Arthritis & Rheumatology
Vol. 75, No. 10, October 2023, pp 1749–1761
DOI 10.1002/art.42528

American College of Rheumatology
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Preclinical Characterization of Pharmacologic NAD$^+$ Boosting as a Promising Therapeutic Approach in Rheumatoid Arthritis

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Objective. We analyzed NAD$^+$ metabolism in patients with rheumatoid arthritis (RA), its association with disease activity and clinical outcomes of RA, and the therapeutic potential of pharmacologic NAD$^+$ boosting.

Methods. Our study included 253 participants. In the first cohort, comprising 153 RA patients and 56 healthy donors, we assessed NAD$^+$ levels and NAD$^+$-related gene pathways. We analyzed 92 inflammatory molecules by proximity extension assay. In the second cohort, comprising 44 RA patients starting anti–tumor necrosis factor (anti-TNF) drugs, we evaluated changes in NAD$^+$ levels and their association with clinical response after 3 months. Mechanistic studies were performed ex vivo on peripheral blood mononuclear cells (PBMCs) from patients with RA to test the beneficial effects of NAD$^+$ boosters, such as nicotinamide and nicotinamide riboside.

Results. Reduced NAD$^+$ levels were found in RA samples, in line with altered activity and expression of genes involved in NAD$^+$ consumption (sirtuins, poly[ADP-ribose] polymerase, CD38), transport (connexin 43), and biosynthesis (NAMPT, NMNATs). Unsupervised clustering analysis identified a group of RA patients with the highest inflammatory profile, the lowest NAD$^+$ levels, and the highest disease activity (as shown by the Disease Activity Score in 28 joints). NAD$^+$ levels were modulated by anti-TNF therapy in parallel with the clinical response. In vitro studies using PBMCs from RA patients showed that nicotinamide riboside and nicotinamide increased NAD$^+$ levels via NAMPT and NMNAT and reduced their prooxidative, proapoptotic, and proinflammatory status.

Conclusion. RA patients display altered NAD$^+$ metabolism, directly linked to their inflammatory and disease activity status, which was reverted by anti-TNF therapy. The preclinical beneficial effects of NAD$^+$ boosters, as shown in leukocytes from RA patients, along with their proven clinical safety, might pave the way for the development of clinical trials using these compounds.

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory diseases and can cause bone and cartilage damage, leading to disability. Although pain and joint alterations are the primary symptoms, patients with RA may also develop extraarticular manifestations, such as vasculitis, pulmonary involvement, and systemic comorbidities (1).

Dr. Lopez-Pedrera's laboratory was supported by ISCIII (PI21/00591 and RICOR-RD21/0002/0033) co-financed by FEDER, Fundacion Andaluza de Reumatología (FAR), and Consejería de Conocimiento, Investigación y Universidad de la Junta de Andalucía (P20_01367). Dr. Barbarroja's laboratory was funded by ISCIII (PI20/00079), co-financed by the European Regional Development Fund (ERDF), A Way of Making Europe, Spain, and by MINECO (RyC-2017-23437). Dr. Villalba's laboratory was supported by the Spanish Ministerio de Ciencia, Innovación y Universidades (MCIU)/Agencia Estatal de Investigación (AEI) grants RTI2018-100695-B-I00 and PID2021-126280OB-I00, RYC2021-033828-I (MCIN/AEI/10.13039/501100011033; "NextGenerationEU"/PRTR), Spanish Junta de Andalucía grants P18-RT-4264, 1263735-R, and BIO-276, the Fondo Europeo de Desarrollo Regional (FEDER) from the European Union, and Universidad de Córdoba. Dr. Sánchez-Mendoza's work was supported by an FPI MINECO contract (PRE2019-087438). Dr. Moreno's laboratory was funded by ISCIII (FIS-FEDER PI20/00375), CIBERCV, Junta de Andalucía (PIGE-0052-2020), and MINECO (RYC-2017-22369). Tomás Cerdó was supported by "Sara Borrel" program (Code CD21/00187) from Spanish Institute of Health Carlos III (ISCIII). Open access funding was provided by Universidad de Córdoba.

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Recent advancements in high-throughput technologies and well-characterized clinical cohorts of RA patients have improved our understanding of the disease. In people who are genetically predisposed, environmental factors, epigenetic modifications, and posttranslational alterations can lead to loss of tolerance and an uncontrolled inflammatory response, characterized by the secretion of proinflammatory cytokines, aberrant oxidative and apoptotic mechanisms, and features of accelerated aging, all of which promote structural damage (2–4).

Modern therapeutic approaches, including conventional, biologic, and novel small molecule disease-modifying antirheumatic drugs (DMARDs), constitute the current options for patients with RA. Methotrexate and anti–tumor necrosis factor (anti-TNF) drugs remain the mainstay of RA treatment. Although these treatments have contributed to significant progress toward achieving disease remission without joint damage, a significant proportion of RA patients do not respond effectively to current therapies; thus, new drugs or therapeutic strategies are urgently needed (5,6).

In this sense, emerging evidence from mouse models and human trials has suggested that elevating NAD^+ levels could delay or even reverse the progression of age-related diseases (7). In addition, accumulating evidence has suggested that the decline of NAD^+ levels contributes to the development of many age-associated pathophysiologies (8–10).

In all living cells, nicotinamide adenine dinucleotide (NAD^+) is a crucial cofactor of key metabolic enzymes such as sirtuins (SIRT), poly(ADP-ribose) polymerases (PARPs), CD38, and CD157, among others, that are involved in fundamental biologic processes, such as metabolism, cell signaling, immune response, oxidative stress, DNA repair, and gene expression (8,10–12).

The NAD^+ status is maintained in cells through a balance of synthesis, degradation, and recycling. Mammalian cells usually cannot import NAD^+ and must synthesize it de novo from tryptophan or from intermediate forms of vitamin B_3 such as nicotinamide (NAM), nicotinamide mononucleotide (NMN), nicotinamide riboside (NR), or nicotinic acid (NA). In the recycling or salvage pathway, NAM is converted to NMN by the rate-limiting enzyme, nicotinamide phosphoribosyltransferase (NAMPT or visfatin). NMN can also be produced from NR via an NR kinase (NRK)–mediated phosphorylation reaction. Finally, NMN is converted into NAD^+ by NMN adenylyltransferases (NMNATs). Another essential player in NAD^+ homeostasis is connexin 43, which forms plasma membrane hemichannels that allow the efflux of NAD^+ (13).

NAD^+ boosting with the intermediate precursors of NAD^+ (boosters) has been shown to be an effective and safe strategy to increase the NAD^+ levels, promoting beneficial effects to prevent and treat several pathologic conditions (14). As a result, several clinical trials are currently underway to evaluate the therapeutic effects of NAD^+ boosters on a wide variety of physiologic outcomes, including insulin sensitivity, glucose metabolism, immune function, autoimmunity, cardiovascular function, and cognitive function (https://ClinicalTrials.gov).

Because the beneficial effects of NAD^+ boosters may include targeting pathologic mechanisms present in RA, we hypothesized that these natural compounds might have a positive impact on the disease. Thus, in this preclinical study, we analyzed for the first time, to our knowledge, the metabolism of NAD^+ in RA patients, its relationship with disease status and clinical outcome of RA, and the positive effects promoted by NAD^+ boosters on leukocytes from RA patients.

PATIENTS AND METHODS

Patients. Our study included 2 groups of 253 total patients and healthy donors. The first cross-sectional cohort comprised 153 RA patients and 56 healthy donors, with clinical and analytic details displayed in Table 1. The second group, a longitudinal cohort comprising 44 RA patients starting anti-TNF therapy, was followed for 3 months, during which the EULAR response was assessed (15). Peripheral blood from all RA patients and healthy donors was collected by direct venous puncture, and an extensive clinical assessment was conducted. The study was conducted in accordance with the ethics principles of the Declaration of Helsinki (see Supplementary Material for further details, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42528).

