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Personalized cardiovascular risk assessment in Rheumatoid Arthritis patients using circulating molecular profiles and their modulation by TNFi, IL6Ri, and JAKinibs

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ABSTRACT

Background & objectives: This study aimed to: 1) analyze the inflammatory profile of Rheumatoid Arthritis (RA) patients, identifying clinical phenotypes associated with cardiovascular (CV) risk; 2) evaluate biologic and targeted-synthetic disease-modifying antirheumatic drugs (b-DMARDs and ts-DMARDs': TNFi, IL6Ri, JAKinibs) effects; and 3) characterize molecular mechanisms in immune-cell activation and endothelial dysfunction.
Patients & methods: A total of 387 RA patients and 45 healthy donors were recruited, forming three cohorts: i) 208 RA patients with established disease but without previous CV events; ii) RA-CVD: 96 RA patients with CV events, and iii) 83 RA patients treated with b-DMARDs/ts-DMARDs for 6 months. Serum inflammatory profiles (cytokines/chemokines/growth factors) and NETosis/oxidative stress-linked biomolecules were evaluated. Mechanistic *in vitro* studies were performed on monocytes, neutrophils and endothelial cells (EC).
Results: In the first RA-cohort, unsupervised clustering unveiled three distinct groups: cluster 3 (C3) displayed the highest inflammatory profile, significant CV-risk score, and greater atheroma plaques prevalence. In contrast,

Abbreviations: B-DMARD, biologic disease-modifying antirheumatic drug; CIMT, Carotid intima-media thickness; CV, Cardiovascular; CVD, Cardiovascular disease; HD, Healthy donor; HUVEC, Human umbilical vein endothelial cell; IL6Ri, Interleukin 6 receptor inhibitor; JAKinib, JAK-STAT signaling pathway inhibitor; NET, Neutrophil extracellular trap; RA, Rheumatoid Arthritis; SJC, Swollen joint count; TJC, Tender joint count; TNFi, Tumor necrosis factor inhibitor; Ts-DMARD, targeted-synthetic disease-modifying antirheumatic drug.

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cluster 1 (C1) exhibited the lowest inflammatory profile and CV risk score, while cluster 2 (C2) displayed an intermediate phenotype. Notably, 2nd cohort RA-CVD patients mirrored C3's inflammation.
Treatment with b-DMARDs or ts-DMARDs effectively reduced disease-activity scores (DAS28) and restored normal biomolecules levels, controlling CV risk. *In vitro*, serum from C3-RA or RA-CVD patients increased neutrophils activity and CV-related protein levels in cultured monocytes and EC, which were partially prevented by pre-incubation with TNFi, IL6Ri, and JAKinibs.
Conclusions: Overall, analyzing circulating molecular profiles in RA patients holds potential for personalized clinical management, addressing CV risk and assisting healthcare professionals in tailoring treatment, ultimately improving outcomes.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease associated with a heightened morbidity and mortality from cardiovascular disease (CVD). This elevated cardiovascular (CV) risk can be attributed to several factors. Clinically, RA patients exhibit premature development of atherosclerosis, characterized by the accumulation of fatty deposits in the arteries, as well as a high prevalence of endothelial dysfunction, impairing proper blood vessel function. At the molecular level, the relationship between RA and CVD involves various underlying mechanisms including shared inflammatory mediators, post-translational modifications of peptides/proteins, and the subsequent immune responses. Furthermore, alterations in the composition and function of lipoproteins, increased oxidative stress, and endothelial dysfunction play significant roles in connecting RA and CVD [1,2].

These factors form a complex network rather than isolated compartments consisting of multiple interacting features, where bidirectional and synergistic effects can take place.

Emerging evidence has unequivocally emphasized the pivotal role of white blood cells in both RA onset and progression, alongside the heightened CV risk witnessed in individuals with this condition [3].

Individuals with RA manifest a notable absence of the co-stimulatory molecule CD28 on CD4 positive T cells. This loss of CD28 is of particular significance as it serves as the crucial "second signal" necessary for T cell activation. Moreover, the presence of elevated levels of circulating CD4+CD28null T cells not only correlates with heightened disease activity but also amplifies the risk of CV events [4–7].

RA is characterized by the expansion of B cells, which undergo differentiation into plasma cells and memory B cells, resulting in the abundant production of autoantibodies. In addition, B cells have the potential to exert influence on CV complications through the production of specific chemokines and cytokines (i.e. B cell activating factor (BAFF) and CCL7 -also known as monocyte-chemotactic protein 3-), which can contribute to the recruitment of monocytes to the myocardium, thereby precipitating heart injury [8–11].

Regarding the role of innate immunity on these processes, it has been widely demonstrated that monocytes are central players in the heightened CV risk evident in patients with RA. Their impact is intricately woven into various facets, spanning chronic inflammation characterized by the upregulation of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 [12]**.** Once infiltrated into the arterial intima, these cells undergo a transformative process—engulfing oxidized LDL and evolving into foam cells, a hallmark of early atherosclerotic lesions. Additionally, monocytes differentiate into macrophages, releasing metalloproteinases and tissue factor (TF), thereby fostering atherosclerotic plaque rupture and thrombus formation [13]. Understanding the intricate involvement of monocytes in the pathophysiology of CV risk in RA is crucial for developing targeted therapeutic strategies to mitigate these risks.

Neutrophils also play a significant role in RA pathogenesis, participating in various immune-related functions, ranging from the recruitment of other leukocytes and regulation of T-cell responses to thrombotic events and autoimmunity [14–17]. In addition, neutrophils undergo NETosis, generating neutrophil extracellular traps (NETs) with proinflammatory effects. NETosis is upregulated in RA, contributing to the inflammatory response. Furthermore, recent investigations have unveiled the proatherogenic role of neutrophils, with NETs being detected in atherosclerotic lesions in both mice and humans [18]. In this context, our recent studies have unveiled the diagnostic potential of NETosis-derived products in evaluating disease activity and atherosclerosis, as well as assessing therapeutic effectiveness in individuals with RA [19–21].

Collectively, a diverse array of immune cell dysfunctions synergistically contributes to the overproduction of inflammatory and prothrombotic mediators, oxidative stress, and heightened NETosis in RA. These dysregulated immune responses culminate in the release of these mediators into the bloodstream, leading to the exacerbation of endothelial dysfunction and organ damage. Furthermore, these RA-specific mechanisms intensify the detrimental effects of well-established CVD risk factors such as tobacco use, diabetes, hypertension, dyslipidemia, obesity, and chronic kidney disease.

