète ou une intolérance aux traitements de première ligne dans un but de rémission ou de moindre activité de leur pathologie **(17–19)**.

2. Le sujet de réflexion

Malgré les nombreuses options thérapeutiques dont le rhumatologue dispose, 30 à 40% des patients atteints de spondylarthrite axiale ou de polyarthrite rhumatoïde ne répondent pas complètement au traitement **(20–22)**. Aujourd'hui, en plus de la compréhension des mécanismes physiopathologiques de ces deux rhumatismes inflammatoires chroniques, il devient nécessaire d'identifier des marqueurs prédictifs de la réponse afin de pouvoir proposer une prise en charge thérapeutique la plus optimale possible.

La métabolomique désigne l'exploration de l'ensemble des métabolites des organismes vivants et fait partie du groupe des « OMICS » (génomique, transcriptomique, protéomique, lipidomique et glucidomique), procédés développés ces dernières années ayant permis d'explorer et de comprendre de nombreux processus physiopathologiques sur le plan moléculaire. L'inflammation locale et systémique retrouvée dans les rhumatismes inflammatoires chroniques est à l'origine de modifications dans le métabolisme local et général, pouvant être objectivées par les analyses métabolomiques **(23)**. Des profils métaboliques particuliers, pouvant correspondre à des « signatures » de ces pathologies ont ainsi pu être mis en évidence **(24–26)**.

Sur le plan thérapeutique, les modifications induites dans le métabolome par les traitements tels que les anti-TNF alpha ont pu être étudiées chez les patients atteints de spondylarthrite d'une part et de polyarthrite rhumatoïde d'autre part, révélant la restauration de l'équilibre de certains métabolites dans le sérum après traitement chez les premiers et la possibilité de différencier les répondeurs et les non répondeurs au traitement selon le profil métabolique urinaire de base chez les seconds par exemple **(27,28)**.

3. Les projets MetAFORA et MetCOMARIS

C'est ainsi que sont nés les projets MetAFORA et MetCOMARIS, consistant en l'analyse post-hoc, en utilisant la spectrométrie de masse, d'échantillons de sérums de patients des études AFORA (Serum Concentration of Adalimumab as a Predictive Factor of clinical Outcomes in Rheumatoid Arthritis, NCT01382160) et COMARIS (Effect of the Combination of

Methotrexate and Adalimumab on Reduction of Immunization in Ankylosing Spondylitis, NCT01895764) dans le but d'observer des trajectoires métaboliques selon la réponse à l'adalimumab afin d'identifier des biomarqueurs prédictifs.

Dans le cadre de ces études, des prélèvements sanguins avaient été réalisés avant et après initiation du traitement par adalimumab à intervalles réguliers (4 semaines, 8 semaines, 12 semaines puis 26 semaines) en parallèle de l'évaluation de la réponse clinique au traitement via les scores d'activité. Les résultats de l'étude COMARIS sont publiés et disponibles **(29)**; ceux de l'étude AFORA sont encore en cours d'analyse.

MetAFORA a initialement fait l'objet de la thèse de médecine du Dr Caroline PETIT, soutenue en septembre 2020. Ses analyses avaient permis de mettre en évidence une différence de concentration de certains métabolites avant traitement par adalimumab selon la réponse EULAR et l'hippurate semblait se démarquer parmi les métabolites potentiellement prédictifs de la réponse thérapeutique. Des éléments cliniques et métabolomiques des patients de l'étude COMARIS étant disponibles, nous avons envisagé de faire le même type de travail sur ces données (naissance de l'étude MetCOMARIS), de reprendre celles de l'étude MetAFORA, puis de comparer les résultats entre les deux études.

Ainsi, dès novembre 2021, nous avons commencé par une première phase de bibliographie afin de faire le point sur la littérature disponible dans le domaine de la métabolomique et les pathologies rhumatologiques inflammatoires chroniques. Puis j'ai collecté les caractéristiques cliniques des patients de l'étude COMARIS afin de déterminer leur réponse thérapeutique à l'adalimumab selon la variation du score ASDAS entre l'inclusion et l'évaluation clinique à 12 et 26 semaines. Les données métabolomiques avaient été analysées à l'inclusion, 4 semaines puis 12 semaines par la plateforme PST ASB de l'INSERM 1253, iBrain, et ont été recueillies au mois de décembre 2021.

Le pourcentage de variation des concentrations relatives des métabolites entre l'inclusion et 4 semaines a été déterminé, et une base de données intégrant les données cliniques et métabolomiques a ainsi été constituée en rapportant la différence brute à la valeur de détection de concentration de métabolite la plus faible à l'inclusion. Les données ont été analysées grâce au logiciel METABOANALYST 5.0 afin d'évaluer les profils métabolomiques des sujets répondeurs et non répondeurs au traitement par adalimumab et d'identifier les principales voies métaboliques potentiellement impliquées aussi bien dans la physiopathologie que dans la réponse au traitement.

Dans le but d'assurer une homogénéité de méthodologie entre les deux études, j'ai repris les éléments de MetAFORA selon le même principe à partir du mois de février 2022 : collecte des données cliniques avec identification des répondeurs et non répondeurs selon la variation du score DAS28 entre l'inclusion et l'évaluation clinique à 12 puis 26 semaines en accord avec les recommandations EULAR ; détermination du pourcentage de variation de la concentration des métabolites avec Microsoft Excel entre l'inclusion et 4 semaines puis analyse métabolomique sur METABOANALYST 5.0 dans le même but que celui précédemment cité pour MetCOMARIS.

L'article rapportant les résultats obtenus est présenté au cours des pages suivantes comme travail de thèse. Celui-ci sera soumis à la revue Metabolites (ISSN 2218-1989, IF 4.932), sur invitation le 15 décembre 2021 à participer au numéro spécial intitulé "How Metabolomics Findings Can Drive New Therapeutic Approaches ? » (Guest Editor: Professor Helene Blasco).

Metabolomic profile and therapeutic response to adalimumab in inflammatory rheumatic diseases

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Abstract

Objectives: To analyse metabolomic changes 4 weeks after initiation of adalimumab, a TNF inhibitor in patients with axial spondyloarthritis (SpA) and Rheumatoid Arthritis (RA) and to study the relationship with clinical response at 12 and 26 weeks.

Methods: We performed post-hoc analyses of serum from patients with SpA from the COMARIS study (NCT01895764) and from patients with RA from the AFORA study (NCT01382160) by liquid chromatography coupled to high-resolution mass spectrometry (LC/HRMS) at inclusion (W0) and 4 weeks (W4) after initiation of adalimumab therapy. Clinical responses were assessed at 12 and 26 weeks. The variations in metabolites concentrations between W0 and W4 were compared between responders and non-responders in univariate, multivariate unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA), using METABOANALYST software. The variables of interest, defined as those with a VIP (Variable Influence on Projection) score greater than or equal to 2 were selected. Finally, we studied the different metabolic pathways in which these variables of interest were involved.

Results: Seventy-four patients were included in the COMARIS cohort, including 43 responders and 31 non-responders. Sixty-three patients were included in the AFORA cohort, including 52 responders and 11 non-responders. In univariate analysis, leucine, hypoxanthine, and n-acetyl-l-alanine were lowered in SpA responders. Carnosine, cortisol, and purine were lowered in RA responders, whereas l-methionine and 5-oxo-l-proline were increased in these subjects. We were unable to differentiate the patients from each cohort according to their response using both PCA and PLS-DA. However, in SpA patients, five metabolites with a VIP-score >2 from the PLS-DA analysis including PAF-C16, a platelet activating factor, and LysoPC(18:0), a component of cell membranes, appeared relevant. Various metabolic pathways were identified in both studies: glycerophospholipid metabolism; arginine and proline metabolism; metabolism of alanine, aspartate, and glutamate; biosynthesis of phenylalanine, tyrosine, and tryptophan; cysteine and methionine metabolism; biosynthesis of pantothenate and CoA, and biosynthesis of aminoacyl-tRNA.

Conclusion: In patients with RA and SpA, adalimumab induced early changes in the metabolome involving pathways such as essential and non-essential amino acid metabolism and oxidative stress. This finding may guide future investigations to find predictive markers of therapeutic response to TNF inhibitors in chronic inflammatory rheumatic diseases.

Keywords: metabolomics; spondylarthritis; rheumatoid arthritis; adalimumab; response prediction; LC/HRMS; amino acids; oxidative stress.

Introduction

Spondylarthritis (SpA) and rheumatoid arthritis (RA) are the two most common chronic inflammatory rheumatic diseases, affecting 0.32 to 1.4% and 0.5 to 1% of the population, respectively, with regional particularities **(1,5)**. These pathologies induce an important functional impact and an alteration of the quality of life **(30–33)** as a result from an inflammatory process involving cytokines such as Tumor Necrosis Factor (TNF) alpha. Adalimumab, a recombinant human monoclonal antibody to TNF, is one of the first molecules to be developed and to have proven its efficacy in both diseases **(34–37)**.

However, despite the availability of therapeutic options and a better knowledge of these diseases, an insufficient clinical response persists in approximately one third of patients treated with TNF inhibitors **(20–22)**. Several factors have been mentioned to explain this disparity in response to treatment between patients such as smoking, female sex **(38,39)**, advanced age **(20)**, or the presence of antibodies against treatment **(21)**. Moreover, a poorer response to TNF inhibitors in obese SpA patients has been reported by Micheroli et *al.* **(40)** and was recently confirmed in patients with RA by Law-Wan J, et *al.* in a meta-analysis **(41)**.

Metabolomics is the study of all metabolites present in living organisms. Metabolites are low molecular weight molecules, involved in multiple processes, as intermediates or final products. The study of pathophysiology through various samples (serum, urine, synovial fluid, stool) from patients suffering from SpA and RA via this field of exploration has allowed the identification of metabolic specific signatures **(23,24)**, essentially in a diagnostic approach given the relatively long time between the onset of symptoms and diagnosis, frequently found in chronic inflammatory rheumatic diseases **(42–44)**.

The contributions of this technique in a process of prediction of response to treatment are still recent although encouraging. Indeed, the analysis of the modification of the metabolome in response to TNF inhibitors has demonstrated the restoration of the balance of certain metabolites in serum in patients with SpA on the one hand and the possibility of differentiating responders from non-responders according to their urinary metabolic profile before treatment in those with RA on the other hand **(27,28)**. Disturbances in lipid metabolism with regulation after TNF inhibitor were identified after analysis of serum, urine, and stool samples from patients with Crohn's disease, suggesting their use as predictive markers of therapeutic response **(45)**.

