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TRANSLATIONAL SCIENCE

Counteracting tryptophan metabolism alterations as a new therapeutic strategy for rheumatoid arthritis

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ABSTRACT

Objectives Alterations in tryptophan (Trp) metabolism have been reported in inflammatory diseases, including rheumatoid arthritis (RA). However, understanding whether these alterations participate in RA development and can be considered putative therapeutic targets remains undetermined.

In this study, we combined quantitative Trp metabolomics in the serum from patients with RA and corrective administration of a recombinant enzyme in experimental arthritis to address this question.

Methods Targeted quantitative Trp metabolomics was performed on the serum from 574 previously untreated patients with RA from the ESPOIR (Etude et Suivi des POlyarthrites Indifférenciées Récentes) cohort and 98 healthy subjects. A validation cohort involved 69 established patients with RA. Dosages were also done on the serum of collagen-induced arthritis (CIA) and collagen antibody-induced arthritis (CAIA) mice and controls. A proof-of-concept study evaluating the therapeutic potency of targeting the kynurenine pathway was performed in the CAIA model.

Results Differential analysis revealed dramatic changes in Trp metabolite levels in patients with RA compared with healthy controls. Decreased levels of kynurenic (KYNA) and xanthurenic (XANA) acids and indole derivatives, as well as an increased level of quinolinic acid (QUIN), were found in the serum of patients with RA. They correlated positively with disease severity (assessed by both circulating biomarkers and disease activity scores) and negatively with quality-of-life scores. Similar profiles of kynurenine pathway metabolites were observed in the CAIA and CIA models. From a mechanistic perspective, we demonstrated that QUIN favours human fibroblast-like synoviocyte proliferation and affected their cellular metabolism, through inducing both mitochondrial respiration and glycolysis. Finally, systemic administration of the recombinant enzyme aminoadipate aminotransferase, responsible for the generation of XANA and KYNA, was protective in the CAIA model.

Conclusions Altogether, our preclinical and clinical data indicate that alterations in the Trp metabolism play an active role in the pathogenesis of RA and could be considered as a new therapeutic avenue.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Alterations in tryptophan (Trp) metabolism have been described in several chronic inflammatory diseases including rheumatoid arthritis (RA).

⇒ Kynurenine (Kyn) pathway has been closely linked with the pathogenesis of autoimmune diseases.

⇒ High serum Kyn levels or high Kyn/Trp ratio has been found to correlate with disease activity in patients with RA.

WHAT THIS STUDY ADDS

⇒ The abundance of xanthurenic acid (XANA) and kynurenic acid (KYNA), two metabolites of the kynurenine pathway, is negatively correlated with inflammation in mice and human RA.

⇒ Quinolinic acid (QUIN) abundance is positively correlated with inflammation in mice and human RA.

⇒ Abundances of kynurenine pathway metabolites strongly correlate with disease activity and quality of life in patients with RA.

⇒ QUIN alters human fibroblast-like synoviocyte proliferation and metabolism.

⇒ The use of the recombinant aminoadipate aminotransferase (AADAT), to restore KYNA and XANA production, is protective in arthritis model.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Manipulating endogenous tryptophan metabolism with AADAT is an attractive new therapeutic strategy in RA.

INTRODUCTION

Besides the breakdown of tolerance, rheumatoid arthritis (RA) could be considered the consequence of immunometabolism disturbances, as metabolic alterations, in particular in T-cells, drive the observed exaggerated immune response.[1 2]

Recently, alterations in tryptophan (Trp) metabolism have been described in several chronic inflammatory diseases,[3 4] including RA.[5] Trp can be metabolised through three major pathways:

Table 1 ESPOIR population studied

	RA (ESPOIR cohort)	Healthy subjects
N	574	98
Age (years)	48.9±12.1	41.8±14.0
% female	45 (77.5%)	35 (35.7%)
DAS28	5.4±1.2	
CRP (mg/L)	21.7±33.5	
ESR (mm at first h)	31.0±25.1	
IgM-RF+	338 (58.9%)	
value (IU/mL)	163±647	
Anti-CCP+	301 (52.4%)	
value (IU/mL)	663.2±1672.3	
IL-1Ra (pg/mL)	1287.4±1013.0	
IL-6 (pg/mL)	13.0±39.8	
IL-10 (pg/mL)	0.9±12.7	
MCP1 (pg/mL)	214.4±146.7	
IL-4 (pg/mL)	0.6±7.8	
IL-17 (pg/mL)	0.5±4.2	
MCP1 (pg/mL)	214.4±146.7	
IFNgamma (pg/mL)	0.2±0.7	
TNF(pg/mL)	2.6±5.8	
IL-1beta (pg/mL)	0.3±2.6	
IL-2 (pg/mL)	1.0±5.2	

Results are presented as mean±SD.
anti-CCP, anti-cyclic citrullinated peptide antibodies; CRP, C-reactive protein; DAS28, Disease Activity Score on 28 joints; ESPOIR, Etude et Suivi des POlyarthrites Indifférenciées Récentes); ESR, erythrocyte sedimentation rate; IL, interleukin; RA, rheumatoid arthritis.

the serotonin, the kynurenine (Kyn) and the indole pathways. Whereas serotonin and kynurenine pathways occur in mammalian cells, the indole pathway is gut microbiota dependent.[6]

Interest in Trp metabolism in RA pathophysiology is not new, as in 1960, Mary McMillan described increased levels of 3-hydroxyanthranilic acid (3-HAA), a metabolite of the Kyn pathway, in the urine of patients with RA.[7] This was further confirmed in 1962 by a seminal work of Isobel M. Bett in an article entitled, 'Metabolism of Tryptophan in Rheumatoid Arthritis', published in *Annals of the Rheumatic Diseases*.[8] Interestingly, Bett demonstrated that patients with RA differently metabolised a loading dose of L-Trp (3 g) compared with control subjects, suggesting an intrinsic metabolic disorder rather than a malabsorption or deficiency issue.

Both the Kyn and the indole pathways of Trp metabolism have been proposed as contributive to the pathogenesis of RA.[8 9] An imbalance between increased circulating Kyn and decreased Trp concentrations is described in patients with RA, illustrating the activation of the Kyn pathway.[9–13] As for indole derivatives, evidence in patients remains scarce.[11] However, a role for microbiota-derived Trp metabolites is supported by an abundant literature indicating that many of these molecules are agonists of the aryl hydrocarbon receptor (AhR), which is considered a therapeutic target in RA.[14]

While previous studies have focused on limited aspects of Trp metabolism in small samples of patients with RA, we here applied targeted quantitative metabolomics to provide a global and accurate overview of Trp metabolism alterations in a large cohort of early untreated patients with RA and relevant RA experimental models. We provide the first evidence of a pathophysiological role of altered Trp metabolism in RA, and demonstrate the therapeutic potential of its preclinical manipulation.

METHODS
Patients
Discovery cohort: ESPOIR cohort
We used the baseline data from patients with early RA included in the ESPOIR (Etude et Suivi des POlyarthrites Indifférenciées Récentes) cohort, details of which have been previously described.[15] Briefly, these patients have had at least two swollen joints persisting for more than 6 weeks but less than 6 months. Diagnosis of RA was defined as fulfilling the American College of Rheumatology–European Alliance of Associations for Rheumatology (ACR-EULAR) 2010 criteria for RA at inclusion.[16] Otherwise and without another defined diagnosis at inclusion, patients were considered to have undifferentiated arthritis and were not included in the current study. The Montpellier (France) Ethics Committee approved the study in July 2002, and all patients provided informed consent. Patients had no history of long-term glucocorticoid or disease-modifying antirheumatic drugs (DMARDs) use. However, glucocorticoid therapy in a mean dosage ≤20 mg/day given for ≤2 weeks and discontinued at least 2 weeks before inclusion was allowed.