Analysis of NAD^+ and NADH levels. Levels of NAD^+ and NADH were determined using the bioluminescent NAD/NADH-Glo assay (Promega) according to the manufacturer's instructions (Supplementary Material, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42528).

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Author disclosures are available at https://onlinelibrary.wiley.com/doi/10.1002/art.42528.

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Submitted for publication March 8, 2023; accepted in revised form April 4, 2023.

Table 1. Clinical characteristic of the subjects included in the study*

	Rheumatoid arthritis (n = 153)	Healthy donors (n = 56)	*P*
Clinical parameters			
No. of women/men	117/36	37/19	
Age, mean ± SD years	53.21 ± 19.24	47.83 ± 10	–
Evolution time, mean ± SD years	10.85 ± 10.04	–	–
Swollen joints, mean ± SD number	3.28 ± 4.15	–	–
Tender joints, mean ± SD number	5.50 ± 7.11	–	–
Radiologic involvement	64 (42)	–	–
DAS28, mean ± SD	3.87 ± 1.78	–	–
RF positivity	97 (64)	4 (8)	<0.001
ACPA positivity	113 (74)	0 (0)	<0.001
Obesity	33 (22)	5 (10)	–
Diabetes mellitus	12 (8)	0 (0)	–
Hypertension	59 (39)	0 (0)	0.047
Smoker	49 (32)	2 (3)	<0.001
Serological assessments			
ESR, mean ± SD mm/h	24.56 ± 17.46	6.88 ± 5.36	0.000
CRP, mean ± SD mg/dl	15.10 ± 26.9	9.02 ± 30.24	–
Total cholesterol, mean ± SD mg/dl	198.53 ± 40.51	174.4 ± 31.08	0.003
HDL-cholesterol, mean ± SD mg/dl	57.22 ± 18.4	59.07 ± 21.14	–
LDL-cholesterol, mean ± SD mg/dl	118.52 ± 31.18	109.8 ± 25.69	–
Apolipoprotein A, mean ± SD mg/dl	150.67 ± 30.39	133.97 ± 38.1	0.032
Apolipoprotein B, mean ± SD mg/dl	86.88 ± 23.88	76.4 ± 21.22	0.022
Triglycerides, mean ± SD mg/dl	112.22 ± 71.23	79.49 ± 45.01	0.003
Treatment			
Corticosteroids	120 (79)	0 (0)	–
Antimalarials	70 (46)	0 (0)	–
NSAIDs	110 (72)	0 (0)	–
Methotrexate	110 (72)	0 (0)	–
Leflunomide	73 (48)	0 (0)	–
Vitamin D	53 (35)	0 (0)	–

* Except where otherwise indicated, values are the number (%) of subjects. DAS28 = Disease Activity Score in 28 joints; RF = rheumatoid factor; ACPA = anti–citrullinated protein antibody; RF = rheumatoid factor; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; HDL = high-density lipoprotein; LDL = low-density lipoprotein; NSAIDs = nonsteroidal antiinflammatory drugs.

Circulating inflammatory profile. We evaluated the Olink Target 96 inflammation panel using proximity extension assays (Cobiomic Bioscience SL) (Supplementary Material, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42528).

Transcriptomic analyses. Expression levels of selected genes involved in NAD^+ metabolism and inflammation were analyzed by real-time quantitative reverse transcription–polymerase chain reaction. Primer sequences (Merck KGaA) are listed in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42528.

In addition, independent public data sets of healthy donors and RA patients were used from the Gene Expression Omnibus database to validate and complement the data from the real-time quantitative reverse transcription–polymerase chain reaction, which included accession numbers GSE120178 (16) and GSE15258 (17) (Supplementary Material, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42528).

NAD^+-consuming enzyme activity. The activity of the overall SIRT and PARP system was analyzed in cell lysates from peripheral blood mononuclear cells (PBMCs) of patients with RA and healthy donors using the Sirtuin Activity Assay Kit (Abcam) and the PARP Universal Colorimetric Assay Kit (R&D Systems), respectively, in accordance with the manufacturer's instructions.

Ex vivo assays. In each set of experiments, PBMCs isolated from 5 healthy donors and 5 RA patients with active disease (Disease Activity Score in 28 joints [DAS28] >5.1) were seeded in 24-well plates and cultured at 37°C and 5% CO_2 with RPMI medium and 10% of autologous serum to maintain activated status of cells. Cells were then treated for 24 hours with different compounds, depending on the specific set of experiments, including 1 m*M* NAM, NR, and NMN; 10 n*M* FK866; 100 μ*M* tannic acid; and 100 μ*M* GAP19 (Sigma-Aldrich) (Supplementary Material, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42528).

Concurrently, we purified monocytes and lymphocytes from 5 RA patients with active disease using immunomagnetic

selection with CD14 and CD3 microbeads (Miltenyi Biotec) and then treated the samples with NAD^+ boosters under the same experimental conditions. We also analyzed the effects of NAD^+ boosters in PBMCs obtained from another group of 5 patients who were in remission with low disease activity (DAS28 <2.6).

Flow cytometry. PBMCs were incubated with 20.5 μ*M* dichlorodihydrofluorescein diacetate (Sigma-Aldrich) for 30 minutes in the dark at 37°C to evaluate the oxidative status and analyze peroxide levels. Apoptotic cells were measured using Annexin V-PE Apoptosis Detection Kit I (BD Biosciences) in accordance with the manufacturer's instructions (Supplementary Material, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42528).

NF-κB (p65) DNA binding activity. The effects of NAD^+ boosters on the activity of the transcription factor NF-κB (p65) were analyzed in nuclear extracts of PBMCs from RA patients using the NF-κB (p65) Transcription Factor Assay Kit (Cayman) in accordance with the manufacturer's instructions.

Data analysis. For statistical analysis and graphical representation of results, we used GraphPad Prism 8 software. For comparisons between quantitative and qualitative variables, we used the Student's *t*-test or, alternatively, the Mann-Whitney U test for nonparametric tests. Relationships between the studied parameters were analyzed using Pearson and Spearman correlations. *P* values less than 0.05 were considered significant (Supplementary Material, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42528).

RESULTS

Alteration of NAD^+ levels and expression of genes associated with NAD^+ metabolism in RA. We first analyzed the levels of NAD^+ and NADH in plasma samples from RA patients and healthy donors. We found that mean levels of both metabolites were decreased in the plasma of RA patients compared with healthy donors, and we also identified a positive correlation between the levels of oxidized and reduced pyridine nucleotides (Figures 1A–C).