In theory, effective treatments for RA should disrupt inflammatory circuits, attenuate the development of inflammation-driven atherosclerosis, and slow the progression of traditional CV risk factors [22,23]. Various inflammatory pathways, including the IL-6 and TNF pathways, and the downstream Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, have a key role in both, RA and atherosclerotic cardiovascular disease.

Several treatments are available that target these shared pathways and are used in the treatment of RA or have either been shown in clinical trials to reduce the risk of CVD [24]

From studies of targeted therapies in RA, evidence suggests that targeting certain pathways might have a more beneficial effect on cardiovascular risk than others [21–23].

ts-DMARDs such as JAK-STAT inhibitors, and b-DMARDs including IL-6 and TNF inhibitors, are two classes of medications used in the treatment RA that aim to modify the course of the disease by targeting specific components of the immune system. Regarding their effects on CV risk in RA patients, TNF inhibitors are generally considered to have a neutral or potentially beneficial effect on CV risk in RA [25]. A role for IL-6 reduction in decreasing the risk of CVD has also been demonstrated in several trials, which further showed that the magnitude of IL-6 reduction correlated with the overall reduction in major CV events [26].

Inhibition of the JAK-STAT pathways by ts-DMARDs, which have been proven in small mechanistic prospective studies to have beneficial effects on atherosclerosis [27] further impacts both, the IL-6 and TNF pathways. However, this broader outcome might also lead to mixed signals regarding its effect on CV risk.

The intricate signaling of these pathways poses a challenge as results can't be seen in isolation. Each cytokine and pathway have diverse functions, inducing chemokines that directly regulate cell migration and influence specific aspects of inflammatory responses in the synovium and vasculature [28] This complexity complicates blocking pro-inflammatory mediators, potentially leading to varied off-target effects. Additionally, the effectiveness of targeting these pathways to reduce CV risk may vary among RA patients.

The heterogeneity of both clinical and molecular profiles among RA patients complicates the implementation of appropriate treatment approaches to further prevent the development of CVD. Individual

variations in disease presentation, response to therapy, and associated comorbidities necessitate a personalized approach to RA management.

Improving risk stratification and optimizing the utilization of current medications for CV risk factors could enhance outcomes. Given the notable variations among patients, utilizing combinations of biomarkers might offer more utility than assessing them individually. Consequently, creating matrices that integrate clinical and laboratory parameters relevant to diagnosis or prognosis could aid in tailoring the most effective treatment for individual patients.

With these premises, we undertook this work to identify and characterize RA patients at high CV risk and to evaluate the effects of b-DMARDs and ts-DMARDs in these processes. Integrative biology by advanced computational analysis and mechanistic *in vitro* studies will be used to identify combinations of clinical and serum parameters underlying this process.

2. Methods

2.1. Patients

Three hundred and eighty-seven patients and forty-five HDs were included in the study (during a 48-month period) after obtaining approval from the ethics committees of the participant hospitals [Reina Sofia University Hospital (Córdoba, Spain), Marques de Valdecilla Hospital (Santander, Spain), Virgen Macarena University Hospital (Sevilla, Spain), Virgen de la Victoria Hospital (Malaga, Spain), Hospital Regional Universitario de Malaga (Malaga, Spain), Virgen de Valme University Hospital (Sevilla, Spain) and Jaen University Hospital (Jaen, Spain)]

The study encompassed three patient cohorts.

Cohort 1 comprised 208 RA patients with established disease and no prior CV events, along with 45 HD (Table 1). Treatment for this group included conventional DMARDs therapy.

Cohort 2 involved 96 RA patients who experienced CV events (myocardial infarction, cerebrovascular accident, or ischemic disease) (Supplementary Table 1).

Table 1
Clinical and molecular profiles of Rheumatoid Arthritis patients and Healthy Donors recruited to the study.

Clinical parameters	RA patients (n=208)	Healthy donors (n=45)	*p value*
Gender (Female/Male)	161 / 47	15 / 30	0,15
Age, years (mean ± SD)	54,2 ± 11,8	49,9 ± 5,1	0,067
Disease Evolution, years (mean ± SD)	10,1 ± 9,2		
TJC (mean ± SD)	6,3 ± 6,6		
SJC (mean ± SD)	4,1 ± 4,6		
DAS28 (mean ± SD)	4,1 ± 1,5		
Pathological CMIT (n, %)	50 (43%)	3 (7%)	<0.001
CV-risk SCORE (mean ± SD)	3,4 ± 6,4	1,2 ± 1,3	0,022
Smoking (n, %)	67 (34%)	11 (28%)	0,428
Arterial hypertension (n, %)	44 (22%)	1 (2%)	0,004
Diabetes (n, %)	10 (5%)	0	0,025
Hypercholesterolemia (n, %)	102 (51%)	16 (36%)	0,065
Laboratory parameters			
CRP, mg/mL (mean ± SD)	13,8 ± 23,4	1,5 ± 2,1	<0,001
ESR, mm/h (mean ± SD)	20,8 ± 16,9	7,9 ± 5,8	<0,001
ACPAs positivity (n, %)	124 (67%)		
RF positivity (n, %)	117 (64%)		
Treatments			
Corticosteroids (n, %)	144 (74%)		
Methotrexate (n, %)	103 (53%)		
Leflunomide (n, %)	75 (38%)		
Salazopyrine (n, %)	13 (8%)		
Hidroxychloroquine (n, %)	50 (26%)		

TJC inicates Tender Joint Count; SJC, Swollen Joint Count; CIMT, Carotid Intima Media Thickness; CRP, C-reactive Protein; ESR, Erythrocyte Sedimentation Rate; ACPA, Anti-citrullinated Protein Antibodies; RF, Rheumatoid Factor.

Cohort 3 encompassed 83 RA patients treated with TNFi (n=46), JAKinibs (n=20), or IL6Ri (n=17) therapy for 6 months. Their laboratory and clinical changes were evaluated (Supplementary Table 2).

The study adhered to the Declaration of Helsinki principles. All patients met the American College of Rheumatology's RA classification criteria. Patients and HD provided informed consent. HD were matched by age/sex and had no history of autoimmune diseases, atherosclerosis or thrombosis.