We used the following baseline data:
- Disease Activity Score on 28 joints (DAS28): Disease activity on 0–100 mm visual analogue scale (VAS) evaluated by the patient or by the physician, tender and swollen joint count.
- Routine inflammatory biomarkers: C reactive protein (CRP) level, erythrocyte sedimentation rate (ESR).
- Cytokine serum level (IL-1beta, IL-1Ra, IL-2, IL-6, IL-10, IL-17, IFNgamma, MCP1, TNF) previously assessed.[17 18]
- Autoantibodies: Rheumatoid factor-IgM (RF) (Menarini France, Rungis Cedex, France; positive >9 IU/mL) and anti-cyclic citrullinated peptide antibodies (anti-CCP) levels (anti-CCP2; Dia-Sorin, Saluggia (Vercelli), Italy; positive >50 U/mL) with ELISA.
- Patient-reported outcomes (PROs): EuroQol-5 Dimension (EQ-D5), a standardised measure of health-related quality of life developed by the EuroQol Group, Mental Health Inventory-5 (MHi5), Short Form 36 (SF36) Physical Functioning and mental health subscores, fatigue on a 0–100 mm VAS.[16 17]

We compared serum metabolite levels of patients with RA from ESPOIR with those of 98 healthy subjects (HS) from the blood bank (Etablissement Français du Sang).

Replication cohort: TrypAFORA
TrypAFORA is a *post hoc* study of AFORA (Serum Concentration of Adalimumab as a Predictive Factor of clinical Outcomes in Rheumatoid Arthritis) that was conducted within the HUGO (Hôpitaux Universitaires du Grand Ouest – Western France University Hospitals) network and whose main objective was to estimate the pharmacodynamic parameters of the concentration–effect relationship of adalimumab in RA (ClinicalTrials.gov NCT01382160). A total of 69 patients (28% male; mean age ~54 years) were included between January 2011 and January 2013 with a 26-week follow-up.

Blood samples were taken at baseline, W4, W12 and W26. Serum was aliquoted, labelled and stored in each investigating centre at −20°C before being sent centrally on dry ice to the Tours biobank where all samples were stored at −80°C until use. CRP and calprotectin measurements were performed at the University hospital of Tours. A total of 320 samples from 63 patients were analysed.

Targeted quantitative metabolomics

In a serum sample collected at the baseline visit for ESPOIR cohort and at four time points for the TrypAFORA cohort, 19 Trp metabolites were assessed. The same approach was performed in the serum of collagen-induced arthritic (CIA) mice collected at arthritis peak (day 35).

The method allowing a quantitative detection of 3-hydroxyanthranilic acid (3HAA); 3-hydroxykynurenine (3HKyn); kynurenic acid (KYNA); kynurenine (Kyn); picolinic acid (Pico); quinolinic acid (QUINA); xanthurenic acid (XANA); 5-hydroxyindole acetic acid (5HIAA); 5-hydroxytryptophan (5HTP); melatonin; N-acetylserotonin (N-AS); serotonin (5-hydroxytryptamine) (5-HT); indole-3-aldehyde (I-3aL); indole-3-acetamide (IAM); indole-3-acetic acid (I-3AA); indole-3-lactic acid (ILA); indoxyl sulfate; tryptamine (TA); tryptophan (Trp) has been described previously.[19]

Trp metabolites were analysed using three different strategies:

▶ Separate analysis at metabolite level.
▶ Sum of each three main Trp metabolite pathways (serotonin, Kyn/IDO and indole/AhR).
▶ Ratio of metabolites on their precursor to have an estimation of enzymatic activity.

Aminoadipate aminotransferase (AADAT) quantification in serum

AADAT was quantified by ELISA following manufacturer's instruction (XpressBio, XPEH1430).

AADAT production

Cloning of the gene encoding for murine AADAT in *Escherichia coli*, expression and purification of the recombinant protein, as well as tests for confirmation of its biological activity have been described previously.[20]

CIA and CAIA

Animal experiments were performed according to the local ethics committee and the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation (MESRI, France) recommendations under agreement APAFIS#19 783-201902181444241. CIA was performed in DBA1/J male mice as previously described.[21] CAIA was induced in C57BL6J male mice by intraperitoneal injection of a cocktail of arthritogenic (CIA-MAB-2C, MD Bioproducts GmbH, Zurich, Switzerland) anti-type II collagen antibodies on day 0. By day 3, mice received a booster injection of lipopolysaccharide from bacterial cells walls of *E. coli* 055:B5. Mice were administered daily with an intraperitoneal injection of recombinant AADAT (5 µL in 100 µL of phosphate buffer saline, PBS) throughout study duration. These experiments have been reproduced twice with 8 mice per group (AADAT treated, or PBS vehicle treated). Clinical assessment (clinical score and hindpaw oedema) and histological score were graded as previously described.[21]

Human fibroblast-like synoviocytes (hFLS) obtention

Specimens of synovial tissues were obtained from patients with osteoarthritis undergoing total knee replacement surgery. The human study described here was conducted in conformity with the Declaration of Helsinki principles and was approved by the local Research Institution. Written informed consent has been obtained from all participants. The synovial tissues were isolated from the underlying connective tissues, finely minced and washed in PBS. hFLS were obtained after an enzymatic digestion followed by at least three subcultures to prevent contamination by macrophage-like cells.

Statistical analysis

Statistical analysis of human data was performed in the R statistical environment (R V.3.6.2). Plotting was performed with ggplot2 (V.3.3.2), Prism V.9 (Graphpad Software, San Diego, California, USA) and Morpheus (https://software.broadinstitute.org/morpheus/). In all statistical analyses, differences with $p<0.05$ were considered significant. The p values were corrected using the Benjamini and Hochberg (BH) procedure to control for the false discovery rate. The murine data were analysed using Prism V.9 (Graphpad Software). Values are expressed as mean±SEM.

RESULTS

Differential analysis revealed drastic changes in serum Trp metabolites in RA

Using Trp metabolites targeted metabolomics, we compared the biological data of 574 patients with early naïve of treatment RA from the ESPOIR cohort with those of 98 HS (table 1). Differential analysis revealed drastic changes (figure 1A and online supplemental figure 1) between RA and HS involving the three pathways of Trp metabolism. While an increase in the sum of serotonin pathway metabolites was observed in RA, a decrease of the kynurenine (Kyn) pathway metabolites and of the indole derivatives was observed (online supplemental figure 2). Within the Kyn pathway, we observed an elevated level of QUIN (as well as the ratio of QUIN to its precursor 3HAA (figure 1) and a decreased level of KYNA (figure 1C) and of the ratio between XANA and its precursor 3-hydroxykynurenine in patients with RA (figure 1D).

Since alterations in Kyn pathway were reminiscent of those observed in inflammatory bowel disease (IBD), we then focused on these metabolites.

KYNA and XANA abundance correlates negatively with disease activity and proinflammatory biomarkers in RA

We then performed correlation analyses with Disease Activity Score (DAS 28), clinical features, inflammatory markers (CRP, ESR and cytokines) and autoantibodies levels (figure 2).