However, the numbers of these studies are limited with a multiplicity of substrates and a variety of analytical methods that could lead to contradictory results, as found in the literature. So, no definite predictive marker of response to TNF inhibitors has been found so far. Thus, through a post-hoc analysis of serum samples from patients with SpA and RA, our study was to identify possible markers associated with response to adalimumab treatment at 12 and 26 weeks in these two populations, according to the early variation of their metabolome after treatment initiation.

Material and methods

1. Study plan

MetCOMARIS is a post-hoc study of the COMARIS study (Effect of the Combination of Methotrexate and Adalimumab on Reduction of Immunization in Ankylosing Spondylitis; NCT01895764) that was conducted within the HUGO (Hôpitaux Universitaires du Grand Ouest - Western France University Hospitals) network from March 2013 to October 2014. The primary objective was to study the effect of the combination of methotrexate with adalimumab on the immunization of patients with axial SpA. A hundred and ten patients were included.

Patients were randomized in a 1:1 ratio to receive either adalimumab 40 mg subcutaneous injection every two weeks as monotherapy; or in combination with 10 mg weekly subcutaneous injection. This was a prospective, multicentre study that lasted 26 weeks. Results were published in 2020 by Ducourau E, et *al*. **(29)**.

MetAFORA is a post-hoc study of AFORA (Serum Concentration of Adalimumab as a Predictive Factor of clinical Outcomes in Rheumatoid Arthritis; NCT01382160) which was also conducted within the HUGO network and whose main objective was to estimate the pharmacodynamic parameters of the concentration-effect relationship of adalimumab in RA. AFORA was conducted from January 2011 to January 2013 with a 26-week follow-up of patients. The results of this study are still under analysis.

In the present study, we performed metabolomic analyses on samples from patients in each cohort according to their clinical response to adalimumab treatment, to assess early metabolome changes potentially involved in the response to treatment.

2. Detailed study methodology

a. Patients

The following inclusion criteria were similar in both cohorts:

- Male or female of age greater than or equal to 18 years.
- With ASAS diagnostic criteria of axial SpA or ACR (1987) diagnostic criteria of RA **(Annexes I)**.
- Justifying treatment with adalimumab in accordance with the marketing authorization.

More specifically to the COMARIS cohort:

- Absence of methotrexate administration in the last 3 months and the absence of previous treatment with a TNF inhibitor.

More specifically to the AFORA cohort:

- Stability of background methotrexate and/or corticosteroid therapy 4 weeks prior to inclusion and at least during the first 12 weeks of the study.

b. Clinical data

Levels of spinal pain, peripheral pain and swelling, and duration of morning stiffness were collected using a visual analogue scale at each visit (W4, W12 and W26) in the COMARIS cohort. A joint index, synovitis and patients' assessment of disease activity using a visual analogue scale were reported at each visit (W4, W12 and W26) in the AFORA cohort.

All these clinical elements associated with the biological data were used to calculate the activity score specific to each cohort: ASDAS in the COMARIS cohort and DAS28 in the AFORA cohort. According to the ASDAS score obtained, SpA was considered as ‘inactive’, ‘moderately active’, ‘active’, or ‘very active’. Similarly, according to the DAS28 score obtained, RA was in ‘remission’, ‘low’, ‘moderate’, or ‘high’ activity **(Annexes II)**.

c. Blood samples and biological data

Blood samples were taken before adalimumab injections and then at each visit at W4, W12 and W26. The samples were centrifuged, aliquoted, labelled and stored in each investigating centre before being sent centrally in dry ice to the Tours centre where all samples were stored at -80°C. The erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were measured in the biochemistry laboratories of the Centre Hospitalier Régional Universitaire de Tours under the responsibility of Eric Piver.

d. Evaluation of the therapeutic response

Therapeutic response was defined according to the change in each activity score between inclusion and the 12- and 26-week clinical assessment.

In the COMARIS cohort, based on the degree of change in ASDAS, therapeutic response was defined as: ‘absent’; ‘significant’ or ‘major’. In the AFORA cohort, according to the variation of DAS28, therapeutic response was defined as: ‘absent’; ‘moderate’ or ‘good’ **(Annexes III)**.

In our study, we chose to group patients into "responders" and "non-responders" according to the following modalities:

- COMARIS cohort: those with a ‘significant’ or ‘major’ response were considered "responders" and those with ‘absent’ were considered "non-responders".
- AFORA cohort: those with a ‘moderate’ or ‘good’ response are considered "responders" and those with ‘absent’ were considered "non-responders".

e. Metabolomic data

One-millilitre serum samples were analysed. They were first thawed for 1 hour 30 minutes and then 50 µL of each serum was collected. The metabolites were extracted with 400µL of methanol added to 50µL of serum. The samples were vortexed for 5 seconds and then incubated at -20°C for 30 minutes to deproteinize the sample. After centrifugation for 25 min at 5000 rpm at 4°C, the supernatant (350 µL) was harvested and transferred to a 96-well plate. After evaporation of the plate under nitrogen, the dry residue was resuspended in 100 µL of methanol/water (75/25) for C18 analysis, in 100 µL of acetonitrile/water (75/25) for hydrophilic interaction liquid chromatography (HILIC) analysis. After vortexing again for 20 seconds, the supernatant was transferred to a 500 µL Eppendorf tube. The tube was centrifuged for 15 minutes at 5000 rpm at 4°C. Five µL of sample was injected for analysis by positive and negative ionization mass spectrometry. The analytical technique was liquid chromatography coupled to high-resolution mass spectrometry (LC/HRMS). The analyses were performed on an Ultimate WPS-3000 UPLC system (Dionex, Germany) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

Liquid chromatography was performed using two different modes:

- HILIC to analyse polar molecules with HILIC Cortecs columns (1.6µm 150x2.10m) maintained at 40°C. Two mobile phases were used, and chromatographic gradients were performed at a flow rate of 0.3mL performed at a flow rate of 0.3mL / min.
- C18 to analyse apolar molecule. C18xb columns (1.7µm 150 x 2.1mm) were maintained at 55°C. Two mobile phases were used, and chromatographic gradients were performed at a flow rate of 0.3mL/min.

Ionization of the molecules was performed using the positive (ESI+) and negative (ESI-) electrospray method. During acquisition, ions were scanned from 58 to 870 m/z at a resolution of 70,000 (m/z = 200). The analyses were processed in a so-called "targeted" manner. A library of 530 molecules has already been characterized with the chromatographic conditions used for this study (C18 and HILIC combined). Signal values were calculated with the Thermo Xcalibur processing configuration software (Thermo Fisher Scientific) by integrating the chromatography peaks corresponding to the selected metabolites. Once the Excel data table with the metabolites was obtained, different data pre-treatments were performed. Analyses were performed by Antoine Lefevre under the supervision of Patrick Emond.

In case the metabolite was not detectable, the lowest measured value within the cohort was used as a surrogate to calculate the variation between the inclusion visit and W4.

3. Statistical analysis

Clinical and biological data from AFORA and COMARIS cohorts at inclusion were analysed using BiostaTGV. Quantitative values were analysed with a student's t test and qualitative variables with a Fisher's test. The significance level was set at 0.05.

Metabolomic profiles of patients from both cohorts were independently analyzed with the online software METABOANALYST 5.0., at baseline and then between W0 and W4 depending on the therapeutic response at 12 weeks (primary objective) and 26 weeks (secondary objective).

First, we performed a univariate analysis, followed by an unsupervised principal component analysis (PCA). Then we performed a partial least squares discriminant analysis (PLS-DA), a supervised method of multiple linear regression to separate the data of each cohort into two groups to find the maximum covariance at baseline and between W0 and W4 according to the therapeutic response at 12 and 26 weeks.

Permutation tests were performed to verify the reliability of our models and then we identified variables of interest, defined as those with a VIP (Variable Influence on Projection) score greater than or equal to 2, from the PLS-DA analysis in each cohort.

From these variables of interest, we proceeded to the analysis of the metabolic pathways involved at the inclusion, then in the early variation of the metabolome according to the therapeutic response at 12 and 26 weeks in each cohort. Those found in the early variation of the metabolome according to the therapeutic response at 12 weeks were then compared between the two cohorts.

Those analysis were performed by Elom Tay under the supervision of Hélène Blasco.

Results

1. Population characteristics

In the COMARIS cohort, at 12 weeks, we excluded 30 patients with no metabolomic data available over the entire study period, 1 patient for whom the ASDAS was missing, 2 patients for whom the metabolomic data at inclusion were missing and 3 patients for whom the clinical response data was missing. At 26 weeks, we excluded 1 patient for whom the ASDAS score at inclusion was missing, 7 patients for whom the clinical response was missing and 1 patient for whom the metabolomic data at inclusion was missing. We therefore studied the sera of 74 patients in the primary analysis and 71 patients in the secondary analysis.

In the AFORA cohort, 69 patients were included. The results of 2 patients could not be studied, 2 patients left the study at 4 weeks due to lack of clinical response, one patient was wrongly included, and we had a duplicate of data for one patient at 4 weeks. In addition, at 26 weeks, clinical response for 7 patients were lacking. We therefore studied the sera of 63 patients in the primary analysis and 56 patients in the secondary analysis.

All these data are shown in **Figure 1**.

In the COMARIS cohort, 69.76% of responders were male ($p = 0.002$). There was no significant difference in age or body mass index between the two groups. CRP was significantly increased at baseline in responders ($p = 0.0002$) and both groups had active disease ($p = 0.002$).

In the predominantly female AFORA cohort, all men appeared to be responders (p = 0.053). There was no significant difference by age, CRP or DAS28. Responders were borderline overweight (p = 0.049).

Finally, there was no difference in the association with methotrexate between responders and non-responders in the two cohorts. These results are shown in **Table 1**.