Blood sample collection, assessment of clinical and biological parameters (including the inflammatory profile, determination of oxidative stress biomarkers and NETosis-derived products in plasma of RA patients), and B-mode ultrasound Carotid intima-media thickness (CIMT) measurements are detailed in online Supplementary Appendix.

2.2. In vitro studies

Neutrophils and monocytes purified from HD and primary human umbilical vein endothelial cells (HUVECs) were subjected to a 12-h treatment (neutrophils) or 24-h treatment (monocytes and HUVECs)at 37 °C. with the serum from HD, the serum of RA patients that had suffered previous CV events (RA-CVD), or the serum from patients belonging to the cluster 3 (RA-C3), either in the presence or absence of TNFi (Etanercept, - European Pharmacopoeia reference standard, Strasbourg, France- 10 ug/mL), IL6Ri (Sarilumab, -Selleckchem, Planegg, Germany- 10 ug/mL,) or JAKinibs (Baricitinib, -Tocris Bioscience, Bristol, UK- 10 ug/mL). These inhibitors were added 1 h before the addition of serum.

Total RNA was purified from neutrophils and changes occurred on several molecules related to neutrophil activity (IL-1b, IL-8, CD26L, CD11b, CD66b, CD15) were evaluated by RT-PCR (Supplementary Table 3). Neutrophil elastase and nucleosome levels were quantified in culture supernatants.

Endothelial cells were harvested by trypsin treatment and cell lysates were obtained as described elsewhere. Proteome assays in cell lysates from monocytes and HUVECs were then performed by proximity extension assay technology (see online supplementary appendix for details).

2.3. Statistical analysis

Statistical analysis employed mean ± SEM or median ± IQR, determined by data distribution (Kolmogorov-Smirnov test). Student's t test or Mann-Whitney rank sum test assessed unpaired data; paired t tests, Wilcoxon matched-pairs signed rank tests evaluated paired data. Chi-square tests associated qualitative variables. Spearman's correlation test gauged correlations. Benjamini Hochberg-based false discovery rate (FDR) adjusted p-values for multiple hypothesis testing. Significance was at p-value <0.05.

To stratify patients with RA according to their molecular profile, Self-Organizing Maps (SOM) was utilized for clustering analysis and PCA for dimensionality reduction and visualization of the data. These analyses were performed using the Metaboanalyst software (https://www.metaboanalyst.ca/MetaboAnalyst/ModuleView.xhtml).

Data analyses were performed using SPSS 24.0 (IBM, Chicago, IL) and GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Unsupervised clustering analysis based on circulating molecular profiles identified three subgroups of RA patients with a distinctive cardiovascular risk

Unsupervised clustering analysis in the RA cohort distinguished 3 clusters representing different molecular profile groups with respect to the serum levels of 27 cytokines, chemokines and growth factors conforming an inflammatory signature in RA patients (Fig. 1A). Among