We observed a negative correlation between several disease activity parameters (especially DAS28, morning stiffness, swollen joint count, CRP and ESR) with KYNA and XANA levels (as well as the ratio of KYNA and XANA to their precursors, Kyn and 3-hydroxykynurenine, respectively). The opposite was observed with Kyn and QUIN (figure 2). Among the biological parameters analysed, and in agreement with what we found with the clinical features, a remarkable correlation was observed, either negative with KYNA and XANA (as well as the ratio of KYNA and XANA to their precursors, kynurenine and 3-hydroxykynurenine, respectively) or positive with Kyn and QUIN, with serum levels of IL-6, TNF, IL-1Ra and CRP and with ESR. Finally, we observed an overall negative correlation between indole-derivative levels and disease activity (including DAS28) or inflammatory markers (CRP, ESR and IL-6). To validate our findings, we performed the same analysis in another independent longitudinal cohort of patients with RA receiving adalimumab (TryAFORA cohort). We observed the same correlations between clinical features and markers of disease severity (DAS28, CRP and calprotectin levels) and Trp metabolites alterations, namely, a decrease of KYNA, XANA, indole derivatives and an increase of QUIN and serotonin levels (online supplemental figure 3).
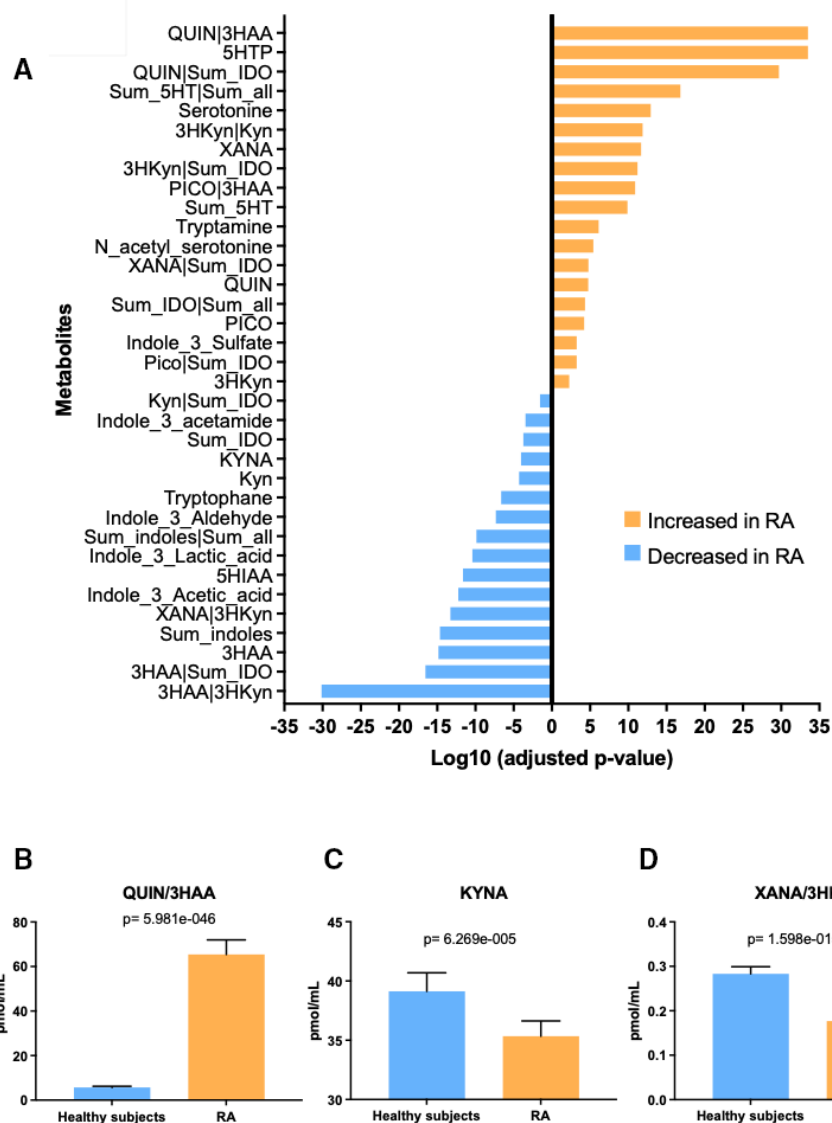
Figure 1 Targeted tryptophan metabolomics analysis: Differential abundance between patients with RA (ESPOIR cohort) and healthy controls. Targeted tryptophan metabolomics analysis was conducted on the serum of 574 patients with early RA from the ESPOIR cohort and on 98 healthy subjects. (A) Differential analysis between RA and HS. (B) Ratio of QUIN over 3HAA in RA and HS serum. (C) Level of KYNA (pmol/ml) in RA and HS serum. (D) Ratio of XANA over 3H-Kyn. 3HAA, 3-hydroxyanthranilic acid; 3H-Kyn, 3-hydroxykynurenine; HS, healthy subject; KYNA, kynurenic acid; QUIN, quinolinic acid; RA, rheumatoid arthritis; XANA, xanthurenic acid.

KYNA and XANA abundance correlates negatively with RA patient-PROs

To investigate whether our findings can be extended to quality of life in RA, we analysed correlations between Trp metabolites and several QoL scores. We observed a positive correlation between QoL scores (especially EQ-D5, MHi5 and SF36) and KYNA and XANA levels (as well as the ratio of KYNA and XANA to their precursors). The opposite, a negative correlation, was noted for Kyn and QUIN levels (figure 3). A positive correlation with QoL was also observed for levels of indole derivatives and serotonin.

Taken together, our results show that the decreased serum levels of XANA and KYNA are associated with a greater disease activity, a higher systemic inflammation and QoL alterations in patients with early RA.

Similar alterations of TRP metabolism in two experimental models of arthritis in mice