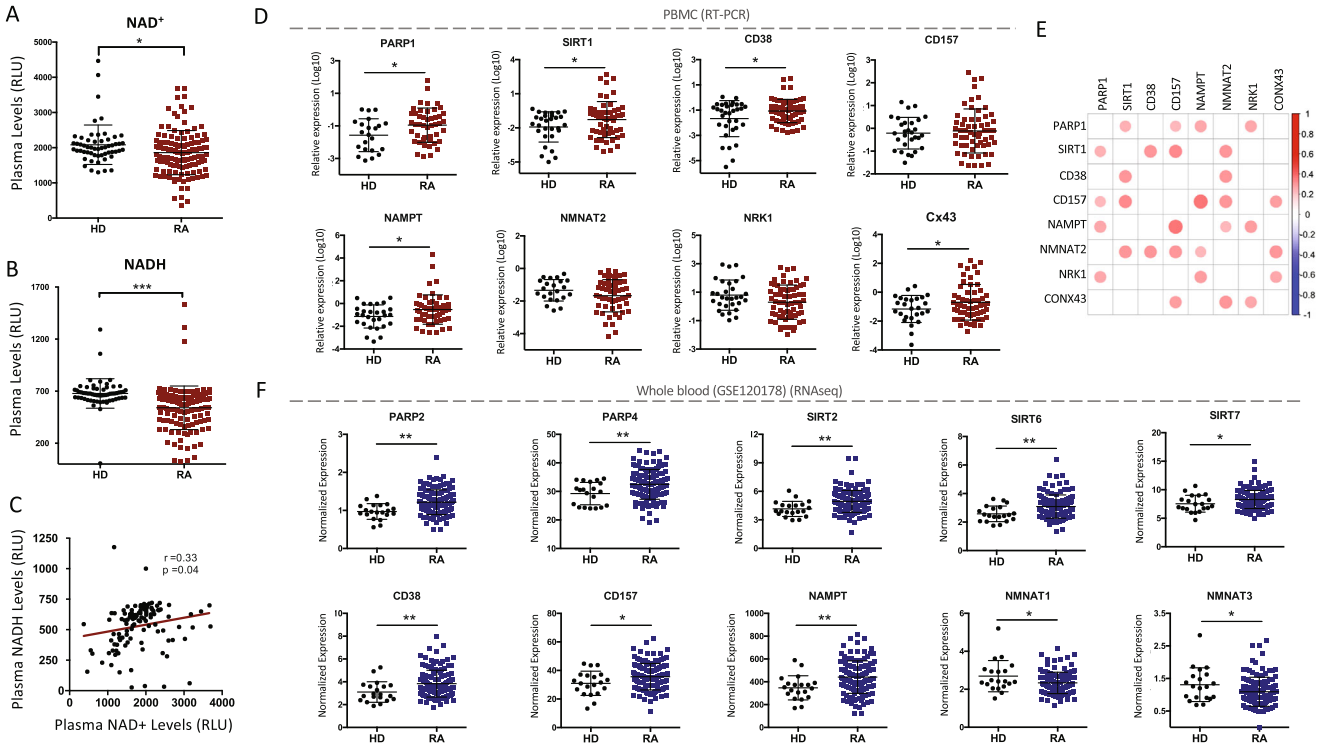

Figure 1. Altered levels of NAD^+ and gene transcripts involved in NAD^+ metabolism in patients with rheumatoid arthritis (RA). **A** and **B**, Levels of NAD^+ (**A**) and NADH (**B**) in plasma samples from patients with RA and from healthy donors (HD), determined by the NAD/NADH-Glo Assay kit (Promega). **C**, Correlation between NAD^+ and NADH levels in plasma from RA patients. **D**, Relative expression levels of genes involved in NAD^+ metabolism, analyzed by real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR), in peripheral blood mononuclear cells (PBMCs) from RA patients and healthy donors. **E**, Heatmap of correlations among the genes involved in NAD^+ metabolism in PBMCs from RA patients. Red dots indicate positive and significant correlations. **F**, Expression levels of genes involved in NAD^+ metabolism in whole blood from an external RNA sequencing (RNAseq) data set (GSE120178). Horizontal lines and whiskers (**A**, **B**, **D**, **F**) represent the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. RLU = relative luminescence unit; PARP = poly(ADP-ribose) polymerase; SIRT = sirtuin; NAMPT = nicotinamide phosphoribosyltransferase; NMNAT = nicotinamide mononucleotide adenylyltransferase; NRK = nicotinamide riboside kinase; Cx43/Conx43 = connexin 43.

These alterations were consistent with the up-regulation of genes involved in NAD+ consumption, including PARP1, SIRT1, and CD38 in PBMCs from patients with RA. In addition, the expression of genes involved in NAD+ biosynthesis, such as NMNAT2 and NRK1, showed a trend toward down-regulation, although the differences did not reach significance. Moreover, key genes, such as NAMPT, which encodes a rate-limiting enzyme in the salvage NAD+ biosynthesis pathway, and connexin 43, which channels the transmembrane NAD+ efflux, were also found to be up-regulated (Figure 1D). The expression levels of these altered genes involved in NAD+ metabolism were strongly correlated with each other in RA patients, suggesting a coordinated alteration of the system (Figure 1E).

To validate and extend our findings, we analyzed an external data set in whole blood. Our analysis showed that RA patients exhibited up-regulation of genes involved in NAD+ consumption, such as PARP2, PARP4, SIRT2, SIRT6, SIRT7, CD38, and CD157. Conversely, the expression of genes involved in NAD+ biosynthesis, such as NMNAT1 and NMNAT3, was reduced in RA patients. NAMPT was also up-regulated in the data set, as previously observed in PBMCs (Figure 1F).

Given the evidence of increased expression of NAD+ consumption systems in RA, we aimed to validate this altered consumption process in PBMCs from RA patients by measuring the activity of the overall SIRT and PARP systems. Our results confirmed that activity levels of both SIRT and PARP were significantly higher in RA patients than in healthy donors (Supplementary Figures 1A and B, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42528).

Direct association between altered levels of NAD+ and genes involved in NAD+ metabolism and the inflammatory and active status of RA patients. Unsupervised clustering analysis of circulating inflammatory proteomes in RA patients identified 3 subgroups. Cluster 1 was characterized by higher levels of inflammatory mediators compared with cluster 3, which showed the lowest levels. Cluster 2 represented RA patients with an intermediate inflammatory profile (Figure 2A).

Of note, NAD+ levels were inversely associated with the inflammatory and clinical profile of RA patients. Patients belonging to cluster 1 showed the lowest levels of NAD+ and the highest disease activity score (DAS28), whereas patients from cluster 3 showed higher levels of NAD+ and lower disease activity. Patients from cluster 2 exhibited an intermediate level of NAD+ and disease activity (Figures 2B and C).