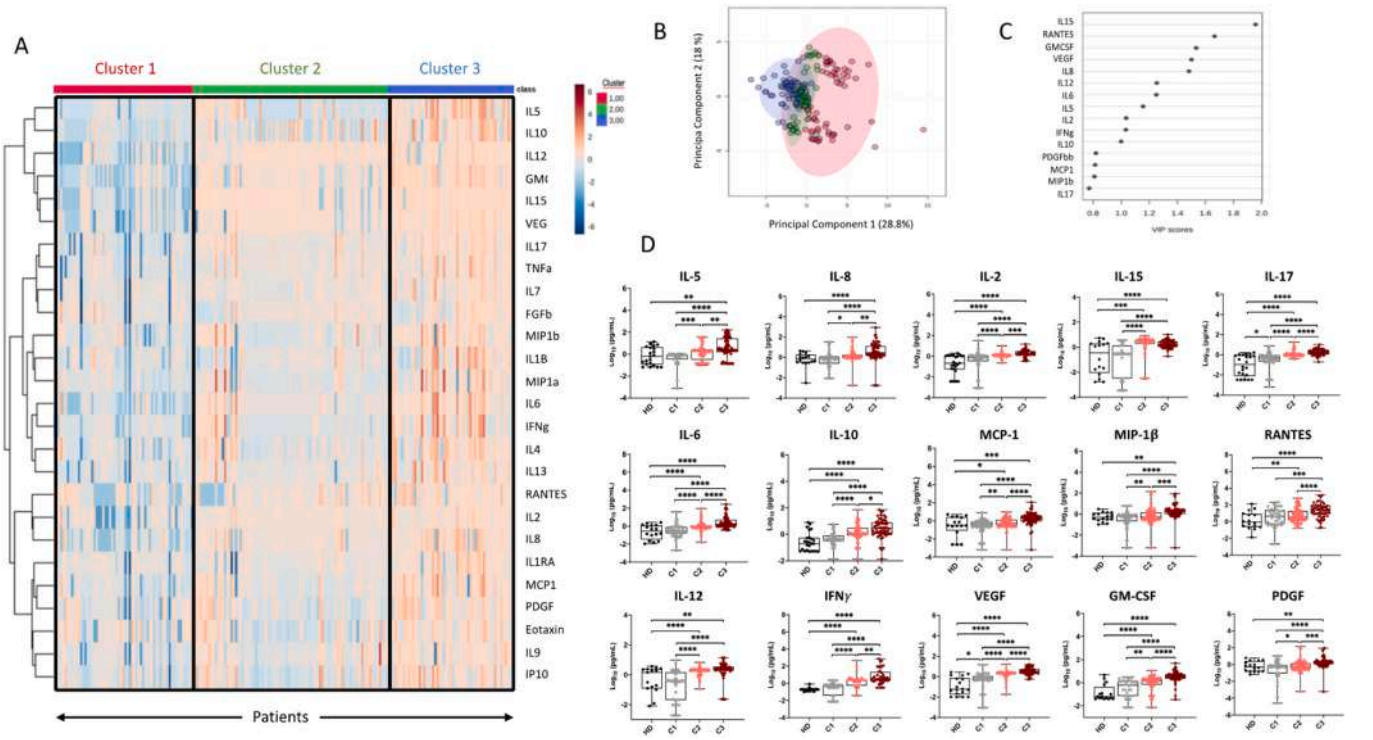

Fig. 1. Cluster analysis of circulating inflammatory proteins in rheumatoid arthritis patients. (A) Self-organization map (SOM) clustering analysis was performed using MetaboAnalyst 5.0 in RA patients belonging to the first cohort analyzed (n=208). The heatmap of the molecular profile of each cluster is depicted, showing the z-score levels of all the proteins analyzed (rows) for each individual patient (columns). (B) Principal Component Analysis (PCA) scatter plot summarizing the differences in the molecular profile of each cluster. Each point on the scatter plot corresponds to an individual sample, and the position of the points reflects their scores on the extracted components. (C) Variable importance in projection plot (VIP): proteins identified by PCA in descending order of importance. The graph represents the relative contribution of these proteins to the variance between the 3 clusters. The blue and red boxes on the right indicate whether the protein levels are increased (red) or decreased (blue) in the serum of RA patients across groups. (D) Boxplots showing comparative expression levels of selected proteins by VIP analysis among clusters and with healthy donors. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Comparison of each mediator among HD and RA patient clusters p-values were calculated using ANOVA, and all reported p-values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure to control the False Discovery Rate (FDR). Points indicate statistically significant differences after correction (FDR-adjusted p-value < 0.05).

them, patients in C3 were characterized by high levels of interleukins, chemokines and growth factors, unlike patients in C1, who showed the least accentuated inflammatory profile, and C2 patients presenting an intermediate profile (Fig. 1A).

Principal component analysis (PCA) confirmed a clear separation between these molecular clusters (Fig. 1B). Besides, to determine which proteins contribute to this discrimination, we conducted the variable importance in projection analysis (VIP score). This analysis identified IL-15, chemokine CC ligand 5 (CCL5 / RANTES), granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF) and IL-8 topping this list with a variable importance in projection score over 1.6. (Fig. 1C-D).

Correlation studies further demonstrated a strong relationship among the levels of the previously identified signature conformed by proteins showing the highest expression in cluster 3 (Supplementary Figure 1A), pointing at a coordinated regulation of their expression and involvement in similar immune pathways as demonstrated by the string analysis of known and predicted protein-protein interaction (Supplementary Figure 1B).

In parallel, increased NETs extrusion, demonstrated by enlarged neutrophil cell-free nucleosomes and cell-free elastase in plasma were also significantly increased in C3 in relation to C1 and C2 (Supplementary Figure 2 A-B). Besides, the most increased oxidative status was demonstrated in the serum of RA patients belonging to C3, involving the highest lipoperoxides levels (Supplementary Figure 2C).

Clinically, even in the presence of similar disease score (DAS28), disease evolution, and acute phase reactants, C3 (28% of the clustered cohort) identified RA-patients expressing the highest CV-risk score -assessed following the 2013 American College of Cardiology/American Heart Association (ACC/AHA) guideline on the assessment of CV risk [29], and a preponderance of atheroma plaques (Fig. 3A-C). Besides, this cluster included the highest prevalence of patients with CV risk factors such as age, arterial hypertension, dyslipidaemia, and diabetes (Fig. 3D). This observation suggests that patients in C3 not only exhibit a distinct inflammatory profile but also present a higher burden of CV risk factors, contributing to their elevated CV risk.

Conversely, RA-patients conforming C1 (30% of the clustered cohort) showed the lowest inflammatory profile and the lowest CV-risk score. Lastly, C2 (42% of the clustered cohort) characterized an intermediate phenotype (Fig. 2A).

3.2. The molecular profile of RA patients included in the higher CV risk cluster showed high similarity to the profile of an independent cohort of RA patients with previous cardiovascular events

To interrogate the relevance of the molecular signature identified in RA patients with higher CVD risk, a comparative study was carried out with a new cohort of RA patients who had suffered CV events (RA-CVD, cohort 2, Fig. 3A. Principal component analysis (PCA) revealed that patients in this new cohort clustered together with C3 from cohort 1 (Fig. 3B). Thus, RA-CVD patients presented increased levels of numerous inflammatory mediators when compared with C1 and C2, while the comparison with C3 revealed little difference in terms of inflammatory mediators' levels (Fig. 3C-E).

Accordingly, levels of both, circulating lipoperoxides and biomolecules related to NETosis were increased in this cohort of patients

A

Clinical parameters	Cluster 1 (n=63)	Cluster 2 (n=88)	Cluster 3 (n=57)	p
Gender (Female/Male)	49/14	67/21	45/12	0,921
Age, years (mean ± SD)	51,28 ± 11,28	53,9 ± 1,66	57,60 ± 11,83	0,015
Disease Evolution, years (mean ± SD)	11,27 ± 9,97	10,30 ± 8,93	9,17 ± 9,46	0,495
DAS28 (mean ± SD)	4,61 ± 1,39	4,26 ± 1,35	4,21 ± 1,31	0,236
Laboratory parameters				
CRP, mg/mL (mean ± SD)	13,41 ± 21,80	11,04 ± 12,29	9,19 ± 11,16	0,352
ESR, mm/h (mean ± SD)	20,75 ± 14,69	20,75 ± 19,19	20,46 ± 16,08	0,994
ACPAs positivity (n, %)	46 (72,5%)	52 (58,4%)	37 (64,8%)	0,210
RF positivity (n, %)	36 (57,1%)	59 (67,1%)	37 (66,1%)	0,420
Treatments				
Corticosteroids (n, %)	51 (81,5%)	55 (63,1%)	49 (85,5%)	0,117
MTX (n, %)	32 (51,9%)	53 (60,7%)	25 (44,4%)	0,423
LFN (n, %)	30 (47,3%)	27 (30,6%)	24 (41,1%)	0,093
SLZ (n, %)	3 (4,1%)	5 (5,3%)	9 (15,2%)	0,037
HXQ (n, %)	11 (17,0%)	27 (30,5%)	17 (28,6%)	0,152