To further investigate whether our findings can be extrapolated from a murine model of experimental arthritis, we performed the same targeted quantitative metabolomics in the serum of CIA (figure 4A) and CAIA mice (online supplemental figure 4a). Interestingly, most of the features of human RA-related metabolic disturbances were found in CIA mice, including a reduced serum levels of indole derivatives (figure 4B), and decreased levels of KYNA and XANA (figure 4C and D) and an increased level of QUIN over the sum of IDO metabolites (figure 4C–E), resulting in a decreased ratio of KYNA+XANA over QUIN+Kyn (figure 4F). As expected in CAIA mice, IDO pathway was activated (online supplemental figure 4b) and similar changes in KYNA and QUIN levels were also found (online supplemental figure 4c–g). Furthermore, we found a negative correlation between KYNA, XANA or indole metabolite levels and clinical
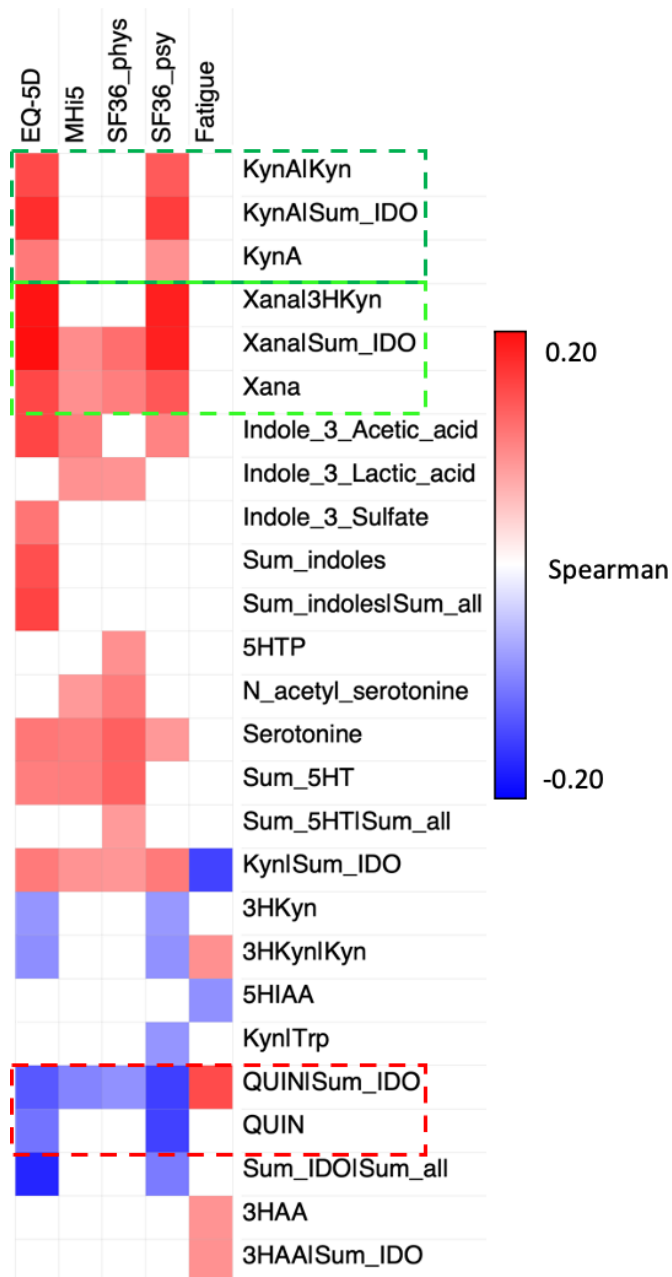
Figure 2 Correlation between RA activity and Trp metabolites in serum. Pairwise correlation using serum samples from the ESPOIR cohort. Statistical significance was determined for all pairwise comparisons using Spearman test; only significant correlations (p value <0.05 after false discovery rate correction, q<0.2) are displayed. Cytokine were measured in serum. DAS28, Disease Activity Score-28 for Rheumatoid Arthritis; RA, rheumatoid arthritis; Trp, tryptophan.

score and circulating IL-6 levels and a positive correlation for kynurenine metabolites (figure 4G). Kinetics of metabolites levels in the course of CAIA showed that maximum QUIN level was reached on day 4 at the same time as the minimum KYNA level (online supplemental figure 4g).

Taken together, these results indicate that Trp metabolism is altered in CAIA and CIA models and that these metabolic changes reflect disease severity, in good accordance with data found in human RA.

Administration of AADAT protects from CAIA in mice

Interestingly, we previously found similar alterations of the Kyn pathway in patients with IBD and we demonstrated that

Figure 3 Correlation between quality of life and Trp metabolites in serum. Pairwise correlation using serum samples from the ESPOIR cohort. Statistical significance was determined for all pairwise comparisons using Spearman test; only significant correlations (p value <0.05 after false discovery rate correction, q<0.2) EQ5D, standardised measure of health-related quality of life developed by the EuroQol Group; MHI5, Mental Health Inventory 5; SF36_phys, physical component of the SF36 score (The Short Form (36) Health Survey); SF36_psy, psychological component of the SF36 score.

rewiring of Trp metabolism towards the production of the anti-inflammatory metabolites XANA and KYNA, while decreasing the production of the proinflammatory metabolite QUIN, was effective in murine colitis models.[20] We thus proposed to use the same therapeutic strategy, which is based on the administration of a recombinant form of Kyn aminotransferase 2, also called AADAT, the common enzyme catalysing the transformation of Kyn and 3OH-kynurenine into KYNA and XANA, respectively (figure 5A). The therapeutic relevance of this approach

is supported by the dramatic decrease in AADAT abundance in the serum of patients with RA compared with HS (figure 5B). In CAIA mice (figure 6A), a validated RA mouse model, the daily administration of AADAT had a strong protective effect, as demonstrated by reduced clinical score and hindpaw oedema (figure 6A). Histological examination confirmed a significant decrease of synovitis (figure 6B and online supplemental figure 6a) and less cartilage damages in AADAT-treated animals. In addition, the systemic administration of AADAT in CAIA mice increased the production of circulating levels of KYNA (figure 6C). The relative expression of IDO and AADAT into the joints were altered during CAIA. IDO maximum expression was reached on day 4 at arthritis onset, whereas AADAT expression decreased but recovering at 10 day during the resolution phase (online supplemental figure 5b). Interestingly, after AADAT treatment, IDO remained unchanged while AADAT expression was significantly reduced, suggesting a feedback loop with metabolites changes (online supplemental figure 6b). In line with the observed anti-inflammatory effects, AADAT decreased IL-1β expression in joint (online supplemental figure 6b).

To investigate the potential involvement of AhR, in AADAT effect, the expression of two AhR target genes, namely, *CYP1A1* and *AhRR*, was analysed in joint and liver of wild-type mice (PBS or CAIA) treated or not with AADAT.

For both genes, a decreased expression was observed in joint and liver during arthritis. Of interest, AADAT treatment restored their expression level in liver with a similar trend in joint. These results suggest that AhR might be involved in the protective effect of AADAT in CAIA, although it is not firmly demonstrated (online supplemental figure 6c).

In summary, these preclinical results show that recombinant AADAT administration has a therapeutic potential in RA, through modulation of endogenous Trp metabolism.

Trp metabolism is altered in synovial fluid of patients with RA
To investigate if Trp metabolite alteration also exist in joint and can directly affect resident joint cells, we performed targeted metabolomics in the synovial fluid from patients with osteoarthritis (controls) and patients with RA. Metabolomic analysis revealed changes in the kynurenine pathway with a downward trend in KYNA and XANA levels, as previously demonstrated in patients' blood. In contrast, an increase in QUIN level and its precursor 3-hydroxyanthranilic acid was observed (online supplemental figure 7a).

However, patients with osteoarthritis (OA) and not healthy subjects were used as controls and we recently showed that a significant proportion of patients with OA have also alterations in Trp metabolism.[22] A comparison with healthy controls would likely have been clearer but synovial fluid sampling from healthy subjects is not feasible due to ethical issues.