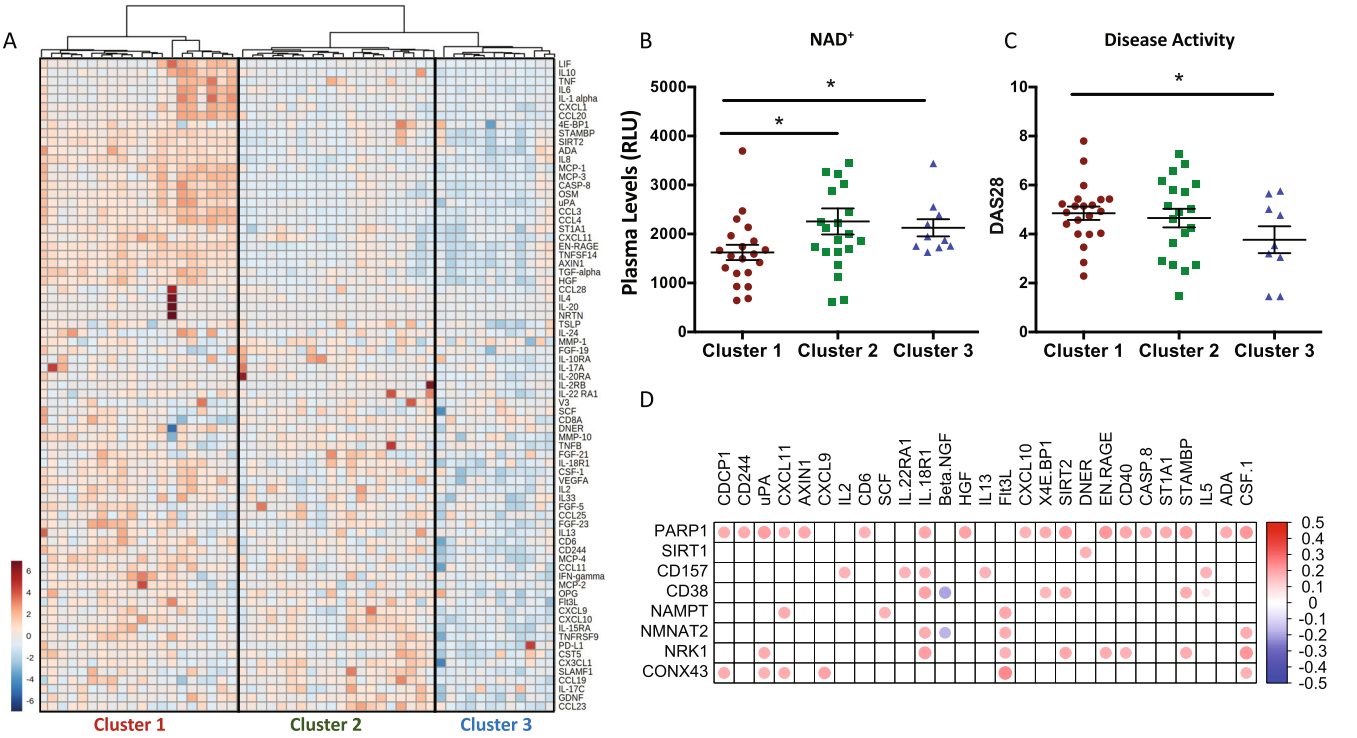

Figure 2. Relationship between NAD+ levels and metabolism and the inflammatory and clinical status of patients with RA. **A**, Unsupervised clustering analysis of a panel of 92 inflammatory mediators using the high-throughput proteomic technique "Proximity extension assay" (Olink) and serum samples from patients with RA. Cluster 1 is characterized by higher levels of inflammatory mediators compared with cluster 3, which showed the lowest levels. Cluster 2 represented RA patients with an intermediate inflammatory profile. **B** and **C**, Association between RA inflammatory clusters and NAD+ levels (**B**) and between RA inflammatory clusters and Disease Activity Score in 28 joints (DAS28) (**C**). Horizontal lines and whiskers represent the mean ± SD. **D**, Heatmap of correlations among the genes involved in NAD+ metabolism and the inflammatory proteome. Red and blue dots indicate positive and negative significant correlations, respectively. * = $P < 0.05$. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42528/abstract.

Moreover, the expression of genes involved in NAD^+ metabolism was significantly correlated with the circulating proinflammatory profile of RA patients, supporting the link between inflammation and NAD^+ metabolism (Figure 2D).

Modulation of NAD^+ levels with anti-TNF therapy and correlation with clinical response in patients with RA. Anti-TNF therapy for 3 months improved the clinical status of this new cohort of RA patients (44 RA patients starting anti–tumor necrosis factor), including a significant reduction of swollen and tender joints and disease activity scores (DAS28, Clinical Disease Activity Index, Simplified Disease Activity Index, Visual Analogue Scale, and Health Assessment Questionnaire) (Figure 3A). Our results also showed that, at the start of anti-TNF therapy, most RA patients had lower NAD^+ levels compared with healthy donors (Figure 3B), confirming our previous observations. However, we also observed that some patients had NAD^+ levels similar to or even higher than those of healthy donors, which could be in accordance with the heterogeneity of the patients previously described in the first cohort that we analyzed (Figure 3B).

With this observation, we thus separately assessed the effects of anti-TNF therapy on NAD^+ levels in these 2 groups of RA patients who started anti-TNF with low or high NAD^+ levels at baseline. In both groups of patients, anti-TNF therapy induced a shift in NAD^+ levels toward the levels seen in healthy donors. In patients with low NAD^+ levels at baseline, anti-TNF therapy increased their NAD^+ levels (Figure 3C), whereas, in patients with high NAD^+ levels at baseline, anti-TNF therapy reduced their NAD^+ levels (Figure 3D). In both groups of patients, the NAD^+ levels after therapy reached those shown in healthy donors.

Interestingly, a significant correlation between the changes in the NAD^+ levels and the disease activity after anti-TNF therapy was shown, pointing to the role of NAD^+ in the clinical response of RA patients (Figure 3E).

The differential NAD^+ levels at baseline were also associated with the therapeutic response to anti-TNF after 3 months of treatment. Thus, the proportion of patients with lower NAD^+ levels at baseline was significantly higher (75%) in the group of nonresponding patients according to EULAR criteria compared with the group of responding patients (25%) (Figure 3F). Moreover, using an external data set in whole blood, we also identified an association between

A

	Before TNFi (T0)	After TNFi (T3)	*p value*
Clinical parameters			
Female/male (n/n)	37/7	-	-
Age (years)	42,49 ± 10,795	-	-
Evolution time (years)	11,14 ± 9,45	-	-
Swollen joints (n)	5,66 ± 4,41	2,07 ± 3,12	0,000*
Tender joints (n)	8,39 ± 6,19	3,41 ± 4,14	0,000*
DAS28	4,94 ± 1,35	3,31 ± 1,32	0,000*
SDAI	30 ± 14,42	14,06 ± 12,34	0,000*
CDAI	27,69 ± 12,87	12,66 ± 11,35	0,000*
VAS	65,78 ± 22,73	35,59 ± 26,97	0,000*
HAQ	1,49 ± 0,67	1,21 ± 0,64	0,044*
Serological assessments			
ACPA levels (U/mL)	373,46 ± 792,72	235,52 ± 395,38	0.121
RF levels (IU/mL)	142,54 ± 233,3	131,27 ± 199,26	0.528
CRP (mg/mL)	18,84 ± 28	13,98 ± 22,02	0.327
ESR (mm/h)	29,61 ± 21,04	25,9 ± 21,03	0.144
Total cholesterol (mg/dL)	202,43 ± 39,8	198,95 ± 36,42	0.439
HDL-cholesterol (mg/dL)	62,12 ± 18,61	59,73 ± 15,93	0.242
LDL-cholesterol (mg/dL)	117,80 ± 29,75	118,4 ± 26,94	0.98
Apolipoprotein A (mg/dL)	154,22 ± 37,21	147,95 ± 35,52	0.122
Apolipoprotein B (mg/dL)	94,97 ± 23,27	92,9 ± 20,32	0.311
Triglycerides (mg/dL)	111,43 ± 50,27	109,29 ± 52,19	0.531