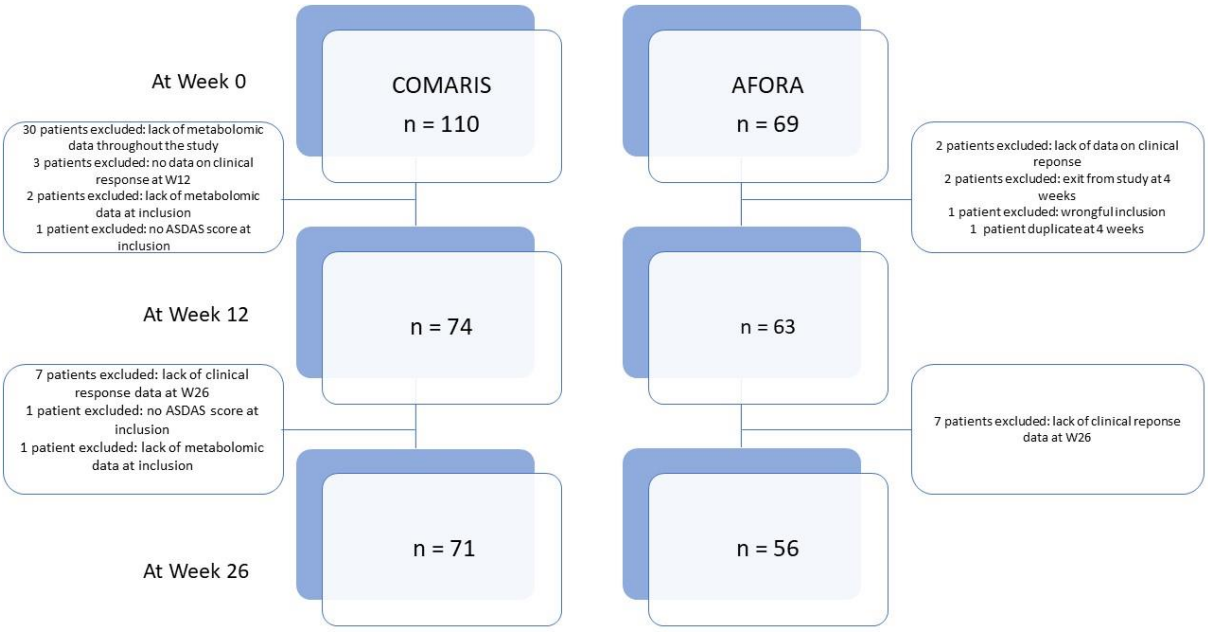

Figure 1. Flow chart of the COMARIS and AFORA cohorts.

Table 1. Baseline characteristics by adalimumab response at 12 Weeks (COMARIS n = 74; AFORA n = 63)

Cohort	COMARIS			AFORA		
Clinical status at 12 weeks	Responders (n= 43)	Non-responders (n= 31)	p-value	Responders (n= 52)	Non-responders (n = 11)	p-value
Male (n, %)	30 (69,76%)	10 (32,25%)	0,002	15 (28%)	0	0,053
Age, mean (years)	41,25	32,25	0,197	54,88	53,36	0,746
BMI, mean (kg/m²)	25,81	25,31	0,623	25,65	23,03	0,049
CRP (mg/l)	13,58	3,22	0,0002	10,87	16,93	0,348
ASDAS-CRP/DAS28	3,42	2,83	0,002	4,8	5,19	0,398
MTX (n, %)	19 (44,18%)	14 (45,16%)	1	35 (67,30%)	7 (63,63%)	1
Corticosteroids	-	-		25 (48,07%)	5 (45,45%)	1

2. Metabolomic analyses

2.1.COMARIS

a. Main analysis: based on therapeutic response at 12 weeks
    - Metabolome analysis at W0 for response prediction

We were not able to separate the metabolomic profiles of patients according to their therapeutic response by either PCA or PLS-DA. There were 2 outliers in PCA and 3 in PLS-DA **(Annexes IV)**. The resulting model can only be considered descriptive (p = 0.46 by permutation test). These analyses revealed higher concentrations of thymidine and theobromine in responders and lower concentrations of L-alanine, L-pipecolic_acid, N-acetyl-l-alanine, sarcosine, methyl_indole-3-acetate, and c4-carnitine (VIP-score ≥ 2) **(Annexes, Figure 1)**.

The following metabolic pathways could be identified: linoleic acid metabolism; glycine, serine, and threonine metabolism; pyrimidine metabolism; and arginine and proline metabolism **(Annexes, Figure 2)**.

- Analysis of the metabolome change between W0 and W4

We were not able to separate the metabolomic profiles of patients according to their therapeutic response at 12 weeks neither in PCA nor in PLS-DA, **Figure 2**. The permutation test showed a p value of 0.65.

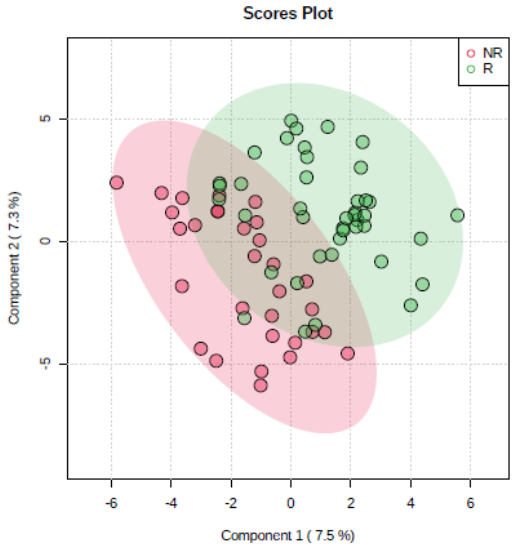

Figure 2. COMARIS. PLS-DA separation of different patient groups according to metabolome change between W0 and W4 based on clinical response at 12 weeks. R = responders; NR = non-responders.

We highlighted 15 metabolites of interest including 5 with a VIP-score ≥ 2 that are: 5,6-dihydrouracil, l-cystine, and retinoate increased in responders whereas LPC(18:0) (LysoPC 18:0) and PAF-C16 (Platelet Activating Factor - C16) were decreased in this subgroup, **Figure 3**.

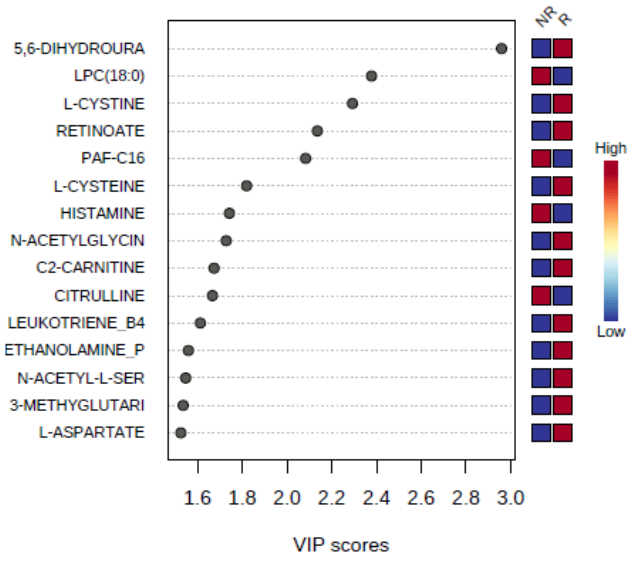

Figure 3. COMARIS. Score-plot of metabolites of interest of different patient groups according to metabolome change between W0 and W4 as a function of clinical response at 12 weeks. R = responders; NR = non-responders.

We were able to identify the following metabolic pathways: arginine biosynthesis; retinol metabolism; alanine, aspartate, and glutamate metabolism; histidine metabolism; cysteine and methionine metabolism; beta-alanine metabolism; and pantothenate and CoA biosynthesis, **Figure 4**.

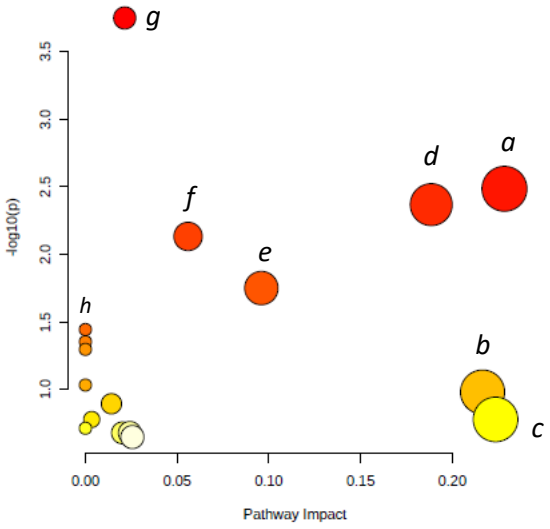

Figure 4. COMARIS. Metabolic pathways involved in the metabolome change between W0 and W4 according to the therapeutic response at 12 weeks. a: arginine biosynthesis; b: retinol metabolism; c: alanine, aspartate, and glutamate metabolism; d: histidine metabolism; e: cysteine and methionine metabolism; f: beta-alanine metabolism; g: pantothenate and CoA biosynthesis; h: aminoacyl-tRNA biosynthesis.

b. Secondary analysis: based on therapeutic response at 26 weeks
    - Metabolome analysis at W0 for response prediction

We did not observe a separation in PCA, nor in PLS-DA between the two groups ($p = 0.53$ by permutation test). There were 3 outliers in both analyses **(Annexes IV)**. Metabolites such as allantoin, theobromine, and cholesteryl acid appeared increased in responders, whereas trans-cinnamate was lowered in these subjects (VIP-score ≥ 2) **(Annexes, Figure 3)**.

The metabolic pathways that could be identified were glycine, serine, and threonine metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; tyrosine metabolism;

arginine and proline metabolism; taurine and hypotaurine metabolism; beta-alanine metabolism; and histidine metabolism. **(Annexes, Figure 4)**.

- Analysis of the metabolome change between W0 and W4

Analyses in PCA and PLS-DA did not separate metabolomic profiles by response status (p = 0.73 by permutation test). There was 1 outlier in PLS-DA **(Annexes IV)**. Among the metabolites of interest from PLS-DA analysis, we found PAF-C16 and LPC (18:0), both lowered in responders. They are shown in **(Figure 5)** in the **(Annexes)**.

Metabolic pathway analysis identified the following pathways: aminoacyl-tNRA biosynthesis; valine, leucine, and isoleucine metabolism; glycine, serine, and threonine metabolism; glutathione metabolism; glyoxylate and dicarboxylate metabolism; arginine and proline metabolism; d-glutamine and glutamate metabolism; and alanine, aspartate, and glutamate metabolism **(Annexes, Figure 6)**.

c. Common metabolic pathways

Metabolites such as PAF-C16 and LPC (18:0) appear to be the only relevant components of the metabolome variation between W0 and W4 between responders and non-responders. Metabolic pathway analysis revealed a few common pathways at both time points represented in **Figure 5**.