QUIN enhances IL-1β induced proliferation of hFLS and deeply impacts synoviocyte metabolism
In RA, fibroblast-like synoviocytes (hFLS) exhibit a deleterious phenotype, contributing to synovial hyperplasia, destruction of the cartilage and bone and are thought as a promising therapeutic target.[23] We therefore evaluated the effects of KYNA, XANA and QUIN on primary hFLS in vitro proliferation (with at least three individuals). No toxicity of Trp metabolites was observed at the concentrations used (1 µM) (online supplemental figure 7b). To mimic arthritis environment, recombinant IL-1β was used as a proinflammatory stimulus at 10 ng/mL. In these experimental conditions, KYNA and XANA showed no effect on
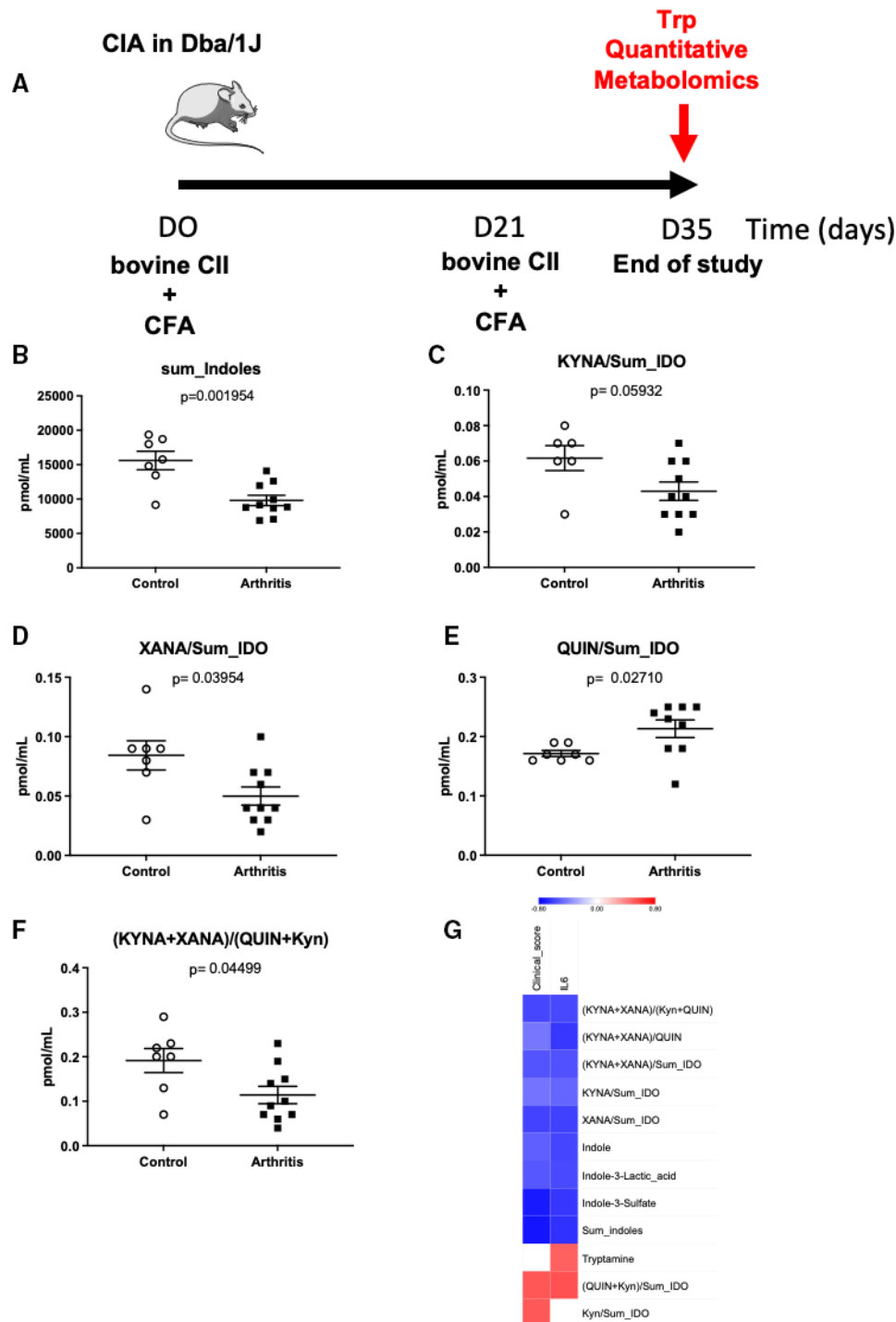
Figure 4 Targeted tryptophan metabolomics analysis in collagen-induced arthritis (CIA). Targeted tryptophan metabolomics analysis was conducted on serum of CIA mice at day 35 and on control mice. (A) Study design of CIA. (B) Level of indole derivatives in the serum of CIA mice and controls. (C–E) Ratio of KYNA, XANA, QUIN over IDO metabolites. (F) Ratio of KYNA+XANA over QUIN+KYN in the serum of CIA mice and controls. (G) Correlation of Trp metabolites and clinical activity and circulating IL-6. Statistical significance was determined for all pairwise comparisons using Spearman test; only significant correlations (p value <0.05 after false discovery rate correction, q<0.2 are displayed). KYNA, kynurenic acid; QUIN, quinolinic acid; Trp, tryptophan; XANA, xanthurenic acid.

cell proliferation both in resting and IL-1β-stimulated cells. In contrast, QUIN demonstrated proinflammatory properties with an increase of hFLS cell proliferation (figure 7A). This increase in proliferation under inflammatory conditions was confirmed by results obtained by flow cytometry. Incubation of cells with QUIN resulted in an increased percentage of cells in G2/M

phase, whereas no changes were noted for KYNA and XANA (figure 7B).

In addition, QUIN increased the basal expression of the IL-1β gene and boosted the stimulating effect of IL-1β (figure 7C).

As proliferation of hFLS is strictly dependent on their metabolic status,[24] we studied mitochondrial respiration and
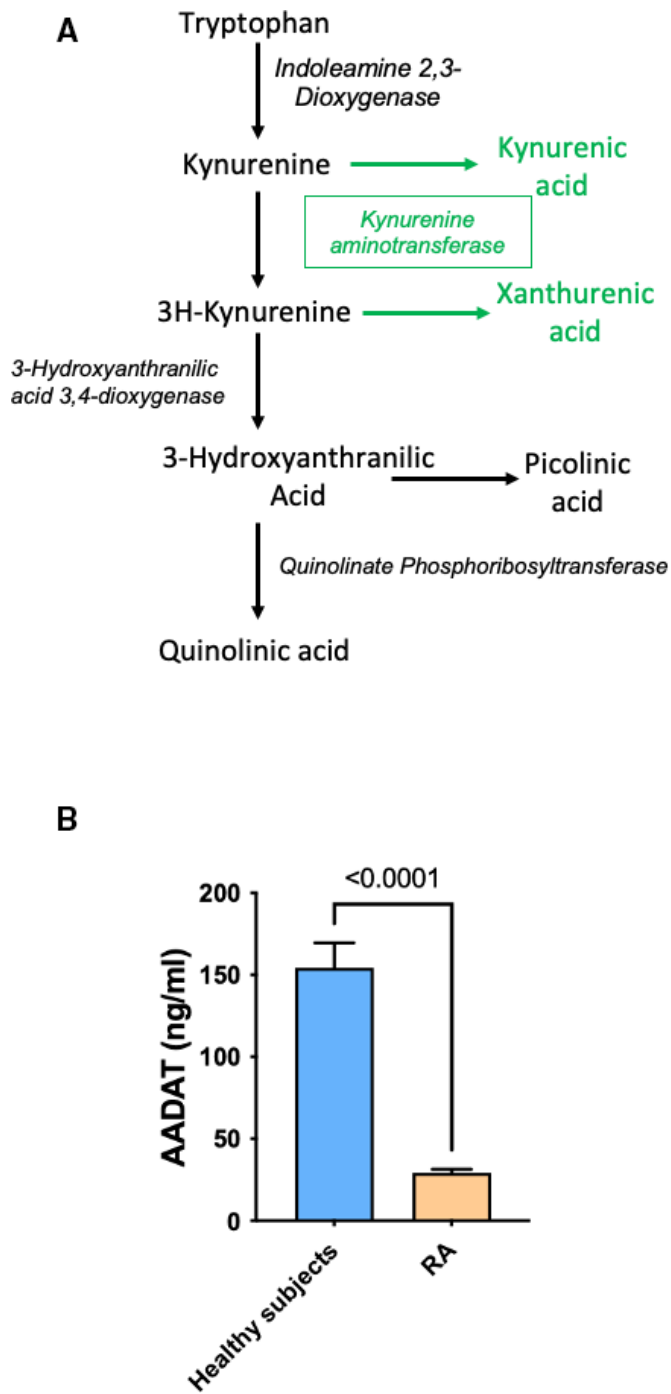
Figure 5 Kynurenine pathway (A) and AADAT level (B). Serum samples from the ESPOIR cohort compared with HS. (A) Kynurenine pathway showing AADAT involvement in KYNA and XANA production. (B) Level of AADAT measured by ELISA in human serum samples from the ESPOIR cohort. P value results from Mann-Whitney (****p<0.0001). AADAT, aminoadipate aminotransferase; HS, healthy subject; KYNA, kynurenic acid; XANA, xanthurenic acid.

glycolysis of hFLS after incubation with Trp metabolites. Using the seahorse XF technology (from at least three individuals), we showed that neither mitochondrial respiration nor glycolysis was altered by KYNA and XANA. In contrast, maximal mitochondrial respiration and glycolysis, estimated from OCR (oxygen consumption rate) and ECAR (extracellular acidification rate), respectively, were increased in QUIN-treated hFLS cells, demonstrating a modulation of cellular energy profile (figure 7D).