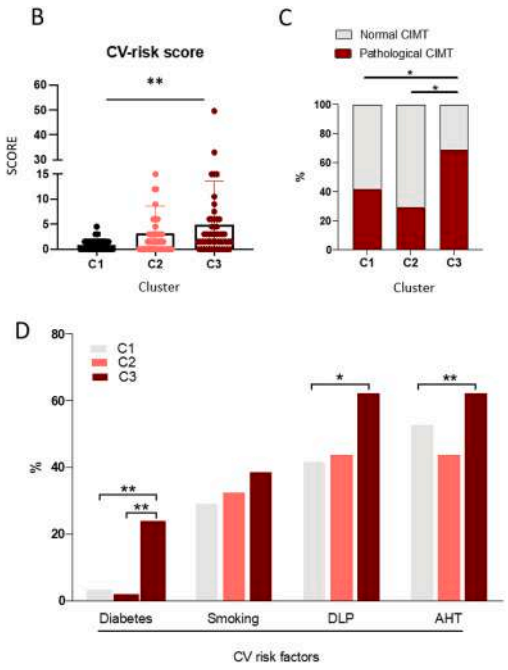

Fig. 2. Demographic and Cardiovascular risk profiles among clusters of RA patients identified by circulating inflammatory proteins levels. (A) Table of demographic and laboratory parameters of RA patients characterizing each cluster (Cluster 1 = 63; Cluster 2 = 88; Cluster 3 = 57). (B) Boxplot showing comparative CV-risk scores among the RA patients of each cluster. (C) Prevalence of pathological carotid intima-media thickness (CIMT) among the RA patients of each cluster. (D) Percentages of individual traditional cardiovascular (CV) risk factors in patients with RA belonging to the 3 clusters. CRP indicates C-reactive Protein; ESR, Erythrocyte Sedimentation Rate; ACPA, Anti-citrullinated Protein Antibodies; RF, Rheumatoid Factor; MTX, Methotrexate; LFN, Leflunomide; SLZ, Salazopyrine; HXQ, Hydroxychloroquine; CIMT, Carotid Intima Media Thickness; DLP, Dyslipidaemia; AHT, Arterial Hypertension. * $p \leq 0.05$, ** $p \leq 0.01$.

with previous CV events (Supplementary Figure 3) emphasizing the intricate interplay between both processes and CV involvement in these patients as well.

3.3. TNFi, IL6Ri and JAKinibs decreased the proinflammatory and NETotic parameters associated with CV risk induction

Therapy of RA patients with either TNFi, IL-6Ri or JAKinibs, promoted a substantial clinical improvement characterized by a reduction in tender and swollen joint count (TJC-SJC), acute phase reactants and Disease Activity Indexes (Supplementary Table 4 and Fig. 4A).

It also fostered a significant decrease in the expression levels of several inflammatory molecules -albeit in a specific way depending on the therapy administered- (Fig. 4B), on oxidative stress, and in NETosis bioproducts (Fig. 4C).

Interestingly, basal levels of various inflammatory molecules were found to be significantly associated with elevated CV risk factors, such as the score cardiovascular, hypertension and dyslipidemia (Supplemental Fig. 4), reflecting the ability of these therapies to modulate the molecular profile associated with enhanced CV risk in parallel to the improvement in global clinical status.

3.4. Serum from RA patients with higher CVD risk, induced the upregulation of CVD markers in monocytes and endothelial cells, which were prevented by TNFi, JAKinibs, and IL6Ri

In order to test the potential damage promoted by the circulating molecular profile of RA patients with higher CVD risk (RA-C3) on the endothelium, in vitro studies were carried out.

Inflammatory mediators present in the serum of RA-C3 patients induced endothelial damage by increasing the intracellular levels of a panel of proteins associated with an enhanced CV-risk including some cytokines and chemokines' ligands growth factors), and coagulation related proteins (Fig. 5A-B).

Interestingly, in monocytes, these inflammatory mediators present in the serum of RA-C3 patients, induced the expression of a similar panel of CV-related proteins (Fig. 5 C-D;).

These effects were further recapitulated by treatment of both HUVECs and monocytes with serum from RA patients that had previously suffered thrombotic events (RA-CVD) (Supplementary figure 5).

Treatment with Biological and ts-DMARDs prevented, in both cell types, the altered expression of promoted by RA-C3 in several interleukins, chemokines and proteins related to atherosclerosis and thrombosis, along with the intracellular pathways associated Nevertheless, the molecular effect was specific for each drug, so that TNFi, IL6Ri and JAKinibs were able to decrease different sets of molecules down to a HD-like level (Fig. 5 E-HSupplementary Tables 5 and 6), among which only eight were commonly reduced by the three inhibitors in endothelial cells (CCL3, CTSL1,CSCL1, GAL-9, HB-EGF, IL6, PGF, SORT1) and in monocytes (GLO1, IDUA, IL16, PARP1, TRAIL-R2, SORT1, STK4 AND TM).

3.5. Serum from RA patients with higher CVD risk induced NETosis and neutrophils activation, which can be prevented by TNFi, JAKinibs, and IL6Ri

Similarly, the impact of the altered circulating molecular profile of patients with higher CVD risk on key immune cells involved in CVD processes like neutrophils was also tested in vitro.

Treatment of HD-neutrophils with serum of RA patients belonging to C3 induced the expression of several activation markers (Fig. 6A-B). Interestingly, serum from C3 also promoted NETosis, identified by fluorescence microscopy (Fig. 6C), as well as by enhanced levels of NETosis bioproducts' in the supernatant of cell cultures (Fig. 6D).

As evident in both HUVECs and monocytes, the aforementioned effects were consistently replicated when neutrophils were subjected to treatment with serum obtained from RA-CVD patients (Supplementary Figure 6).