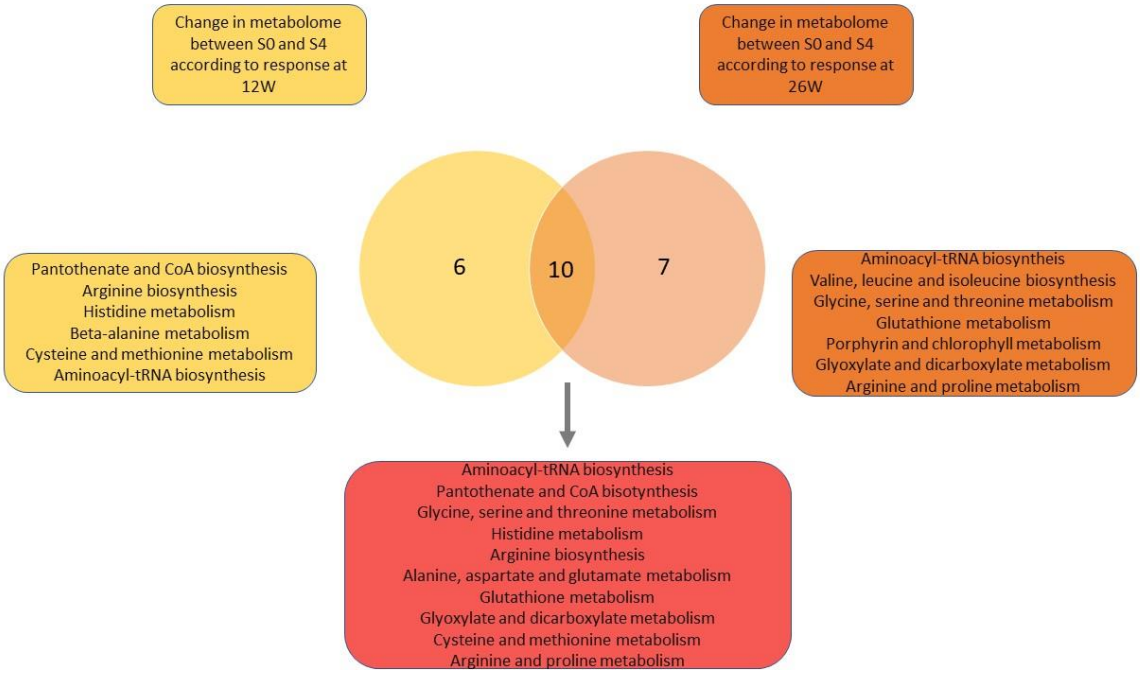

Figure 5. COMARIS. Venn diagram representing common metabolic pathways in metabolome analyses between W0 and W4 according to therapeutic responses at 12 and 26 weeks

2.2.AFORA

a. Main analysis: based on therapeutic response at 12 weeks
   - Metabolome analysis at W0 for response prediction

The analysis in PCA did not allow us to separate the metabolomic profiles of patients according to their therapeutic response. This separation was possible in PLS-DA but with a p = 0.70 in the permutation test **(Annexes, Figure 7)**. There were 4 outliers in PCA and 3 in PLS-DA **(Annexes IV)**.

The metabolites of interest are shown in **(Figure 8)** in the **(Annexes)**. Those with a VIP-score ≥ 2 are paraxanthin, theobromine, catechol, pantothenic acid, n-acetylglycine, o-acetyl-l-carnitine, rac-glycerol_1 and lauroylcarnitine.

Among the metabolic pathways possibly involved at W0, we identified: caffeine metabolism; ketone body synthesis and degradation; and butanoate metabolism **(Annexes, Figure 9)**.

- Analysis of the metabolome change between W0 and W4

The metabolomic profiles of the patients could not be separated by either PCA or PLS-DA analysis (p = 0.23 by permutation test) as shown in **Figure 6**. There were 4 outliers in PLS-DA **(Annexes IV)**.

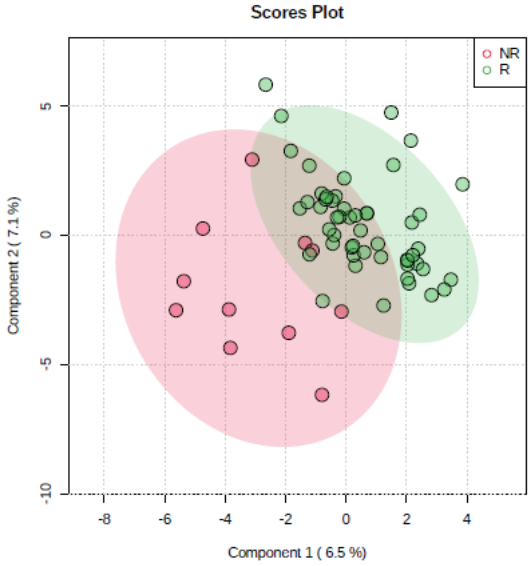

Figure 6. AFORA. PLS-DA separation of different patient groups according to metabolome change between W0 and W4 based on clinical response at 12 weeks. R = responders; NR = non-responders

15 metabolites of interest could be identified in PLS-DA, including 7 with a VIP-score ≥ 2: carnosine; l-ornithine; l-valine; l-glutamic_acid; n-acetyl-serine; l-serine; l-threonine, all of which were lowered in responders, **Figure 7**.

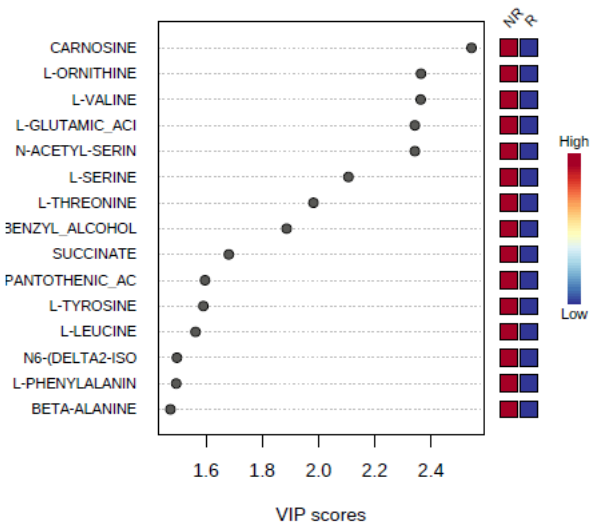

Figure 7. AFORA. Score-plot of metabolites of interest of different patient groups according to metabolome change between W0 and W4 according to clinical response at 12 weeks. R = responders; NR = non-responders.

Part of the highlighted metabolic pathways are shown in **Figure 8**, to which are added the pathways of butanoate metabolism; histidine metabolism; propanoate metabolism; glutathione metabolism and glyoxylate and dicarboxylate metabolism.

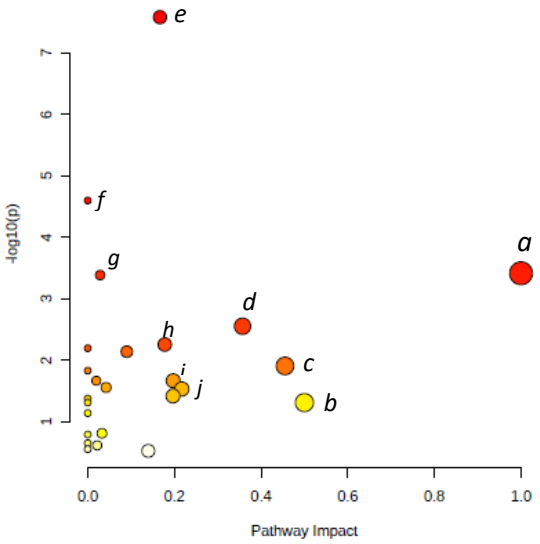

Figure 8. AFORA. Metabolic pathways involved in the metabolome change between W0 and W4 according to therapeutic response at 12 weeks. a: phenylalanine, tyrosine, and tryptophan biosynthesis; b: D-glutamine and D-glutamate metabolism; c: beta-alanine metabolism; d: phenylalanine metabolism; e: Aminoacyl-t-RNA biosynthesis; f: valine, leucine, and isoleucine biosynthesis; g: pantothenate and CoA biosynthesis; h: arginine biosynthesis; i: alanine, aspartate, and glutamate metabolism; j: glycine, serine, and threonine metabolism.

b. Secondary analysis: based on therapeutic response at 26 weeks
    - Metabolome analysis at W0 for response prediction

The two groups of patients could be correctly separated by the PLS-DA analysis although it included 2 outliers **(Annexes IV)**. However, these results can only be interpreted as descriptive due to a p value = 1 in the permutation test. Of the 15 metabolites identified, 6 had a VIP-score ≥ 2 and were increased in responders: l-lysine; 5-aminopentanoate; glyceraldehyde; l-threonine; guanine; and l-phenylalanine **(Annexes, Figure 10)**.

The metabolic pathways involved at this stage are shown in **(Figure 11)** in the **(Annexes)**, including aminoacyl-tRNA biosynthesis; lysine degradation; glycine, serine, and threonine metabolism; and phenylalanine, tyrosine, and tryptophan biosynthesis.

- Analysis of the metabolome change between W0 and W4

There was a slight overlap between the two groups in PLS-DA analysis. The metabolites of interest are shown in **(Figure 12)** in the **(Annexes)**. Gluconic acid, n-acetylglycine, and glycine were all increased in responders with a VIP-score ≥ 2.

The metabolic pathways that could be demonstrated were the pentose phosphate pathway; glutathione metabolism; glyoxylate and dicarboxylate metabolism; glycine, serine, and threonine metabolism; and arginine biosynthesis. They are shown in **(Figure 13)** in the **(Annexes)**.

c. Common metabolic pathways

We were not able to identify metabolites whose variation over time appeared to be related to therapeutic response, but several metabolic pathways could be identified, shown in **Figure 9** below.