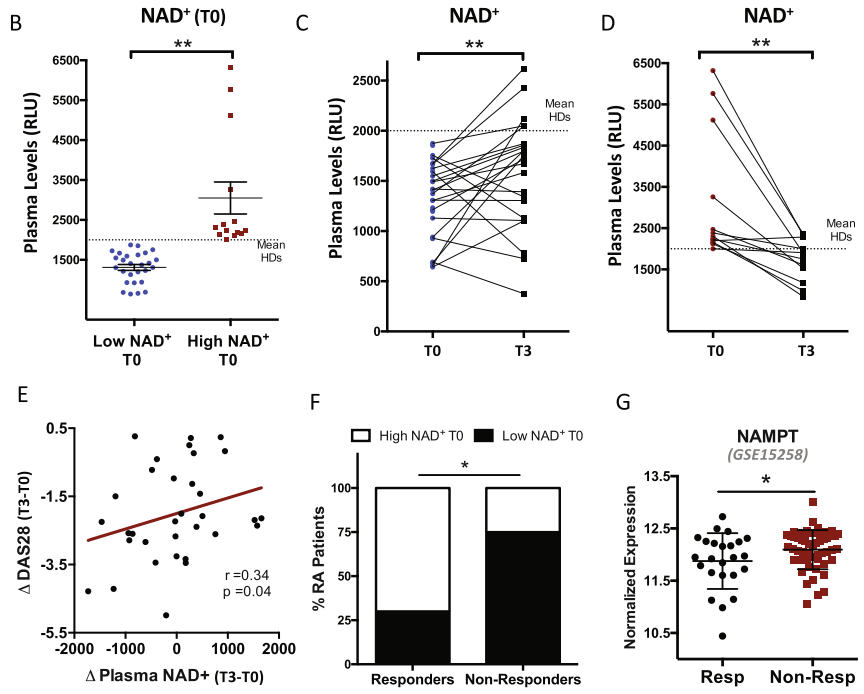

Figure 3. Modulation of NAD^+ levels after treatment with anti–tumor necrosis factor (anti-TNF) agents (i.e., tumor necrosis factor inhibitor [TNFi]) and its association with clinical response in patients with RA. **A**, Clinical parameters modulated after 3 months of anti-TNF therapy. **B**, Plasma levels of NAD^+ in RA patients with low or high levels at baseline (T0). **C** and **D**, Modulation of the NAD^+ levels after 3 months of anti-TNF therapy in RA patients with low (**C**) or high (**D**) NAD^+ levels at baseline. **E**, Correlation between changes in NAD^+ and the Disease Activity Score in 28 joints (DAS28) in RA patients after anti-TNF therapy. **F**, Clinical response of RA patients to anti-TNF therapy and proportion of patients belonging to the groups of lower or higher NAD^+ levels at baseline. **G**, Expression levels of NAMPT in whole blood from RA patients, using an external microarray data set (GSE15258), who had a clinical response versus no response at 3 months. Horizontal lines and whiskers (**B**, **G**) represent the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$. SDAI = Simplified Disease Activity Index; CDAI = Clinical Disease Activity Index; VAS = Visual Analogue Scale; HAQ = Health Assessment Questionnaire; ACPA = anti–citrullinated protein antibody; RF = rheumatoid factor; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; HDL = high-density lipoprotein; LDL = low-density lipoprotein; see Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42528/abstract.

key genes involved in the metabolism of NAD+, such as NAMPT, and the clinical outcome of RA patients treated with anti-TNF therapy. Patients who did not respond at 3 months of anti-TNF therapy showed significantly higher levels of NAMPT at baseline (Figure 3G).

Increase of NAD+ boosters and amelioration of oxidative, apoptotic, and inflammatory status in PBMCs isolated from active RA patients. Ex vivo treatment of PBMCs isolated from RA patients with active disease with NR or NAM promoted significant up-regulation of NAD+ levels, whereas treatment with NMN resulted in a nonsignificant upward trend (Figure 4A).

We further explored the effects of these biosynthetic NAD+ precursors on the oxidative status of PBMCs from RA patients and found that NAM and NR ameliorated the prooxidant status that characterize these cells, as demonstrated by the reduction of peroxide levels. Conversely, changes elicited by NMN did not reach statistical significance (Figure 4B). In addition, treatment with NAD+ boosters prevented the spontaneous apoptosis of PBMCs from RA patients in culture, with the effects of NAM and NR being more prominent than those of NMN (Figure 4C).

Analyses of the intracellular expression of key inflammatory mediators associated with the pathogenesis of RA showed that NAD+ boosters have potent antiinflammatory effects. NR and NAM significantly down-regulated the expression of interleukin-1β (IL-1β), IL-6, TNF, IL-8, monocyte chemotactic protein 1 (CCL2), and STAT3 (Figure 4D). Although NMN also exhibited some antiinflammatory properties, its effects were less prominent compared with NR and NAM.

Promotion of up-regulated NAD+ levels by NR and NAM in PBMCs from active RA patients through NMNATs and NAMPT. To gain insight into the intracellular mechanisms associated with the up-regulation of NAD+ levels mediated by the NAD+ boosters, we conducted in vitro mechanistic studies using inhibitors of NAD+ biosynthesis enzymes of the salvage NAD+ pathway, such as NMNATs (tannic acid) and NAMPT (FK866) (Supplementary Figures 2A and C, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42528).

The inhibition of NMNATs with tannic acid significantly blocked the up-regulation promoted by NR and NAM (Supplementary Figure 2B, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42528), highlighting the role of these enzymes in the generation of NAD+ promoted by both boosters.

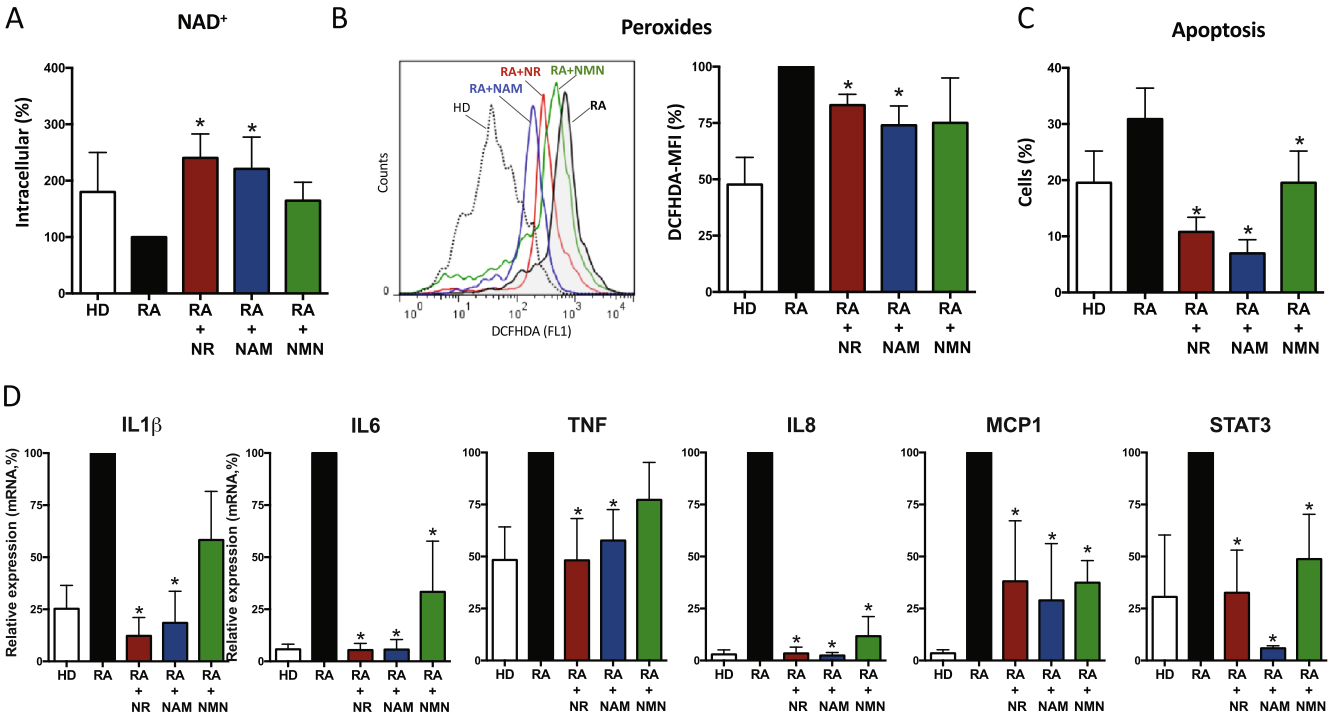

Figure 4. Effects of NAD+ boosters on the oxidative, apoptotic, and inflammatory status of PBMCs from active RA patients. **A**, Intracellular NAD+ levels in PBMCs from RA patients with active disease after the treatment with 1 m*M* of nicotinamide (NAM), nicotinamide riboside (NR), or nicotinamide mononucleotide (NMN) for 24 hours. **B**, Peroxide levels of PBMCs from RA patients treated with NAD+ boosters using dichlorodihydrofluorescein diacetate (DCFH-DA) through flow cytometry. **C**, Spontaneous apoptosis level of RA PBMCs treated with NAD+ boosters using the Annexin V-PE kit through flow cytometry. **D**, Relative expression levels of inflammatory mediators associated with the pathogenesis of RA by RT-PCR after the treatment of RA PBMCs with NAD+ boosters. * = $P < 0.05$. IL = interleukin; MCP-1 = monocyte chemotactic protein 1; see Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42528/abstract.