DISCUSSION

Understanding the pathogenesis of RA to propose new therapeutic options remains a current need more than 20 years after the avenement of TNF inhibitors. Indeed, unmet needs include partial response and non-response to treatment in many patients, failure to achieve immune homeostasis or drug-free remission and inability to repair damaged tissues.[25] Several actors involved in the pathogenesis are being actively investigated, including the systemic immune dysregulation,which probably begins at mucosal surfaces, particularly in the gastrointestinal tract in contact with the gut microbiota. Another recent pathophysiological advance is the understanding of the metabolic dysregulation occurring in immune cells and notably in T cells[26] and macrophages.[27] Also, altered Trp metabolism has long been described and notably in RA.[7][8] Recently, alterations in Trp metabolism have been reported in several chronic inflammatory diseases[3][4] including RA.[5] In particular, the Kyn pathway has been closely linked with the pathogenesis of autoimmune diseases.[11][28] High serum Kyn levels or high Kyn/Trp ratio have been found to correlate with disease activity in patients with RA,[10][29] whereas high levels of Kyn in the synovial fluid of patients with RA[30] were associated with levels of inflammatory markers, rheumatoid factor and anti-CCP antibody.[11] However, the role of specific metabolites of Kyn pathway in the pathogenesis of RA remains unclear.

Here, we focused on Trp metabolism, which has been recognised as a crucial actor in intestinal homeostasis through the generation of active end products both by host cells and gut microbiota.[6] Using targeted quantitative metabolomics on samples from a large human cohort of early RA (574 patients), we identified XANA and KYNA as candidate anti-inflammatory metabolites that are defective in active RA, closely to what occurs in IBD. We showed that XANA and KYNA were negatively associated with disease activity, as well as circulating levels of TNF, IL-6 and IL-1Ra. Corollary, a positive correlation between these biological markers and disease activity was found with QUIN and Kyn levels in the serum of patients with RA. Moreover, lower levels of XANA and KYNA and high levels of QUIN and Kyn were associated with quality-of-life assessments, suggesting a relevant active role of these metabolites in symptoms and disease burden. Importantly, we exploited the demographic characteristics of the ESPOIR cohort of well-phenotyped patients with early RA (largely higher than in previous studies investigating Trp metabolites in RA) without any treatment. Importantly, these results were similar to the one we recently described in patients with IBD, suggesting that these alterations of Trp metabolism are not specific to RA but more likely associated with systemic inflammation. We recently showed that KYNA and XANA (and many other Trp metabolites) can be detected in the stool of healthy subjects and patients with IBD, with alteration associated with intestinal inflammation.[20] In the current study, we did not have access to stool samples, but it will be important to evaluate this aspect in patients with RA in the future. Another strength of our study is that we demonstrate that similar changes in Trp metabolism were observed in another independent cohort and in the CIA and CAIA mouse models and that these metabolic disturbances were correlated with disease severity. These results are critical since CIA is the gold standard animal model of RA and the similarity of metabolic changes between patients with RA and arthritic mice demonstrate that preclinical models have some pathophysiological relevance to test the therapeutic potential of Trp metabolism correction.
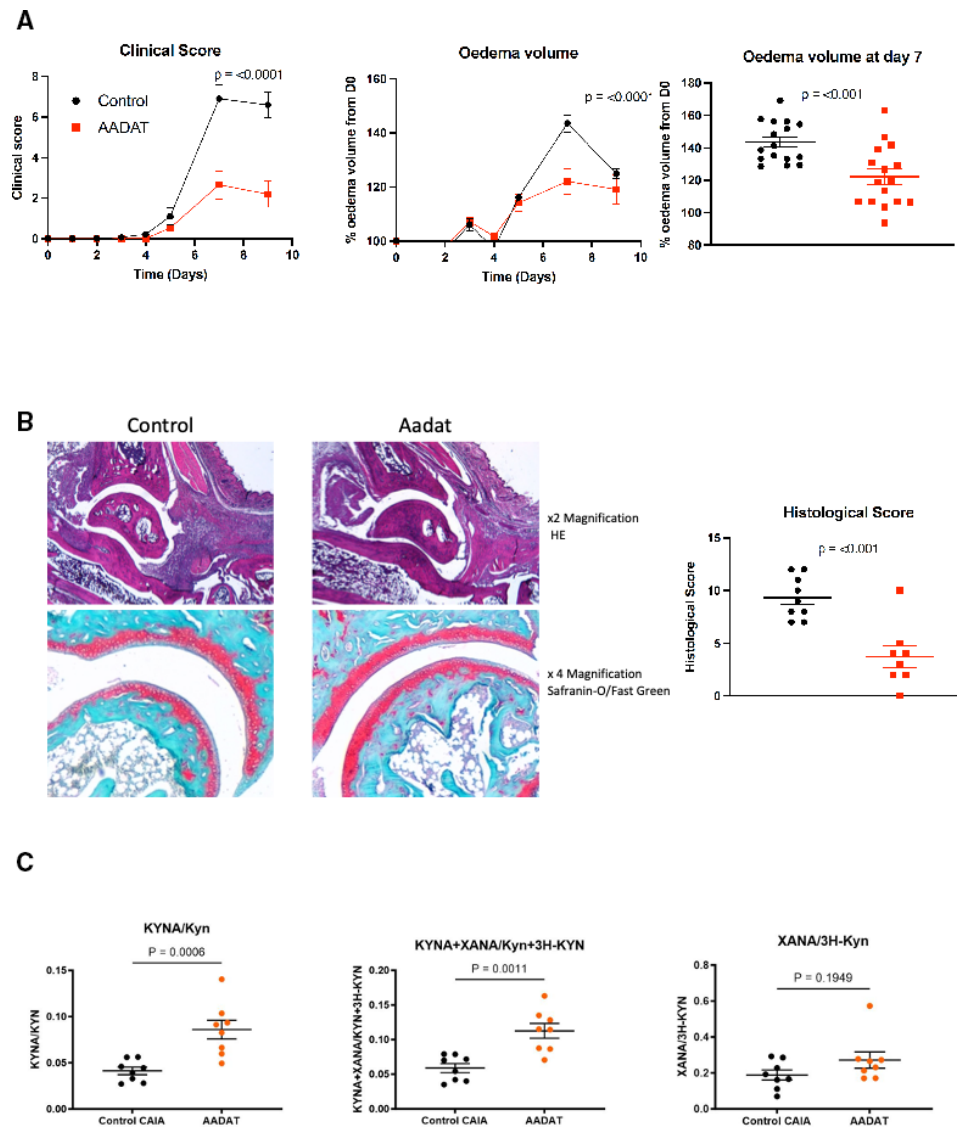
Figure 6 AADAT protects from collagen-antibody induced arthritis (CAIA) in mice. (A) Clinical effect of AADAT administration in collagen antibody-induced arthritis (CAIA) mice. (B) Histological analysis confirms AADAT protective effects in CAIA on synovial inflammation (H&E) and cartilage lesions (safranin O staining). Data are the pool of two independent experiments, n=16 mice per group. Clinical score and oedema volume: p value results from analysis of variance followed by Sidak correction for multiple comparison (for each time point); Oedema volume: p value results from unpaired t-test; Histological score: p value results from Mann-Whitney. (C) Ratio of KYNA/Kyn, KYNA+XANA over Kyn+3H-Kyn and XANA/3H-Kyn in the serum of CAIA mice treated or not with AADAT, p value results from unpaired t-test. 3H-Kyn, 3-hydroxykynurenine; AADAT, aminoadipate aminotransferase; Kyn, kynurenine; KYNA, kynurenic acid; XANA, xanthurenic acid.