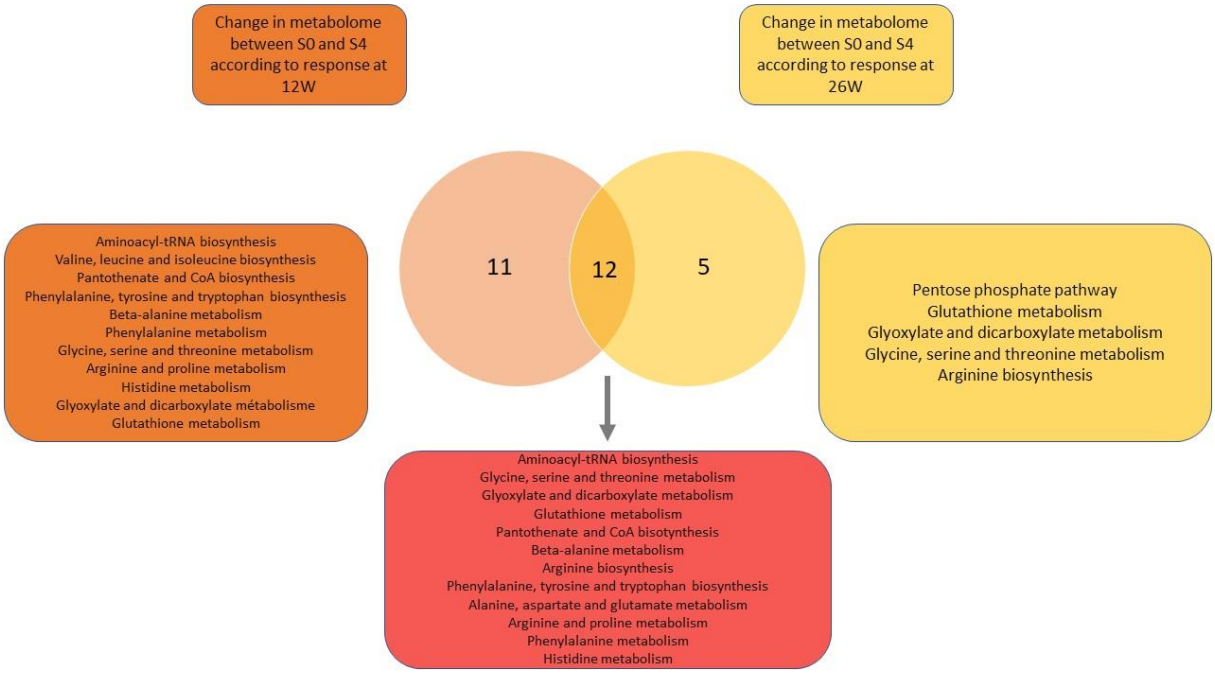

Figure 9. AFORA. Venn diagram representing common metabolic pathways for metabolome analyses between W0 and W4 according to therapeutic responses at 12 and 26 weeks.

2.3.Comparison of the two cohorts

The pathways that appear to be involved in early metabolome variation in response to adalimumab treatment in both cohorts are shown in **Figure 10**.

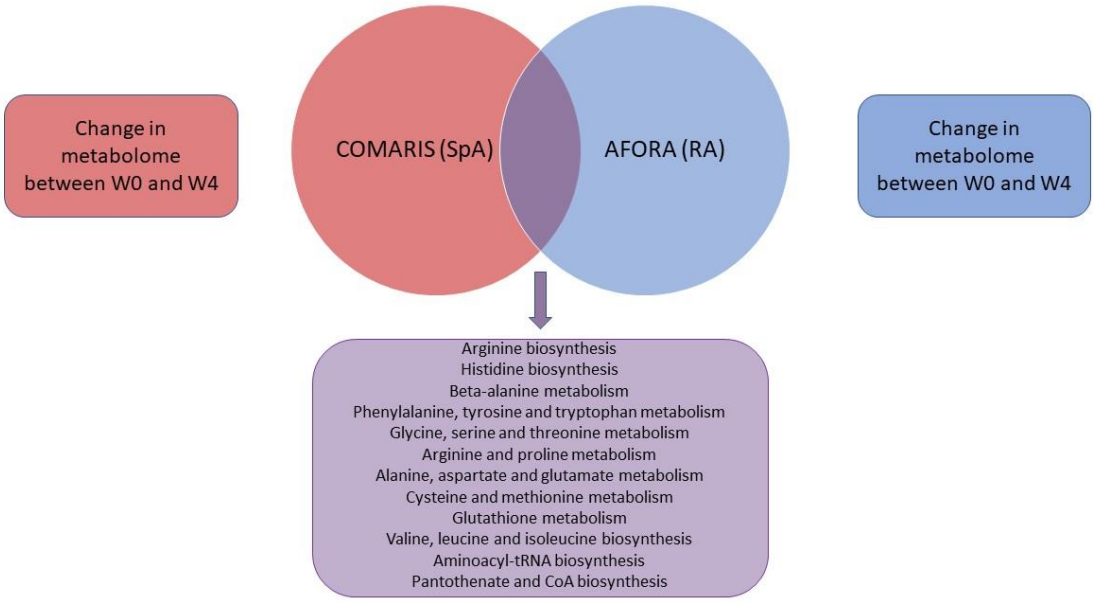

Figure 10. Metabolic pathways common to both cohorts based on metabolome change between W0 and W4.

Discussion

In our study, we tried to identify metabolites whose early variation could differentiate responders from non-responders to adalimumab treatment in patients with SpA and RA by analysing their metabolomic profiles by LC/HRMS. Therapeutic responses were collected at 12 and 26 weeks (respectively primary and secondary endpoints) based on the different clinical activity scores of these conditions in accordance with the recommendations of scientific societies. The characteristics of our model allow us to consider it only as descriptive without the ability to differentiate patients according to their clinical response. However, we were able to highlight relevant metabolites and metabolic pathways already well described in the literature of these two diseases in relation with clinical response, such as the metabolism of glycine, serine, and threonine; arginine and proline metabolism; the metabolism of alanine, aspartate, and glutamate; biosynthesis of phenylalanine, tyrosine, and tryptophan; biosynthesis of valine, leucine, and isoleucine; cysteine and methionine metabolism; glutathione metabolism and glycerophospholipid metabolism.

Metabolome analysis at treatment initiation according to therapeutic response at 12 weeks in the COMARIS cohort showed high levels of thymidine and theobromine in responders, while the following metabolites: l-alanine, l-pipecolic_acid, n-acetyl-l-alanine, sarcosine, methyl_indole-3-acetate, and carnitine were lowered. Low serum carnitine concentrations were indeed found in subjects with SpA **(24,26)** while alanine appeared to be increased in these patients **(26,46)**. We did not find any data concerning thymidine and theobromine in the literature, nor on the possible effect of TNF inhibitors on the regulation of the concentrations of all these metabolites.

When studying the variation of the metabolome between W0 and W4 in the COMARIS cohort, we noticed a decrease in PAF-C16 and LysoPC (18:0) concentrations in relation with the therapeutic response at 12 and 26 weeks. PAF-C16 (also known as PAF) is a ubiquitous molecule belonging to the phospholipid family that acts as a potent activator and mediator in inflammatory processes and cardiovascular diseases. Its involvement in autoimmune diseases, linked or not to its receptor (PAF receptor) was reported by Edwards LJ et *al*. in a review where its concentration was increased in the intestinal mucosa and stool of patients with inflammatory bowel diseases compared to healthy patients **(47)**.

In its storage form, lipo-PAF, it appeared to be higher in patients with inflammatory rheumatic diseases and in those with osteoarthritis or chondrocalcinosis compared to healthy subjects with the particularity of being also elevated in the synovial fluid of patients with RA **(47) (48)**. An interdependence between TNF and PAF has been demonstrated in numerous studies, the former acting at the level of endothelial cells to induce PAF synthesis **(49,50)** ; the second acting on the peripheral monocytes resulting in a production of TNF **(51)**. The consequence was a synergistic inflammatory action at the synovial level **(52,53)**. Thus, PAF appears to be lowered in responders to TNF inhibitors.

LysoPC(18:0) is a lysophospholipid, belonging to the family of lysophosphatidylcholines, molecules involved in fatty acid metabolism. It is present in small quantities in many tissues. An elevated concentration of LysoPC (18:0) was found in patients with SpA compared to healthy subjects without modification after treatment with TNF inhibitor. High concentrations of this metabolite could also be found in the stool of patients with rheumatoid arthritis compared to healthy subjects, supporting the disruption of glycerophospholipid metabolism in these two conditions **(27,54)**.

Interestingly, retinoate was one of the metabolites of interest in the study of metabolome variation between W0 and W4 in relation to therapeutic response at 12 weeks in the COMARIS cohort. It was increased in responders and is known as all-trans-retinoic acid (ATRA), an important regulator of gene expression during growth. Retinoids are known to be involved in bone formation and structure, and in patients with SpA, in whom retinol concentration was disturbed **(55)**. In addition, the in vitro study of the action of ATRA, an active metabolite of vitamin A, on T lymphocytes made it possible to identify its regulatory action on the pathway of Th17 and TNF-alpha synthesis, which were lowered in the cultures studied after treatment **(56)**. Finally, Wu J. et *al*. were able to demonstrate the effect of adalimumab in restoring the concentration of certain proteins such as Retinol Binding Protein 4 (RBP4), whose increase could be used as a predictive marker of a good response to treatment in patients with ankylosing spondylitis **(57)**.

In our RA cohort, metabolome analysis at W0 according to therapeutic response at 12 weeks found high levels of paraxanthin, theobromine, glyceraldehyde, catechol, n-acetylglycine, pantothenic acid, o-acetyl-l-carnitine, and rac-glycerol_1 in responders. Pantothenic acid was described in the 1960s as a potential marker for RA but this has not been confirmed by further studies. Glyceraldehyde is known to be increased in patients with RA **(24,25)**. High levels of xanthine have been found in the urine of patients treated with TNF inhibitors **(58)**; n-acetylglycine was found in increased concentration in patients with RA compared to healthy subjects in numerous studies **(58)** and was increased in etanercept responders in the study by Priori et *al*. **(59)**. Glycerol is also known to be increased in patients with RA **(60–62)** with a decrease in glycerol 3 phosphate after 12 weeks of treatment with a TNF inhibitor **(63)**. Finally, carnitine appears to be lowered in patients with RA **(24)**.

Analysis of the metabolome variation between W0 and W4 according to the therapeutic response at 12 weeks did not identify relevant metabolites. Only n-acetylglycine appeared to be increased in responders according to therapeutic response at 26 weeks, consistent with the data from the study by Priori et *al*. **(59)**

Disturbances in amino acid metabolism have been well described in SpA and RA. A strong association between the combination of concentrations of phenylalanine, tryptophan, threonine, aspartic acid, histidine and the number of painful, swollen joints as well as DAS28 in RA has been reported by Smolenska et *al*. **(64)**. Serine, proline and alanine, non-essential amino acids, have been described as associated with SpA activity **(26)**. More specifically, proline, an

essential element in the synthesis of many human proteins, has been found to be increased in both diseases. Dysfunctions of its metabolism can induce abnormalities in the citric acid cycle, disrupting cellular metabolism and can cause an inflammatory state damaging cartilage and bone **(25,26)**. Its synthesis involves arginine, described as increased in subjects with SpA and rather decreased in those with RA **(25,26)**.