The inhibition of NAMPT with FK866 significantly reduced the generation of NAD^+ promoted by NAM, whereas the induction by NR was not affected (Supplementary Figure 2D, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42528). These findings are consistent with the specific role of NAMPT in the prior processing of NAM in the NAD^+ biogenesis pathway.

Reduced secretion of inflammatory mediators of PBMCs from active RA patients by NR and NAM, with enhancement by connexin 43 blockade. To evaluate in depth the antiinflammatory effects of NR and NAM, we analyzed the secreted inflammatory proteomes of RA in PBMC. We also tested the blockade of connexin 43 with GAP19 in combination with NAM and NR, as a novel strategy to further increase intracellular NAD^+ levels by reducing transmembrane NAD^+ efflux.

The up-regulation of the NAD^+ levels promoted by NR and NAM was significantly enhanced by treatment with GAP19 (Figures 5A and D). The strong antiinflammatory effects of NR and NAM were identified, as evidenced by the significant down-regulation of global levels of inflammatory mediators in the supernatant of PBMC cultures from RA patients (Figures 5B and E). The specific signature of the secreted inflammatory mediators modulated by NR and NAM revealed that crucial RA cytokines, such as TNF, IL-1, and IL-6, were the most down-regulated for both NR and NAM, whereas other cytokines with antiinflammatory properties, such as IL-4, IL-5, and IL-13, were up-regulated (Figures 5C and F).

Remarkably, the antiinflammatory effects of NR and NAM in PBMCs from RA patients were further enhanced in cells treated with the connexin 43 blocker GAP19, which agrees with the up-regulation of NAD^+ levels (Figures 5B and C and Figures 5E and F).

Although the number of inflammatory mediators significantly modulated by NR and NAM was distinctive, a specific signature of 33 secreted proteins was commonly regulated by both of the NAD^+ boosters. Interestingly, an enrichment analysis of this common profile showed that they were specifically associated with RA and key pathways involved in its pathophysiology, such as chemokines IL-17, TNF, and NF-κB signaling (Figure 5G). We were able to confirm experimentally that the activity of NF-κB was significantly reduced after treatment of PBMCs from RA patients with both NR and NAM, suggesting a potential mechanism associated

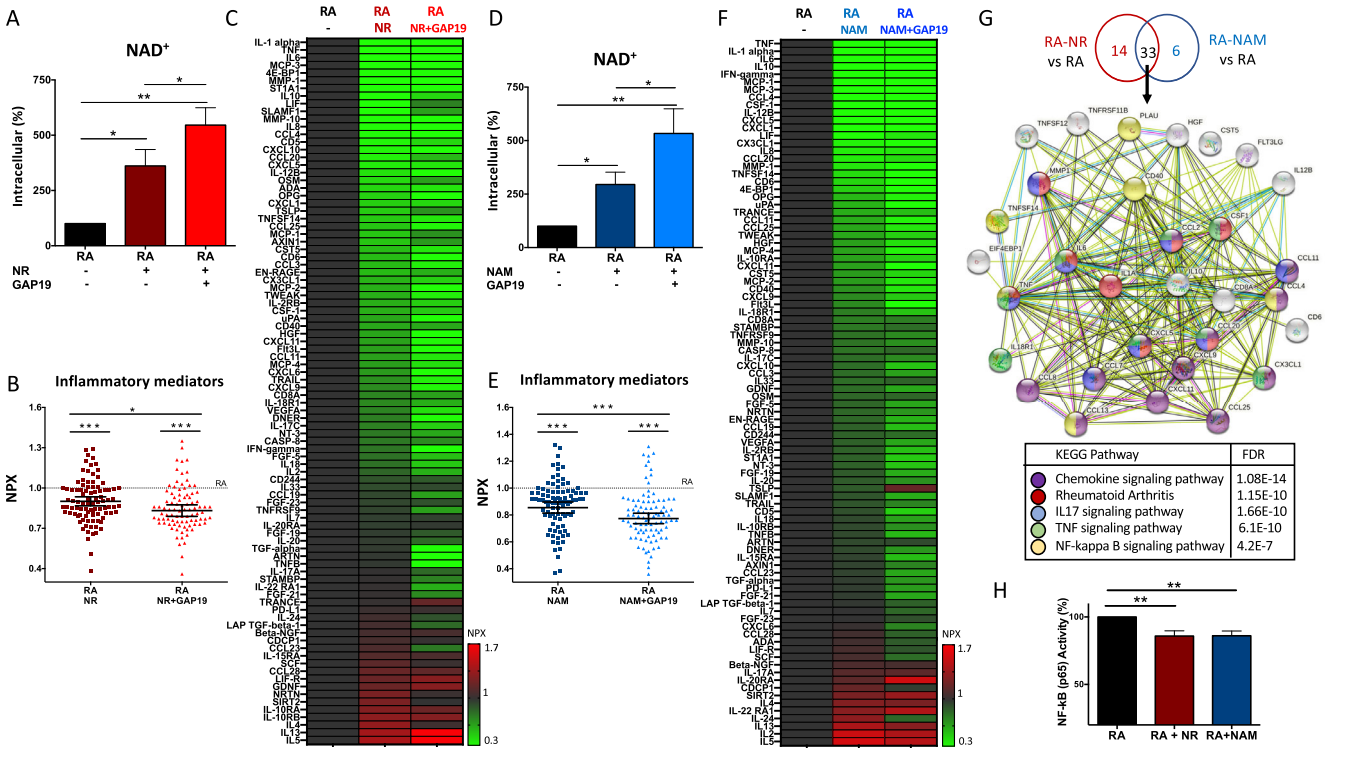

Figure 5. Antiinflammatory combined effects of NAD^+ boosters and GAP19 at protein level. **A** and **D**, NAD^+ levels of PBMCs from RA patients treated with 1 m*M* of nicotinamide riboside (NR) (**A**) or nicotinamide (NAM) (**D**) and 100 μ*M* of Gap19, the blocker of connexin 43, for 24 hours. **B** and **E**, Reduction of the global inflammatory profile in the supernatant of the cultures after treatment with NR (**B**) or NAM (**E**) and GAP19, analyzed with the "Proximity Extension Assay" (Olink). Horizontal lines and whiskers (**B**, **E**) represent the mean ± SD. **C** and **F**, Heatmap showing the individual changes promoted by NR (**C**) or NAM (**F**) and GAP19 in the secreted levels of inflammatory mediators. **G**, Comparative analysis of the proteins significantly modulated by NR and NAM and functional enrichment analysis (KEGG). Network of the common signature modulated by both boosters was obtained through STRING platform. **H**, NF-κB activity in PBMCs treated with NR and NAM. *= $P < 0.05$. NPX = normalized protein expression; IL = interleukin; see Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42528/abstract.

with the antiinflammatory effects of these NAD+ boosters (Figure 5H).