We therefore decided to explore this hypothesis in the mouse CAIA arthritis and demonstrated that exogenous administration of recombinant AADAT, an enzyme that favours the production of XANA and KYNA and can restore the deficiency occurring in active disease, was able to protect mice from CAIA.

We chose the CAIA model since arthritis occurs quicker than in CIA and is therefore more appropriate to investigate a short-term therapeutic effect. Moreover, CAIA will be more appropriate for subsequent experiments since it can be induced in most mouse strains, including transgenic and knockout mice, which is less easy in CIA due to H2 MHC susceptibility. Finally, CAIA is ideal for screening and evaluating anti-inflammatory agents without interference of complete Freund's adjuvant (used in CIA to trigger the immune response against native type II collagen), which strongly affect the host immune response.

Of note, it is important to optimise the route and dosage of AADAT treatment before proposing more translational approaches. Second, AADAT has a broad substrate specificity and its activity is not restricted to the production of KYNA and XANA from kynurenine and hydroxykynurenine (3H-kynurenine), respectively. Indeed, AADAT has transaminase activity towards aminoadipate, kynurenine, methionine and glutamate, but also shows activity also towards tryptophan, aspartate and hydroxykynurenine. AADAT also accepts a variety of oxo acids as amino group acceptors, with a preference for 2-oxoglutarate, 2-oxocaproic acid, phenylpyruvate and alpha-oxo-gamma-methiol butyric acid.

During experimental arthritis, the expression of AADAT is altered. AADAT expression either in the liver or in the joint is decreased during CAIA but rebounds during resolution phase in joint. Interestingly, recombinant AADAT administration restored liver expression of AADAT gene. Taken together and considering other data generated in other models of inflammation including colitis, these results suggest that decreased
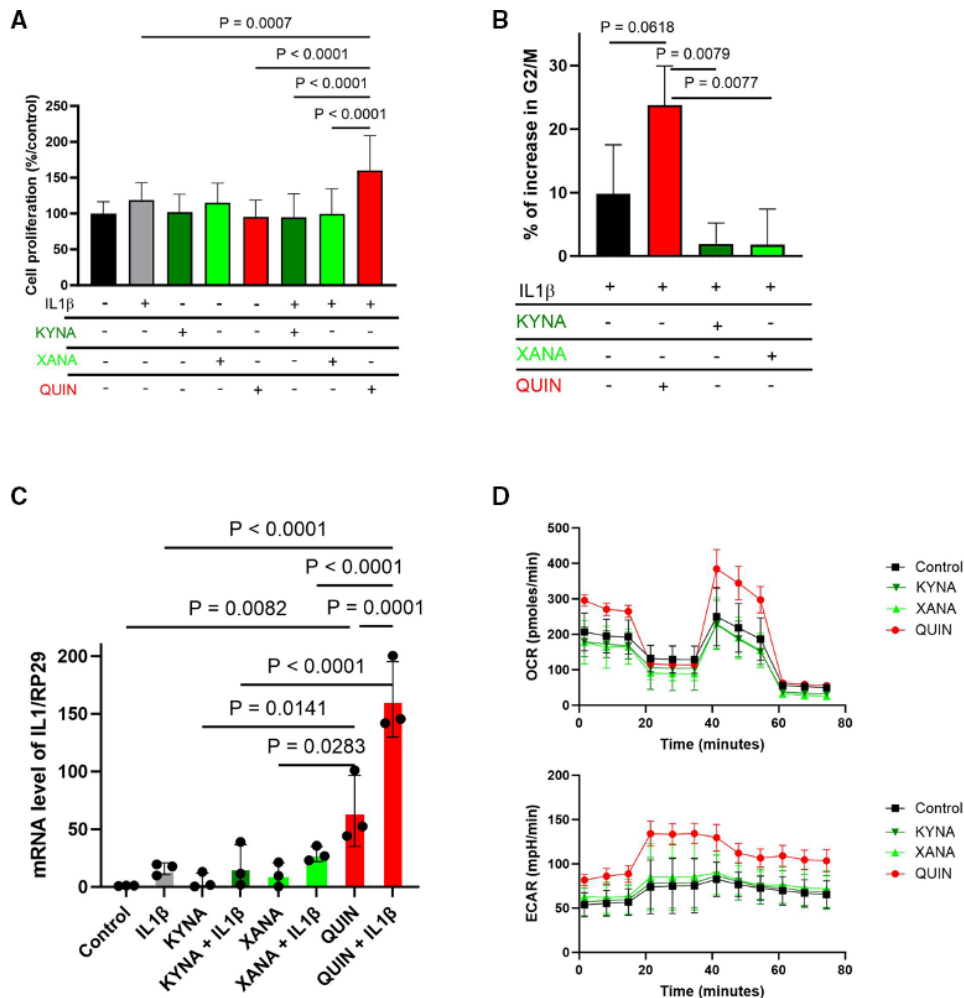
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Figure 7 Effects of KYNA, XANA and QUIN on human synoviocyte metabolism. (A) Proliferation of hFLS after incubation with Trp metabolites at 1 µM in normal and inflammatory conditions. Control corresponds to hFLS proliferation without stimuli. (B) Cell cycle analysis by using DRAQ5 DNA intercalating agent in flow cytometry on hFLS. (C) Relative expression of IL-1β in hFLS. (D) Real-time oxygen consumption rates (OCR) and real-time extracellular acidification rates (ECAR) of incubated hFLS. Data are representative of three individuals. P value results from analysis of variance followed by Sidak correction for multiple comparisons (for each point). KYNA, kynurenic acid; hFLS, human fibroblast-like synoviocyte; QUIN, quinolinic acid; XANA, xanthurenic acid.

AADAT level is a consequence of inflammation rather RA specific.

Finally, it is still unknown how Trp metabolism impacts RA pathogenesis, but we can suggest that these metabolites may modulate the cellular energy of targeted cells as we recently demonstrated in IBD.[20] Indeed, hFLS are responsive to Trp metabolites treatments. In in vitro inflammatory context, QUIN exhibited proinflammatory properties with a modulation of cell proliferation, cytokine secretion and cellular energy. Despite that the measurement of AhR target genes in joint and liver suggests that AADAT treatment might restore this signal pathway, involvement of AhR in these effects remains unclear and depends on the cell type, cellular context and developmental period for mice model.[31] Therefore, further investigations are needed to understand AhR involvement in the mechanism of action of Trp metabolites.