Arginine is needed for the synthesis of nitric oxide, involved in the functioning of endothelial cells with consequences on atherosclerosis and the development of cardiovascular pathologies when this pathway is disrupted. This phenomenon has already been studied in inflammatory rheumatic diseases with encouraging results **(65–67)**. Changes in the metabolic pathways of glycine, serine, and threonine as well as arginine and proline have been reported by Kamleh et *al*. after treatment with etanercept in patients with psoriasis where it was observed a return to the equilibrium state of these pathways **(68)**.

Ou et *al*. described glutamate as being significantly increased in serum in patients with SpA with a strong correlation with disease activity. It is an amino acid derived from glutaminolysis involved in the production of energy for cells including T lymphocytes with a favor of the development of the Th17 pathway. The regulation of its metabolism after treatment with TNF inhibitor was observed in their study **(27)**. In addition, data exist on the key role of glutaminolysis in the cell growth of fibroblast-like synoviocytes in RA **(69)**. The decrease in glutamate after treatment was also found by Gupta et *al*. in patients with SpA **(46)**.

High concentrations of valine, leucine and isoleucine, essential amino acids, have been found in patients with SpA, with a recovery of balance of their metabolism after treatment **(27,46)**. Disturbances in the concentration of phenylalanine were found in both diseases and were influenced by TNF inhibitors **(25,27)**. Low concentrations of tryptophan, another essential amino acid, have been reported in both diseases with a correlation between disease activity in SpA and the breakdown of the bond between tryptophan and its binding protein **(25,70)**. In addition, recent studies have demonstrated some disruptions in tryptophan metabolism in connection with dysregulation of the intestinal microbiota in both SpA and RA patients **(54,71)**.

Cysteine and its precursor, methionine are involved in oxidative stress metabolism with the participation of glutathione. Oxygen and nitrogen reactive species are known to participate in acute or chronic inflammatory processes by interacting with proteins, lipids and DNA thus being able to generate autoantibodies such as citrullinated cyclic peptide antibodies in RA. An increase in these reactive species at the synovial level in RA has also been reported, resulting in local hypoxia and joint destruction **(23,72–74)**. In patients with SpA, a hostile serum microenvironment containing inflammatory markers and advanced oxidative protein products with deleterious action (senescence and apoptosis) on mesenchymal stem cells known to have immunoregulation properties has been reported **(75)**. These advanced oxidative protein products are also known to be linked to disease activity **(76)**. TNF alpha is known to be involved in oxidative stress by inducing the inappropriate production of nitric oxide, a highly reactive species **(77)**. The anti-oxidant effect of TNF inhibitors could be demonstrated by Yamamoto K et *al*. in patients with Crohn's disease treated with infliximab (another TNF inhibitor) and adalimumab without inducing the formation of antioxidant molecules **(78)**.

The main strength of our study is its longitudinal methodology and the novelty of such metabolomic analyses coupled to the clinic for predictive purposes on relatively large samples. We were able to study the metabolomic profile of a total of 137 patients with the two most common inflammatory rheumatic diseases. In addition, we were able to maintain a certain homogeneity of treatment by using the same TNF inhibitor, namely adalimumab.

However, it also has some limitations. Owing to its post-hoc design, blood samples were not intended for metabolomic analyses. As a result, transport and storage conditions have not been standardized. In addition, we used serum as a substrate and not plasma in which the blood clots, a mechanism involving the metabolism of glycerophospholipids **(79)**. We did not control for other factors that may alter patients' metabolism such as diet or cardiovascular comorbidities (dyslipidemia, high blood pressure, obesity/sarcopenia) which is common in patients with inflammatory rheumatic diseases **(80,81)**. Finally, we did not have a unisex population as specific metabolomic profiles by sex were described in the literature both in general population and inflammatory rheumatic diseases **(82–84)**.

Conclusion

Our study aimed to identify metabolites associated with early response to adalimumab treatment in patients with SpA and RA, the two most common inflammatory rheumatic diseases. We were able to identify relevant elements aligned with current data in the literature regarding the metabolism of amino acids (essential and non-essential) and oxidative stress that could serve as a basis for future investigations in the search for predictive markers of response to treatment.

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Annexes

I. Diagnostic criteria for Spondylarthritis by Assessment of Spondyloarthritis international Society (ASAS) and diagnostic criteria for Rheumatoid Arthritis by American College of Rheumatology (ACR) 1987

1. Diagnostic criteria for SpA:

The diagnosis can be made at onset **in subjects < 45 years of age with back pain for at least 3 months** if the following are respected:

- **Sacroiliitis on imaging* + ≥ 1 SpA feature OR HLA-B27 + ≥ 2 other SpA features**

SpA features: inflammatory back pain; arthritis; enthesitis (heel); uveitis; dactylitis; psoriasis; Crohn's/colitis; good response to non-steroid anti-inflammatory drugs (NSAIDs); family history for SpA; HLA-B27; elevated CRP

*: sacroiliitis on imaging means: active (acute) inflammation on MRI highly suggestive of sacroiliitis associated with SpA OR definite radiographic sacroiliitis according to the modified New York criteria.

2. Diagnostic criteria for Rheumatoid Arthritis by ACR 1987

A patient is considered to have rheumatoid arthritis if he meets 4 of the 7 criteria.

Table 1. ACR 1987 diagnostic criteria for Rheumatoid Arthritis.

1. Morning stiffness	Morning stiffness in and around the joint lasting at least one hour before maximal improvement, persisting > 6 weeks.
2. Arthritis of 3 or more joint areas	At least three joints' areas simultaneously have had soft tissue swelling for fluid observed by a physician, persisting > 6 weeks.
3. Arthritis of hand joints	At least one swollen joint in a wrist, MCP joint or IPP joint persisting > 6 weeks.
4. Symmetrical arthritis	Simultaneous involvement of the same joint area on both sides of the body persisting > 6 weeks.
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces or in juxta-articular regions, observed by a physician.
6. Serum rheumatoid factor	Positive serum RF
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions and bony decalcification

II. Spondylarthritis (ASDAS) and rheumatoid arthritis (DAS28) activity score with assessment of disease activity according to this score.

The ASDAS score is a composite score calculated with the following formula:

ASDAS-CRP = 0.12 x spinal pain + 0.06 x duration spinal stiffness + 0.11 x overall patient assessment + 0.07 x peripheral pain/swelling + 0.58 x Ln (CRP+1).

Depending on the result obtained, one could speak of:

- ASDAS-CRP < 1.3: inactive disease
- 1.3 < ASDAS-CRP < 2.1: disease of moderate activity
- 2.1 < ASDAS-CRP < 3.5 : active disease
- ASDAS-CRP > 3.5 : very active disease

The DAS28 score is also a composite score considering the articular index (painful joints) and the synovitis index (swollen joints) of the 28 joints of the EULAR, the sedimentation rate, the evaluation of the overall condition of the patient on a visual analogue scale. It is calculated according to the following formula:

DAS28 = 0.55 x √ (tender joint count 28) + 0.284 x √ (swollen joint count 28) + 0.37 log (ESR) + 0.0142 x (General health).

Depending on the result obtained, the disease was defined as:

- DAS28 < 2.6 : remission
- 2.6 < DAS28 < 3.2 : low activity
- 3.2 < DAS28 < 5.1 : moderate activity
- DAS28 > 5.1 : high activity

III. Classification of patients into two groups according to ASDAS response and DAS28 response (EULAR criteria).

ASDAS at each visit	Improvement of ASDAS compared to W0		
	< 1.1	≥ 1.1 and < 2	≥ 2
< 1.3		Good responder	Very good responder
≥ 1.3 et < 2.1		Moderate responder	Good responder
≥ 2.1 et < 3.5	No responder	Moderate responder	Moderate responder
≥ 3.5	No responder	No responder	No responder

Table 2. Classification of patients according to the change in ASDAS score at each visit compared to W0.

DAS28 at each visit	Improvement of DAS28 compared to W0		
	> 1.2	> 0.6 and < 1.2	≤ 0.6
≤ 3.2	Good responder		
> 3.2 and ≤ 5.1		Moderate responder	
> 5.1			Non-responder

Table 3. Classification of patients according to the change in DAS28 at each visit compared to W0 by EULAR criteria.

IV. Outliers at different analysis times

COMARIS

Analysis of the metabolome at W0 according to the therapeutic response at W12:

- PCA: a woman, responder, treated with adalimumab + methotrexate; a woman, non-responder, treated with adalimumab alone.
- PLS-DA: both PCA patients + one man, responder, treated with adalimumab alone.

Analysis of the metabolome at W0 according to the therapeutic response to W26:

- PCA: one man, responder, treated with adalimumab alone; three women including 2 responders, treated with adalimumab + methotrexate and one, non-responder treated with adalimumab alone.
- PLS-DA: the man responder of the PCA; a female responder, treated with adalimumab + methotrexate; a non-responding woman, treated with adalimumab alone (patients found in PCA).

Analysis of the variation of the metabolome between W0 and W4 according to the therapeutic response to W12:

- PCA : none.
- PLS-DA: a woman, non-responders, treated with adalimumab alone; a female responder, treated with adalimumab + methotrexate.

Analysis of the variation of the metabolome between W0 and W4 according to the therapeutic response to W26:

- PCA: a woman, non-responder, treated with adalimumab alone.
- PLS-DA: a woman, responder, treated with adalimumab + methotrexate; a man responder, treated by adalimumab alone.

AFORA

Analysis of the W0 metabolome according to the therapeutic response to W12:

- PCA: 4 women, 3 responders treated with either adalimumab alone, adalimumab + methotrexate or adalimumab + corticosteroids and one non-responder treated with adalimumab + methotrexate + corticosteroids.
- PLS-DA: 3 women, 2 responders treated with adalimumab alone and one non-responder treated with adalimumab + methotrexate + corticosteroids.

Analysis of the W0 metabolome according to the therapeutic response to W26:

- PCA: 4 women, responders, treated with adalimumab alone, adalimumab + methotrexate, adalimumab + corticosteroids and adalimumab + methotrexate + corticosteroids.
- PLS-DA: two women including one responder and one non-responder, treated with adalimumab + corticosteroids; one man, responder, treated with adalimumab + methotrexate + corticosteroids.

Analysis of the variation of the metabolome between W0 and W4 according to the therapeutic response to W12:

- PCA: 2 women, responders, treated with adalimumab + methotrexate and adalimumab alone respectively.
- PLS-DA: 4 women, responders including 3 treated with adalimumab + methotrexate and the last with adalimumab alone.

Analysis of the variation of the metabolome between W0 and W4 according to the therapeutic response to W26:

- PCA: a woman, responder, treated with adalimumab + methotrexate
- PLS-DA: 2 women, responders including one treated with adalimumab + methotrexate (same as PCA) and the other treated with adalimumab alone.