To gain more insight about the antiinflammatory properties of these compounds in the immune system, we conducted separate studies on monocytes and lymphocytes from active RA patients. In lymphocytes, both NR and NAM increased the intracellular levels of NAD+, with NR appearing to be slightly more effective (Supplementary Figure 3A, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42528). In parallel, both boosters reduced the secretion levels of several proinflammatory mediators, with NR having a significantly greater impact on the inflammatory profile than NAM in this cell type (Supplementary Figures 3B and C, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42528). On the other hand, in monocytes, both boosters also increased intracellular NAD+ levels (Supplementary Figure 3D) and reduced the secretion of several proinflammatory markers. However, in this cell type, NAM showed higher antiinflammatory effects than NR (Supplementary Figures 3E and F).

Despite the demonstrated efficacy of NR and NAM in increasing NAD+ levels and reducing proinflammatory markers in lymphocytes and monocytes from RA patients, each booster demonstrated distinct modulation profiles in each cell type.

Finally, we aimed to explore the antiinflammatory effects of these NAD+ boosters in an opposite group of RA patients, that is, RA patients in remission with low disease activity. We observed that both boosters were able to increase intracellular NAD+ levels in the PBMCs of these patients (Supplementary Figure 4A, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42528), which was consistent with a significant decrease in the secretion of several proinflammatory mediators (Supplementary Figures 4B and C). Notably, although the level of modulation of each proinflammatory marker differed between the groups of patients with high and low activity, we found that TNF, IL-6, and IL-1, which play crucial roles in the development and progression of RA, were the most effectively down-regulated proteins by NAD+ boosters in both groups of patients (Supplementary Figure 4C).

DISCUSSION

We have identified for the first time an alteration in NAD+ metabolism in RA patients that is directly associated with the inflammatory status of the disease and can be reversed by anti-TNF therapy. Furthermore, our results suggest that restoring NAD+ levels with NAD+ boosters as a potential new antiinflammatory clinical strategy for these patients.

We found that NAD+ and NADH levels were significantly reduced and correlated in the plasma of a large cohort of RA patients and healthy donors. Our data are in line with previous studies that analyzed the metabolomic profile of RA patients in plasma, which identified alterations in NAD+ metabolites associated with the clinical features of the disease (18,19). For example, tryptophan levels were significantly reduced in the serum of RA patients with active disease (20). Moreover, the reduction of NAM levels in the synovial fluid of RA patients has also been demonstrated and shown to be negatively correlated with disease activity (21).

The decreased levels of NAD+ appeared to be linked to the up-regulation of genes involved in its consumption, such as PARPs, SIRTs, CD38, and CD157, as well as the down-regulation of genes involved in the NAD+ biosynthesis, such as NMNATs (13). The levels of PARPs, SIRTs, and CD38 have also been shown to be up-regulated in other inflammatory conditions, and their roles as key modulators of immune and inflammatory processes have been extensively documented (22). In line with these findings, we also confirmed the increased activity of NAD+-consuming systems in PBMCs from RA patients.

The expression of SIRT in RA is controversial due to potential variations in different conditions, cell types, and locations (23,24). Nevertheless, our findings are consistent with previous research that has shown an up-regulation of SIRT1 in RA synovial tissues, which is promoted by TNF in vitro and correlates positively with proinflammatory mediators (25–27). This up-regulation may represent an adaptive response to counteract the damaging effects of oxidative stress and inflammation in RA.

Furthermore, we observed in RA patients an up-regulation of connexin 43 expression, which is the hemichannel that regulates the transmembrane NAD+ efflux (28), potentially contributing to the altered NAD+ metabolism. Previous studies have also reported an increase in connexin 43 expression in synovial tissue of RA patients, and in vitro experiments have shown that TNF can up-regulate its expression. Moreover, the inhibition of connexin 43 has been demonstrated to reduce the release of proinflammatory cytokines induced by TNF (29). In fact, the silencing of connexin 43 in a mice model of RA reduced inflammation and joint destruction (30). Hence, blocking connexin 43 has been proposed as a promising therapeutic target for inflammatory diseases (31).

Another relevant gene in NAD+ metabolism that was overexpressed in PBMCs from RA patients was NAMPT, previously known as visfatin (32). NAMPT is the rate-limiting enzyme in the conversion of NAM to NAD+ (33). The increased expression of NAMPT in PBMCs from RA patients might suggest a compensatory effect of the biosynthetic system to increase NAD+ levels and counteract the up-regulation of the genes involved in its consumption. Interestingly, NAMPT is also up-regulated extracellularly in RA and acts as a proinflammatory cytokine in the serum of these patients. Thus, it has also been postulated as a potential therapeutic target in RA (34,35).

The overall coordinated alteration of genes associated with NAD+ metabolism in PBMCs was also externally validated in whole blood of RA patients using public data sets, which reinforced and supported our findings.

We also identified the relationship between NAD^+ metabolism and the inflammatory and clinical status of RA patients. An unsupervised clustering analysis of the inflammatory plasma proteome stratified RA patients into 3 subgroups based on their differential inflammatory profile. Patients with the highest levels of inflammatory mediators had the lowest levels of NAD^+ and the highest disease activity (DAS28). Furthermore, a strong correlation was observed between inflammatory mediators and several genes involved in NAD^+ metabolism, highlighting the direct link between inflammation and NAD^+ metabolism.

Our data are in concordance with a key novel finding reported by Covarrubias et al, who identified chronic inflammation as a driver of NAD^+ decline via activation of CD38 (36). Furthermore, this novel mechanism of NAD^+ depletion via inflammation and CD38 has been recently validated in another inflammatory and autoimmune disease, such as systemic sclerosis (37,38), where a key role for NAD^+ in the pathogenesis of the disease and a main clinical manifestation like fibrosis has also been demonstrated.

Another fact that supports the role of inflammation in NAD^+ depletion is the restoration of NAD^+ levels to normal values after anti-TNF therapy, which also reduces the inflammatory and active status of the disease (tender and swollen joints, DAS28, Clinical Disease Activity Index, Simplified Disease Activity Index, Visual Analogue Scale, and Health Assessment Questionnaire). Moreover, the significant correlation found between the modulation of NAD^+ levels after anti-TNF therapy and the clinical response (changes in DAS28) suggests a key involvement of NAD^+ in the therapeutic response of RA patients. This might represent a novel mechanism of action not previously described for anti-TNF drugs and could have important clinical implications in other inflammatory diseases where this drug has a central role in the clinical management of patients (39).

NAD^+ metabolism was not only associated with the therapeutic response to anti-TNF drugs but also with the prediction of the clinical outcome at baseline. In this regard, patients who did not respond to anti-TNF therapy after 3 months according to EULAR criteria showed lower levels of NAD^+ at baseline. Similarly, we also identified in external data sets that a higher expression of key NAD^+ metabolic genes such as NAMPT at baseline was associated with a poor clinical outcome after anti-TNF therapy. These findings might provide an opportunity to explore NAD^+ metabolism as biomarkers for predicting the response of anti-TNF therapy in RA and other related diseases that use the same drug, as approximately 20–40% of patients do not respond to these costly biologic therapies (40).

Because we identified a global impairment of NAD^+ metabolism and a depletion of NAD^+ levels, which were directly associated with an active and inflammatory status, our next goal was to test mechanistically the capacity of several NAD^+ boosters (NR, NAM, and NMN) (41) to increase NAD^+ levels in PBMCs from active RA patients and modulate their activated status.