Dedicated studies analysing the metabolic impact of KYNA, XANA, QUIN and other Trp-derived metabolites and of AADAT modulation on cells involved in RA aetiology (T cells, B cells and macrophages) are needed.

In summary, our study identified a new mechanism linking Trp metabolism to RA. The skewed metabolism in the overactivated kynurenine pathway is associated with a relative deficiency of the anti-inflammatory metabolites XANA and KYNA. Correcting back these metabolites through AADAT administration has protective effects in animal models. In addition to providing key evidence of the importance of Trp metabolism in RA, this study paves the way for new therapeutic strategies aiming to pharmacologically correct its alterations in RA by directly manipulating the metabolic pathway with AADAT.

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting or dissemination plans of this research.

Patient consent for publication Obtained.

Ethics approval This study involves human participants and was approved by ESPOIR cohort. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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REFERENCES

1 Weyand CM, Goronzy JJ. Immunometabolism in the development of rheumatoid arthritis. *Immunol Rev* 2020;294:177–87.
2 Hooftman A, O'Neill LAJ. The immunomodulatory potential of the metabolite Itaconate. *Trends Immunol* 2019;40:687–98.
3 Lamas B, Richard ML, Leducq V, *et al*. Card9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat Med* 2016;22:598–605.
4 Liu M, Nieuwdorp M, de Vos WM, *et al*. Microbial tryptophan metabolism tunes host immunity, metabolism, and extraintestinal disorders. *Metabolites* 2022;12.
5 Pongratz G, Lowin T, Sewerin P, *et al*. Tryptophan metabolism in rheumatoid arthritis is associated with rheumatoid factor and predicts joint pathology evaluated by the rheumatoid arthritis MRI score (RAMRIS). *Clin Exp Rheumatol* 2019;37:450–7.
6 Agus A, Planchais J, Sokol H. Gut Microbiota regulation of tryptophan metabolism in health and disease. *Cell Host Microbe* 2018;23:716–24.
7 McMillan M. The identification of a fluorescent reducing substance in the urine of patients with rheumatoid arthritis. *J Clin Pathol* 1960;13:140.
8 BETT IM. Metabolism of tryptophan in rheumatoid arthritis. *Ann Rheum Dis* 1962;21:63–9.
9 Schroecksnadel K, Kaser S, Ledochowski M, *et al*. Increased degradation of tryptophan in blood of patients with rheumatoid arthritis. *J Rheumatology* 2003;30:1935–9.
10 Schroecksnadel K, Winkler C, Duftner C, *et al*. Tryptophan degradation increases with stage in patients with rheumatoid arthritis. *Clin Rheumatol* 2006;25:334–7.
11 Kang KY, Lee SH, Jung SM, *et al*. Downregulation of tryptophan-related metabolomic profile in rheumatoid arthritis Synovial fluid. *J Rheumatol* 2015;42:2003–11.
12 Forrest CM, Mackay GM, Stoy N, *et al*. Inflammatory status and kynurenine metabolism in rheumatoid arthritis treated with melatonin. *Brit J Clinical Pharma* 2007;64:517–26. 10.1111/j.1365-2125.2007.02911.x Available: https://bpspubs.onlinelibrary.wiley.com/toc/13652125/64/4
13 Forrest CM, Kennedy A, Stone TW, *et al*. Kynurenine and neopterin levels in patients with rheumatoid arthritis and osteoporosis during drug treatment. *Adv Exp Med Biol* 2003;527:287–95.
14 Nguyen NT, Nakahama T, Nguyen CH, *et al*. Aryl hydrocarbon receptor antagonism and its role in rheumatoid arthritis. *J Exp Pharmacol* 2015;7:29–35.
15 Combe B, Benessiano J, Berenbaum F, *et al*. The ESPOIR cohort: A ten-year follow-up of early arthritis in France methodology and baseline characteristics of the 813 included patients. *Joint Bone Spine* 2007;74:440–5.
16 Aletaha D, Neogi T, Silman AJ, *et al*. Rheumatoid arthritis classification criteria: an American college of rheumatology/European League against rheumatism collaborative initiative. *Arthritis Rheum* 2010;62:2569–81.
17 Gottenberg J-E, Dayer J-M, Lukas C, *et al*. Serum IL-6 and IL-21 are associated with markers of B cell activation and structural progression in early rheumatoid arthritis: results from the ESPOIR cohort. *Ann Rheum Dis* 2012;71:1243–8.
18 Baillet A, Gossec L, Paternotte S, *et al*. Evaluation of serum Interleukin-6 level as a surrogate marker of synovial inflammation and as a factor of structural progression in early rheumatoid arthritis: results from a French national multicenter cohort. *Arthritis Care & Research* 2015;67:905–12. 10.1002/acr.22513 Available: https://acrjournals.onlinelibrary.wiley.com/toc/21514658/67/7
19 Lefèvre A, Mavel S, Nadal-Desbarats L, *et al*. Validation of a global quantitative analysis methodology of tryptophan metabolites in mice using LC-MS. *Talanta* 2019;195:593–8.
20 Michaudel C, Danne C, Agus A, *et al*. Rewiring the altered tryptophan metabolism as a novel therapeutic strategy in inflammatory bowel diseases. *Gut* 2023;72:1296–307.
21 Hablot J, Peyrin-Biroulet L, Kokten T, *et al*. Experimental colitis delays and reduces the severity of collagen-induced arthritis in mice. *PLoS One* 2017;12:e0184624.
22 Binvignat M, Emond P, Mifsud F, *et al*. Serum tryptophan metabolites are associated with erosive hand osteoarthritis and pain: results from the DIGICOD cohort. *Osteoarthritis and Cartilage* 2023;31:1132–43.
23 Wu Z, Ma D, Yang H, *et al*. Fibroblast-like synoviocytes in rheumatoid arthritis: surface markers and phenotypes. *Int Immunopharmacol* 2021;93.
24 de Jong TA, Semmelink JF, Denis SW, *et al*. Altered lipid metabolism in synovial fibroblasts of individuals at risk of developing rheumatoid arthritis. *J Autoimmun* 2023;134.
25 Alivernini S, Firestein GS, McInnes IB. The pathogenesis of rheumatoid arthritis. *Immunity* 2022;55:2255–70.
26 Masoumi M, Alesaeidi S, Khorramdelazad H, *et al*. Role of T cells in the pathogenesis of rheumatoid arthritis: focus on immunometabolism dysfunctions. *Inflammation* 2023;46:88–102.

27 Yang X, Chang Y, Wei W. Emerging role of targeting macrophages in rheumatoid arthritis: focus on polarization, metabolism and apoptosis. *Cell Prolif* 2020;53:e12854.

28 Maria NI, van Helden-Meeuwsen CG, Brkic Z, *et al*. Association of increased treg cell levels with elevated Indoleamine 2,3-dioxygenase activity and an imbalanced Kynurenine pathway in Interferon-Positive primary Sjögren's syndrome. *Arthritis Rheumatol* 2016;68:1688–99.

29 Igari T, Tsuchizawa M, Shimamura T. Alteration of tryptophan metabolism in the synovial fluid of patients with rheumatoid arthritis and osteoarthritis. *Tohoku J Exp Med* 1987;153:79–86.

30 Ozkan Y, Mete G, Sepici-Dincel A, *et al*. Tryptophan degradation and neopterin levels in treated rheumatoid arthritis patients. *Clin Rheumatol* 2012;31:29–34.

31 Larigot L, Juricek L, Dairou J, *et al*. Ahr signaling pathways and regulatory functions. *Biochim Open* 2018;7:1–9.