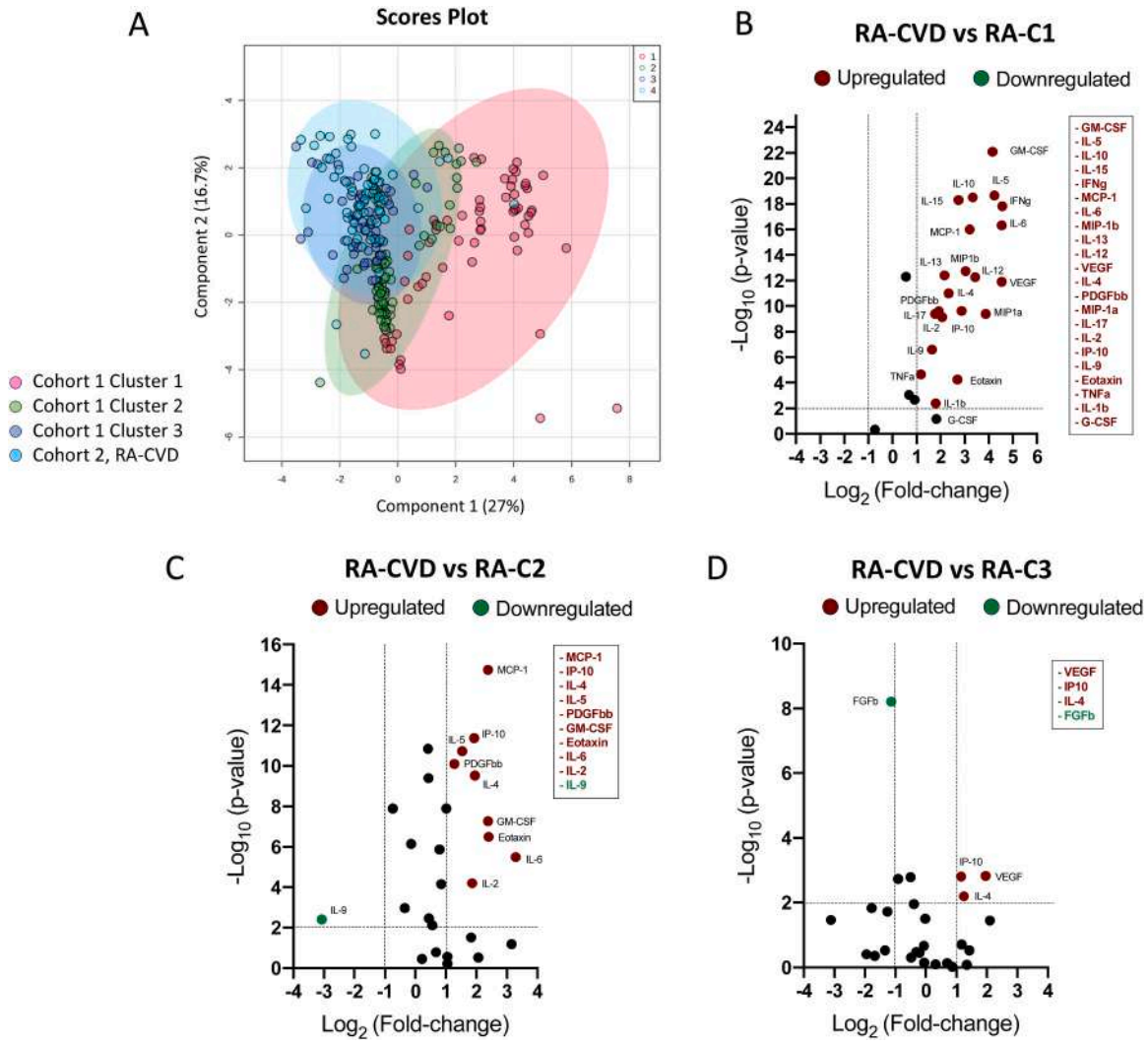

Fig. 3. Relationship among the molecular profiles of RA patients with or without previous cardiovascular events.- (A) Principal component analysis (PCA) summarizing the differences among the protein profiles RA patients with previous CV events and those RA patients without CV events grouped into three clusters (non CVD-RA) encompassing the first RA cohort. (B-D) Volcano plots where the log2 (Fold Change) of total differentially expressed proteins are plotted against the –log10 (p-value) of the Fisher's Exact Test to assay differentially expressed proteins between RA-CVD patients and non-CVD RA patients belonging to cluster 1 (B), cluster 2 (C) and cluster 3 (D). Significative differences in molecular expression levels between the RA-CVD cohort and each individual cluster are pictured in red (upregulated in RA-CVD) or green (downregulated in RA-CVD), whereas non-significative differences are pictured in black. TJC indicates Tender Joint Count; SJC, Swollen Joint Count; CIMT, Carotid Intima Media Thickness; CRP, C-reactive Protein; ESR, Erythrocyte Sedimentation Rate; ACPA, Anti-citrullinated Protein Antibodies; RF, Rheumatoid Factor.

These effects were prevented by the preincubation of neutrophils with either TNFi, JAKinibs or IL6Ri.

4. Discussion

Remarkable advances have been made in our understanding of the intricate mechanisms linking CVD to RA, alongside their interplay with traditional CV risk factors. Nevertheless, identifying the optimal strategies for preventing and treating this complex condition continues to pose a challenge. By refining risk stratification techniques and maximizing the potential of existing medications to target CV risk factors, we hold the potential to significantly improve patient outcomes [30].

In our current study, we made an innovative discovery by identifying distinct CV risk subgroups among a diverse cohort of RA patients. Through comprehensive analysis of clinical and molecular profiles, including prothrombotic and proinflammatory proteins, oxidative stress markers, and NETosis bioproducts, we gained valuable insights into the unique characteristics of each subgroup. Additionally, we investigated the impact of b-DMARDs and ts-DMARDs on these biomarkers, shedding light on the specific changes induced by these treatments' interventions.

Firstly, through the application of unsupervised clustering analysis to the molecular profiles of a diverse cohort of RA patients, we successfully classified them into three distinct groups. These groups exhibited contrasting inflammatory, oxidative, and NETotic profiles. Notably, the identification of these patient groups was not based on parameters conventionally associated with the pathophysiology of the disease, such as disease activity scores, levels of acute phase reactants, or the presence of ACPAs or RF autoantibodies.