V. Figures

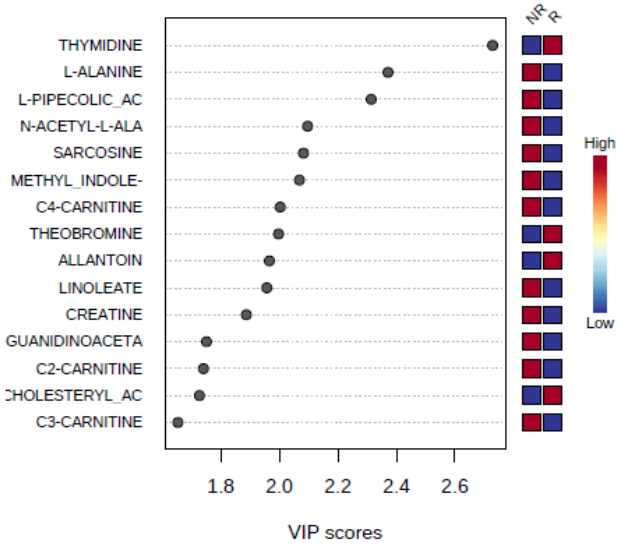

Figure 1. COMARIS. VIP-scores in PLS-DA analysis of the metabolic profile at W0 according to the therapeutic response at 12 weeks. R = responders; NR = non-responders.

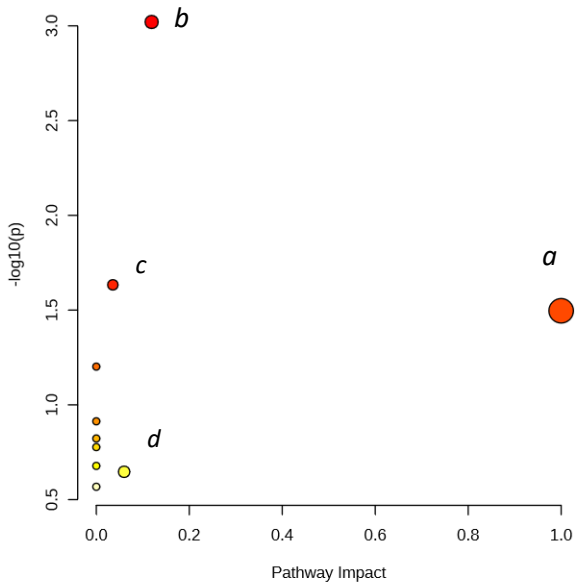

Figure 2. COMARIS. Impact of W0 metabolic pathways between responder and non-responder patients at 12 weeks. a: linoleic acid metabolism; b: glycine, serine, and threonine metabolism; c: arginine and proline metabolism; d: pyrimidine metabolism.

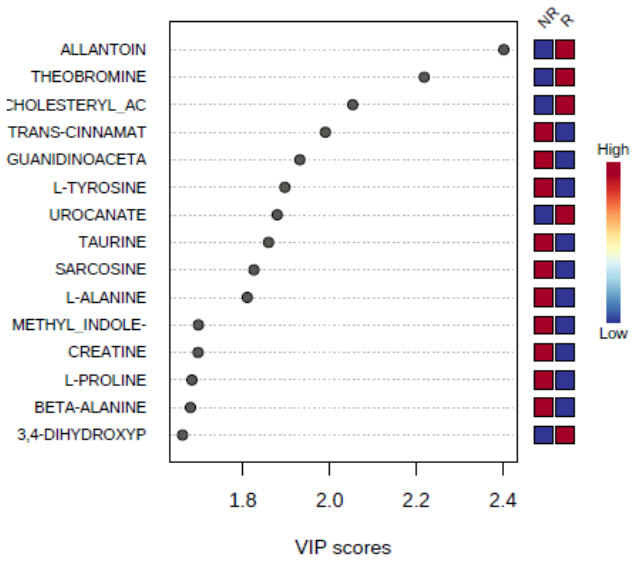

Figure 3. COMARIS. VIP-scores in PLS-DA analysis of the metabolic profile at W0 according to the therapeutic response at 26 weeks. R = responders; NR = non-responders.

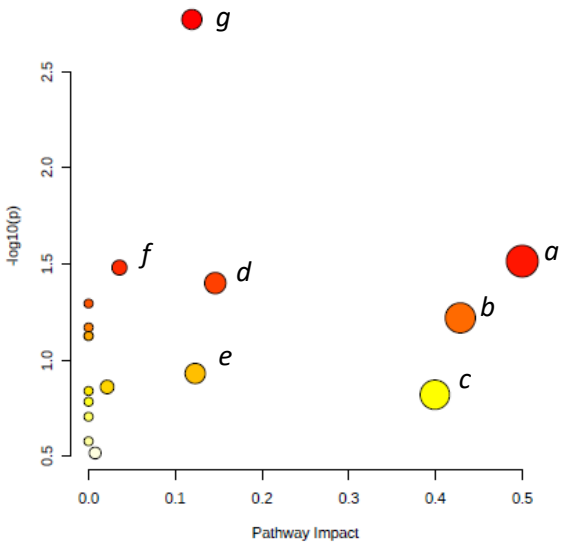

Figure 4. COMARIS. Impact of metabolic pathways involved in W0 between responder and non-responder patients at 26 weeks. a: phenylalanine, tyrosine and tryptophan biosynthesis; b: taurine and hypotaurine metabolism; c: beta-alanine metabolism; d : tyrosine metabolism ; e : histidine metabolism ; f : arginine and proline metabolism ; g : glycine, serine and threonine metabolism.

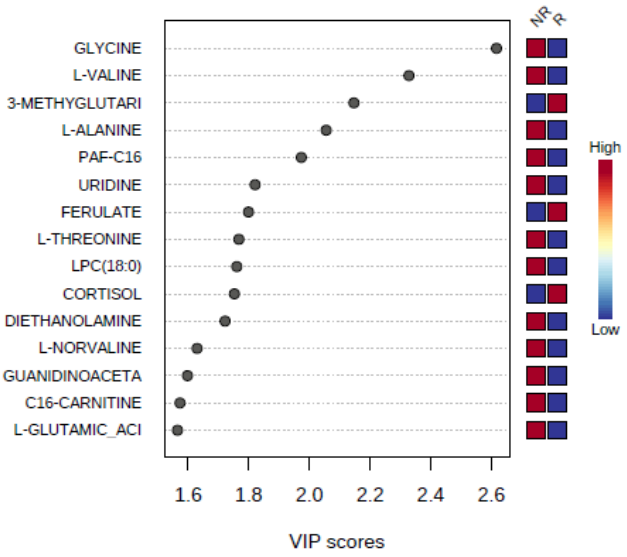

Figure 5. COMARIS. VIP-scores in PLS-DA analysis of the change in metabolome between W0 and W4 according to therapeutic response at 26 weeks. R = responders; NR = non-responders.

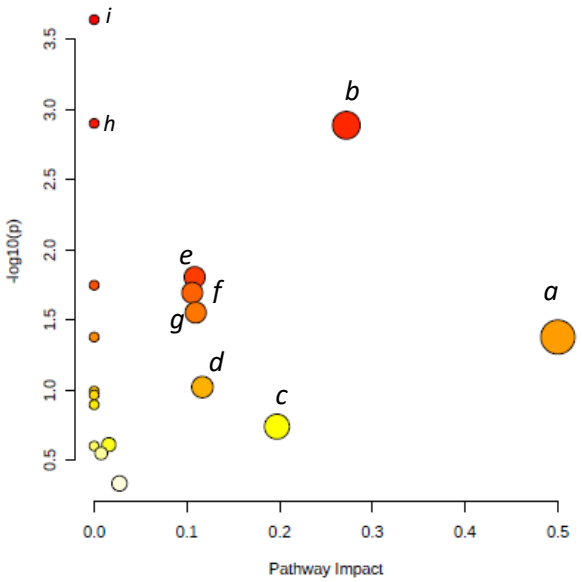

Figure 6. COMARIS. Impact of metabolic pathways involved in metabolome modification between W0 and W4 according to therapeutic response at 26 weeks. a: D-glutamine and D-glutamate metabolism; b: glycine, serine and threonine metabolism; c : alanine, aspartate and glutamate metabolism ; d : arginine biosynthesis ; e : glutathione metabolism ; f : glyoxylate and dicarboxylate metabolism ; g : arginine and proline metabolism ; h : valine, leucine and isoleucine biosynthesis ; i : aminoacyl-t-RNA biosynthesis.

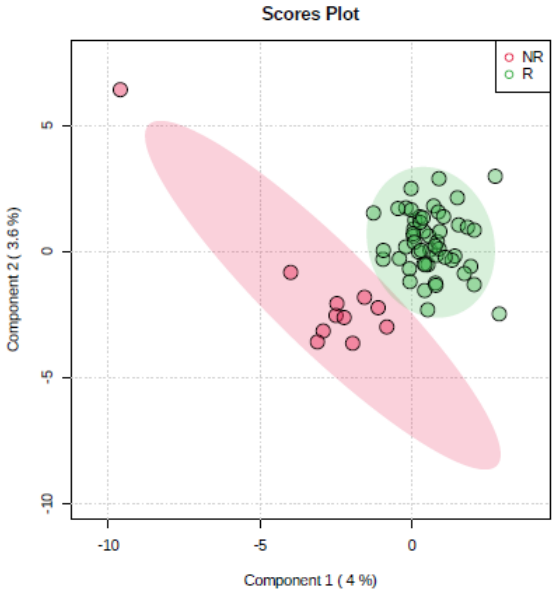

Figure 7. AFORA. Distribution of the W0 metabolome of different patient groups to PLS-DA according to response at 12 weeks. R = responders; NR = non-responders.

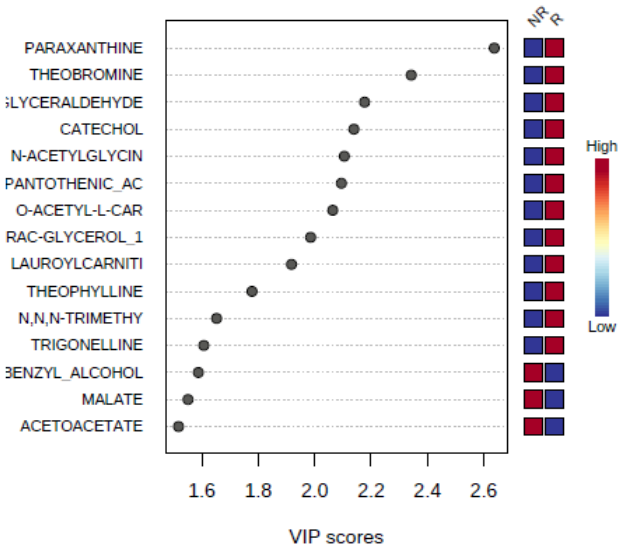

Figure 8. AFORA. VIP-score in PLS-DA analysis of the W0 metabolome in different patient groups according to therapeutic response at 12 weeks. R = responders; NR = non-responders.