Our results showed that NR and NAM significantly increased the intracellular levels of NAD^+ and promoted the reduction of the oxidative, apoptotic, and inflammatory status of PBMCs from RA patients. However, these effects were not identified at the same level when the PBMCs from RA patients were treated with NMN, as this latter substrate did not significantly increase NAD^+ levels. This might be in line with the fact that Slc12a8, the main NMN transporter, is mainly expressed at high levels in the gut (42). In fact, NMN supplementation in a rat model of RA did not show any beneficial effects (43). Conversely, 2 studies conducted on mouse models of RA demonstrated that NAM supplementation significantly reduced the severity of the disease (44,45). These findings could be valuable in determining the most suitable NAD^+ precursors for future clinical studies in RA and related diseases.

We identified possible mechanisms associated with the up-regulation of NAD^+ with NR and NAM in RA. NMNATs were responsible for the generation of NAD^+ with NR, whereas NAMPT and NMNATs were both involved in the up-regulation of NAD^+ levels with NAM. Although we identified, for the first time to our knowledge, the intracellular mechanisms associated with the generation of NAD^+ using these boosters in PBMCs from RA patients, our findings are in line with previous studies showing that both NAM and NR are converted into NMN via NAMPT and NRKs, respectively, and then converted into NAD^+ via NMNATs (salvage pathway) (13).

The antioxidant and antiapoptotic properties of NAD^+ boosters have also been previously described in other cell types and disease contexts (46–48) and may be also relevant in RA, as processes related to oxidative stress and apoptosis have been directly associated with the pathogenesis of the disease (49,50).

The antiinflammatory properties of NAD^+ boosters have been well-documented in vitro (51,52) and in vivo (14,53) in various settings, owing to their role as a cofactor of many enzymes with immunomodulatory and antiinflammatory properties (54). However, we identified for the first time, to our knowledge, that NAM and NR have the potential to significantly decrease the intracellular expression of key inflammatory mediators associated with the pathophysiology of the disease in leukocytes from patients with active RA.

Thus, the deeper understanding obtained in this study of the full potential of these compounds in reducing the hallmark features of RA, such as oxidative stress, apoptosis, and inflammation, will allow the development of precise therapeutic strategies involving NAD^+ boosters.

To gain deeper insight into the antiinflammatory effects of these boosters in RA, we evaluated inflammatory secretomes using novel strategies to further boost NAD^+ levels in PBMCS from RA patients by inhibiting the NAD^+ transmembrane efflux through the blocking of connexin 43 with GAP19 (55). Our results supported and expanded on our previous findings, showing the remarkable antiinflammatory properties of NAM

and NR in RA leukocytes at the protein level in the culture supernatant. Whereas NAM and NR reduced the secreted levels of distinctive proteins, we also identified a common signature of 33 inflammatory mediators that were significantly enriched in biologic pathways and functions crucial in the pathogenesis of RA. Among them, we were able to experimentally validate the potential of NAM and NR to reduce NF-κB activity in PBMCs from RA patients. Although it has been previously shown that NAD^+ boost can suppress NF-κB signaling in other contexts (56–58), this is the first evidence of such suppression in immune cells from RA patients.

Treatment with NAM and NR in combination with GAP19 inhibition promoted a deeper reduction of the inflammatory secreted proteins, which was in line with a further increase in the intracellular NAD^+ levels. Thus, the inhibition of NAD^+ transmembrane efflux in RA leukocytes represents a complementary potential tool to increase and store NAD^+ intracellularly, enhancing their beneficial effects.

We also analyzed the antiinflammatory effects of NAD^+ boosters in purified immune cell types with key roles in the pathogenesis of RA, such as monocytes and lymphocytes. Although these agents were generally effective in both cell types, each booster appeared to be more effective in a particular immune cell type than the other. These new findings reinforce our previous observations in PBMCs and offer novel insights into the specific antiinflammatory capabilities of NR and NAM in different cell types. Overall, these findings highlight the unique potential of these boosters to precisely control the inflammation present in RA patients.

Because RA is a heterogeneous disease, we also tested the effects of NAD^+ boosters in patients in remission, with low disease activity. Our results validated the potential antiinflammatory effects of NR and NAM in RA patients, highlighting their shared role in modulating key proinflammatory mediators that contribute to the development of RA. Thus, the control of subclinical inflammation in patients in remission using NAD^+ boosters may be beneficial in maintaining a low disease status and reducing disease progression.

Overall, our data showed the therapeutic potential of NAD^+ boosters such as NR and NAM in RA. Recent findings have provided new insights related to the up-regulation of NAD^+ levels and the beneficial effects of these compounds in other autoimmune and immune-mediated diseases. For example, in mouse models, NAM supplementation has been shown to prevent the development of multiple sclerosis by modulating the inflammatory response and differentiation of CD4 T cells. This was achieved by decreasing the circulating levels of interferon (IFN) and IL-17 and reducing the activity of the NF-κB pathway (58,59). NAM treatment has also been shown to significantly ameliorate the course of another inflammatory disease, inflammatory bowel disease, in a dextran sulfate sodium–induced colitis model, by controlling body weight, histologic damage, and myeloperoxidase activity (60). In addition, in monocytes from lupus patients, NR was found to mediate the suppression of autophagy and attenuate the type I IFN signature, one of the main drivers of lupus pathogenesis (61). These findings suggest that NAD^+ boosters such as NR and NAM may have broad therapeutic potential for various autoimmune and immune-mediated diseases.

In a placebo-controlled, randomized, double-blind, crossover trial of older men, supplementation with NR for 21 days resulted in the activation of antiinflammatory transcriptomic signatures and decreased circulating levels of proinflammatory mediators, including IL-6, TNF, IL-2, and IL-5 (53). Multiple complementary studies and clinical trials in humans have also demonstrated the capacity of NAD^+ boosters to increase NAD^+ levels to promote beneficial effects in different contexts (53,62–64).

Given the proven safety of NAD^+ boosters from the vitamin B_3 family in clinical trials with humans and the significant antiinflammatory effects observed in our in vitro study in leukocytes from active RA patients, we may have an unprecedent opportunity to conduct clinical trials to evaluate the therapeutic potential of NR and NAM in RA patients. In addition, the positive effects of NAM demonstrated in a double-blind, placebo-controlled clinical trial with 72 patients with osteoarthritis (65), a closely related disease to RA, in which disease activity, joint flexibility, and inflammation were reduced, may support and strengthen the notion for further studies.

Taken together, our study identified a reduction of NAD^+ levels in RA patients and an altered expression of genes involved in NAD^+ biosynthesis and degradation. The alteration of the NAD^+ metabolism was directly linked to the inflammatory and activated status of the disease, which was reversed by anti-TNF therapy. The restoration of the NAD^+ levels via NMNAT and NAMPT using the boosters NAM and NR reduced the oxidative, apoptotic, and proinflammatory status of RA leukocytes. Combining NAD^+ boosters with blocking transmembrane NAD^+ efflux may represent a complementary strategy to further reduce the inflammatory secretome of leukocytes from RA patients. Thus, our findings could pave the way for the development of clinical trials to evaluate the therapeutic effects of NAD^+ boosters in patients with RA and related diseases.

ACKNOWLEDGMENTS

We thank the patients who participated in the study.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Perez-Sanchez had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Perez-Sanchez, Escudero-Contreras, Barbarroja, Moreno, Burón, González-Reyes, Collantes-Estevez, Lopez-Pedrera, Villalba.

Acquisition of data. Perez-Sanchez, Cerdó, Sánchez-Mendoza, Llamas-Urbano, Arias-de la Rosa, Pérez-Rodriguez, Muñoz-Barrera, del Carmen Abalos-Aguilera, Calvo, Ortega-Castro, Ruiz-Vilchez.
Analysis and interpretation of data. Perez-Sanchez, Cerdó, Escudero-Contreras, Collantes-Estevez, Lopez-Pedrera, Villalba.

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