However, when examining parameters specifically associated with CVD, we discovered that molecular profiles effectively distinguished RA patients with distinct CV risks. Particularly, within the identified subgroups (C1, C2, and C3), there was a noteworthy disparity in the prevalence of pathological CIMT. C3 exhibited a significantly higher percentage of patients (70%) with pathologic CIMT compared to C2 and C1, where the figures ranged from 30% to 40%. Furthermore, when assessing the CV risk using the SCORE calculation for each patient

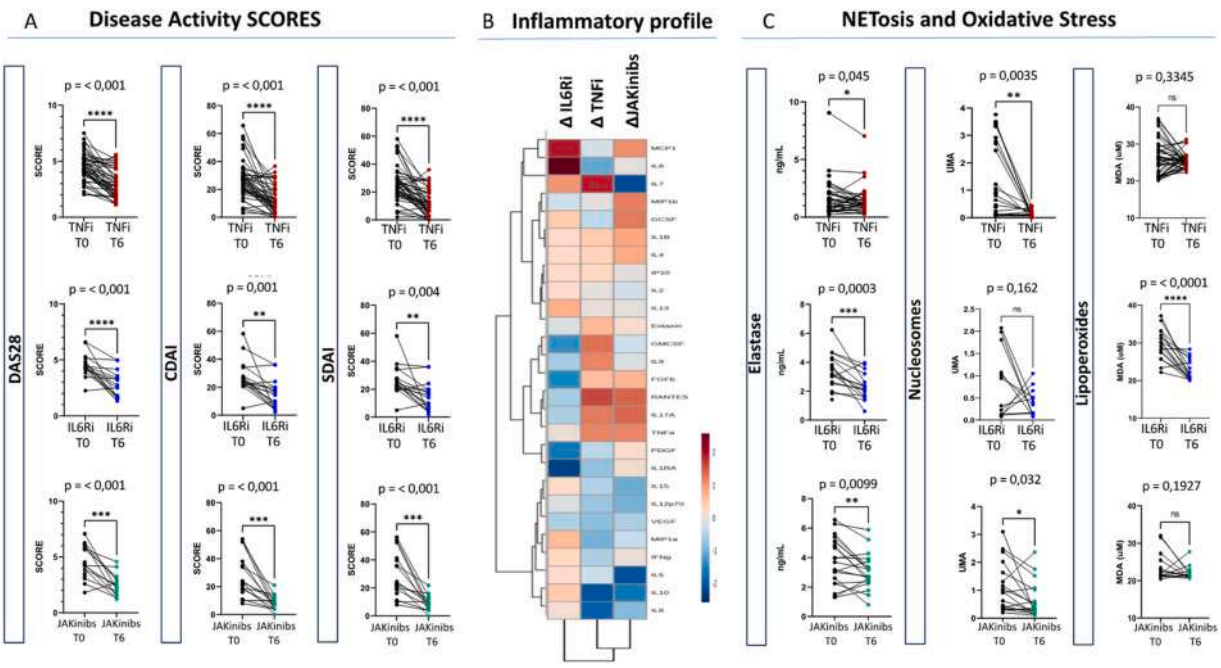

Fig. 4. Changes in clinical and molecular profiles of rheumatoid arthritis patients treated with b-DMARDs or ts-DMARDs. (A) RA patients' (n = 83) changes in clinical features at 6 months of TNFi, IL6Ri and JAKinibs therapies, including Disease Activity Score (DAS28), Clinical Disease Activity Index (CDAI), and Simple Disease Activity index (SDAI). At the initial timepoint (t0), each data point corresponds to an individual patient, with a subsequent connection established to the respective measurement obtained at the 6-month interval. (B) Heat map showing differential levels of circulating inflammatory proteins in plasma of patients with RA after 6 months of TNFi, IL6Ri and JAKinibs therapies (Δ T6-T0). Levels of inflammatory proteins are expressed as log 2,and have undergone a clustering analysis to aid interpretation. (C) RA patients' changes in NETosis bioproducts (elastase and nucleosomes) and lipoperoxides after 6 months of TNFi, IL6Ri and JAKinibs therapies. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

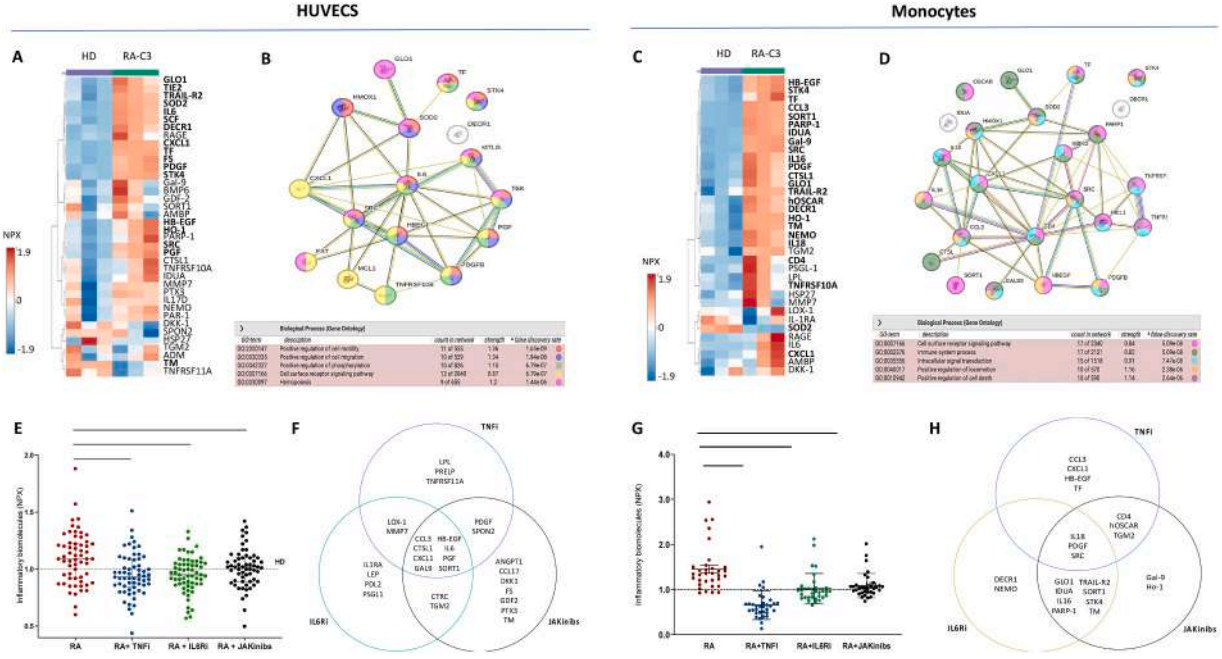

Fig. 5. Serum from RA patients with high CVD risk induced the upregulation of CVD-related markers in endothelial cells (ECs) and monocytes, which was prevented by biological and targeted synthetic DMARDs.- (A and C) Heat map showing the changes promoted in CV- related proteins on endothelial cells (A) and monocytes (C) treated in vitro with serum from RA patients belonging to cluster 3 (showing the highest inflammatory profile) in comparison with serum from HD. Levels of inflammatory proteins are expressed as NPX (normalized protein expression, arbitrary units on a Log 2 scale), and have undergone a clustering analysis to aid interpretation. Proteins showing significant alterations (FDR-adjusted p value <0.05) are detached in bold. (B and D) Predicted and validated protein-protein interactions among proteins potentially modulated by RA-C3 serum, using the STRING platform. Protein networks showing the relationship between differentially expressed proteins are displayed. Below are displayed tables containing functional enrichment analysis of biological processes by using the Gene ontology platform. (E and G) Changes in cardiovascular mediators' levels in cell lysates of endothelial cells and monocytes cultured with HD or RA-C3 serum, either, in the presence or in the absence of TNFi, IL6Ri or JAKinibs. *p<0.05, **p<0.01, ***p<0.001. (F and H) Venn diagram showing the common and differential proteins modulated in endothelial cells and monocytes by TNFi, IL6Ri or JAKinibs.