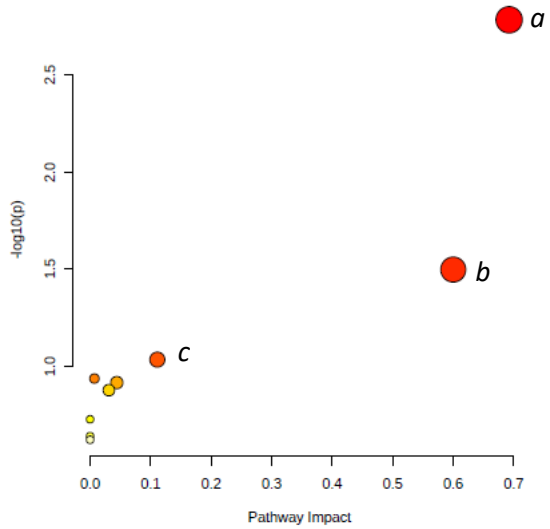

Figure 9. AFORA. Impact of metabolic pathways involved at W0 between responder and non-responder patients at 12 weeks. a: caffeine metabolism; b: synthesis and degradation of ketone bodies; c: butanoate metabolism.

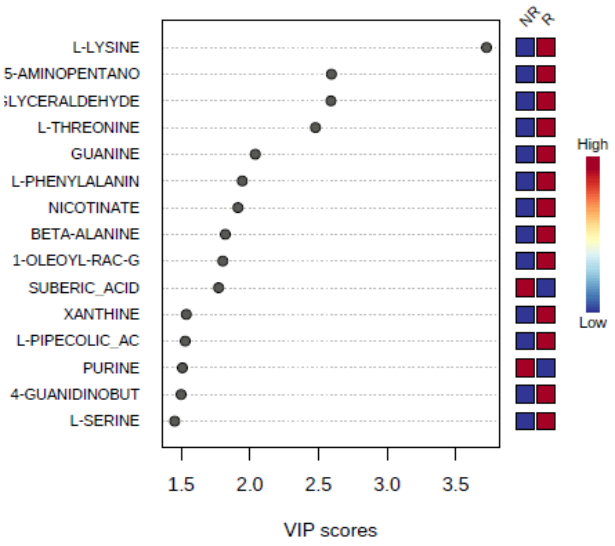

Figure 10. VIP-score in PLS-DA analysis of the W0 metabolome in different patient groups according to therapeutic response at 26 weeks. R = responders; NR = non-responders.

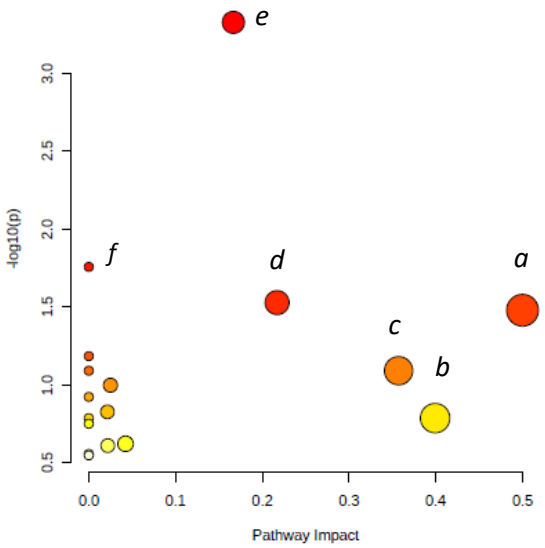

Figure 11. AFORA. Impact of metabolic pathways involved at W0 between responder and non-responder patients according to therapeutic response at 26 weeks. a: phenylalanine, tyrosine, and tryptophan metabolism; b: beta-alanine metabolism; c: phenylalanine metabolism; d: glycine, serine, and threonine metabolism; e: aminoacyl-tRNA biosynthesis; f: lysine degradation.

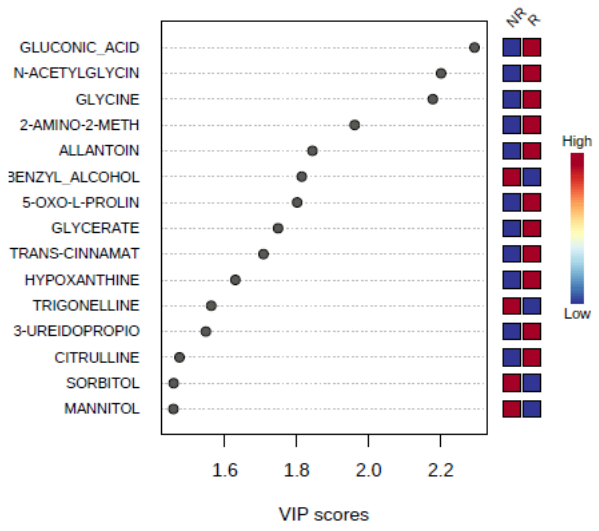

Figure 12. AFORA. VIP-score in PLS-DA analysis of the change in the metabolome between W0 and W4 in different patient groups according to the therapeutic response at 26 weeks. R = responders; NR = non-responders.

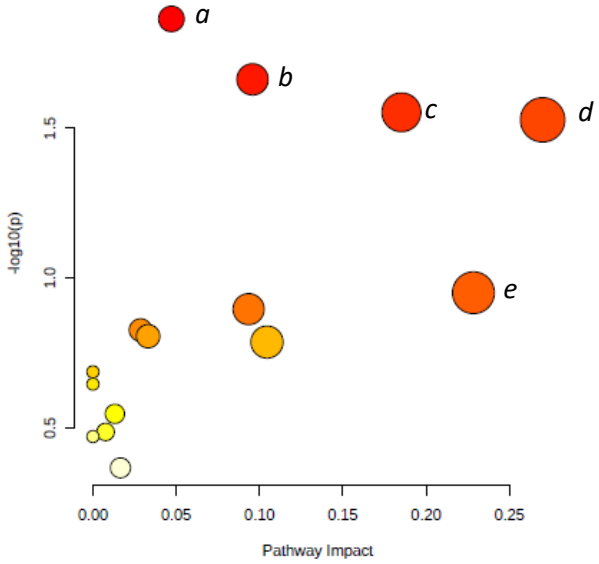

Figure 13. AFORA. Impact of metabolic pathways involved in metabolome modification between W0 and W4 according to therapeutic response at 26 weeks. a: pentose phosphate pathway; b: glutathione metabolism; c: glyoxylate and dicarboxylate metabolism; d: glycine, serine, and threonine metabolism; e: arginine biosynthesis.

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52 pages – 1 tableau – 10 figures – 5 annexes.

RESUME

Objectifs : Analyser les changements métabolomiques 4 semaines après l'initiation de l'adalimumab, un anti-TNF chez les patients atteints de spondylarthrite axiale (SpA) et de polyarthrite rhumatoïde (PR) et étudier la relation avec la réponse clinique à 12 et 26 semaines.

Méthodes : Nous avons effectué des analyses en post-hoc du sérum de patients atteints de SpA de l'étude COMARIS (NCT01895764) et de patients atteints de PR de l'étude AFORA (NCT01382160) par chromatographie liquide couplée à la spectrométrie de masse à haute résolution (LC/HRMS) à l'inclusion (S0) et 4 semaines (S4) après l'initiation du traitement par adalimumab. Les réponses cliniques ont été évaluées à 12 et 26 semaines. Les variations des concentrations de métabolites entre S0 et S4 ont été comparées entre les répondeurs et les non-répondeurs en analyse univariée, multivariée non supervisée en composantes principales (PCA) puis multivariée supervisée par des analyses discriminantes partielles des moindres carrés (PLS-DA), à l'aide du logiciel METABOANALYST. Les variables d'intérêt, définies comme celles ayant un score VIP (Variable Influence on Projection) supérieur ou égal à 2 ont été sélectionnées. Enfin, nous avons étudié les différentes voies métaboliques dans lesquelles ces variables d'intérêt étaient impliquées.

Résultats : Soixante-quatorze patients ont été inclus dans l'étude COMARIS, dont 43 répondeurs et 31 non-répondeurs. Soixante-trois patients ont été inclus dans l'étude AFORA, dont 52 répondeurs et 11 non-répondeurs. En analyse univariée, la leucine, l'hypoxanthine et la n-acétyl-l-alanine étaient abaissées chez les répondeurs atteints de SpA. La carnosine, le cortisol et la purine étaient abaissés chez les répondeurs atteints de PR, tandis que la l-méthionine et la 5-oxo-l-proline étaient augmentées chez ces sujets. Nous n'avons pas été en mesure de différencier les patients de chaque étude en fonction de leur réponse en utilisant la PCA et la PLS-DA. Cependant, chez les patients atteints de SpA, cinq métabolites avec un score VIP >2 de l'analyse PLS-DA, y compris le PAF-C16, un facteur d'activation plaquettaire, et le LysoPC (18:0), un composant des membranes cellulaires, semblaient pertinents. Diverses voies métaboliques ont été identifiées dans les deux études : métabolisme des glycérophospholipides ; métabolisme de l'arginine et de la proline; métabolisme de l'alanine, de l'aspartate et du glutamate; biosynthèse de la phénylalanine, de la tyrosine et du tryptophane; métabolisme de la cystéine et de la méthionine; biosynthèse du pantothénate et du CoA et biosynthèse de l'aminoacyl-ARNt.

Conclusion : Chez les patients atteints de PR et de SpA, l'adalimumab induit des changements précoces dans le métabolome impliquant des voies telles que le métabolisme des acides aminés essentiels et non essentiels et le stress oxydatif. Ce résultat pourrait guider les investigations futures afin de trouver des marqueurs prédictifs de la réponse thérapeutique aux anti-TNF dans les rhumatismes inflammatoires chroniques.

Mots-clés : métabolomique ; spondylarthrite axiale ; polyarthrite rhumatoïde ; adalimumab ; prédiction de la réponse ; LC/HRMS ; acides aminés ; stress oxydatif.

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