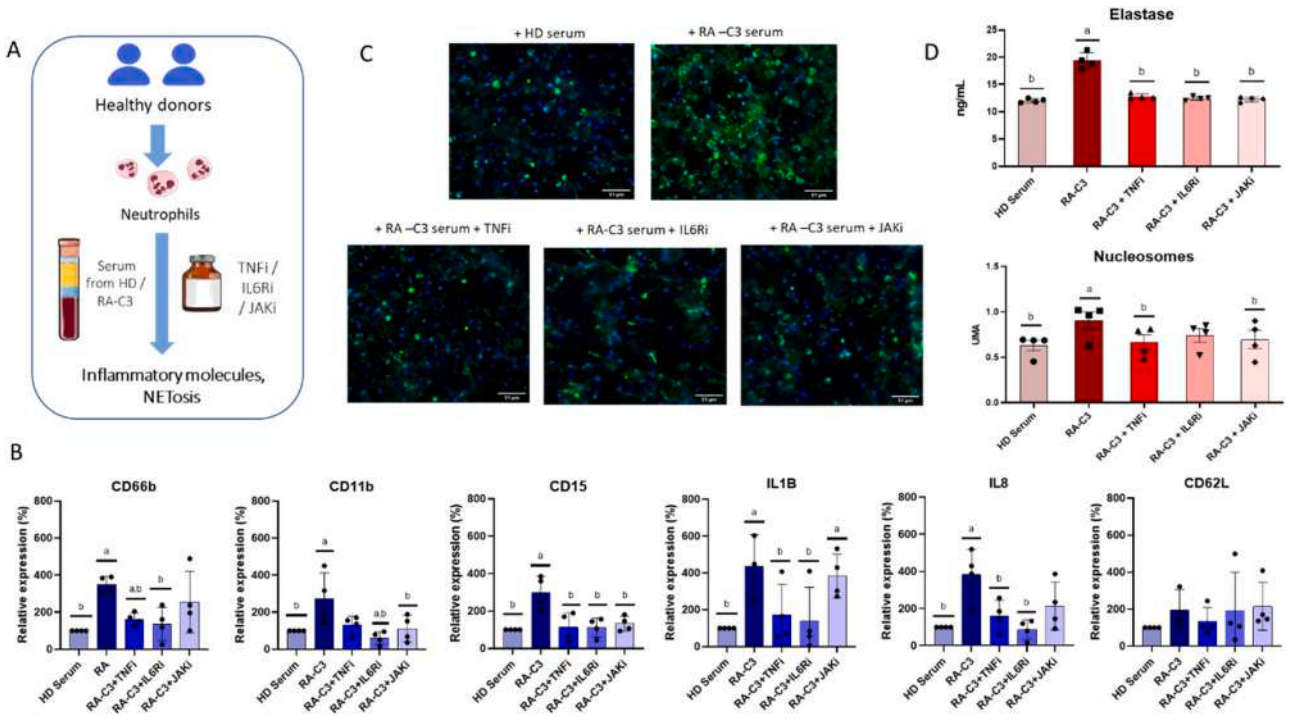

Fig. 6. Serum from RA patients with high CVD risk induced NETosis and neutrophils activation, which was prevented by b-DMARDs and ts-DMARDs.- (A-B) Bar graphs showing the changes promoted in gene expression levels of several activation markers on neutrophils treated *in vitro* with serum from RA patients belonging to cluster 3 (showing the highest inflammatory profile) either in the presence or in the absence of TNFi, IL6Ri or JAKinibs inhibitors. All experiments were compared with neutrophils treated with HD serum, set at 100% in each panel. (Significance at $p<0.05$, *a*, vs neutrophils treated with HD serum; *b*, vs neutrophils treated with RA-C3 serum). (C) Representative micrographs of neutrophil extracellular traps (NETs) from HD neutrophils treated *in vitro* with serum from RA patients belonging to cluster 3 (showing the highest inflammatory profile) either in the presence or in the absence of TNFi, IL6Ri or JAKinibs inhibitors. NETs were visualized by using a Nikon Eclipse-Ti-S fluorescence microscope 20x objective. Scale bar 10 micrometers. (D) Concentration of cell-free nucleosomes and cell-free elastase in supernatants from cell cultures of HD neutrophils performed as above described (Significance at $p<0.05$, *a*, vs neutrophils treated with HD serum; *b*, vs neutrophils treated with RA-C3 serum).

group, C3 patients displayed a statistically significant increase in CV risk compared to C1, while C2 patients exhibited an intermediate SCORE between the two groups. Consistent with these findings, the evaluation of other traditional CV risk factors, such as diabetes, smoking, dyslipidemia, and hypertension, revealed a significantly higher presence of these factors among C3 patients in comparison to both C2 and C1 patients.

Our findings also draw attention to the positive correlation observed between the levels of a specific signature of circulating inflammatory molecules in a subgroup of RA patients (those belonging to C3). This association extends to biomolecules linked to related processes such as oxidative stress and NETosis, and further demonstrates the varying degrees of CV risk among the RA patients, independent of disease activity. These results carry clinical significance, since the measurement of disease activity, which primarily considers local parameters such as the number of painful and swollen joints, may not suffice to identify an elevated CV risk.

To further support these findings, a comparative analysis was conducted using a separate cohort of RA patients who had experienced previous CV events. Notably, their inflammatory profile closely resembled that of the C3 subgroup, thus reinforcing the association between this distinct molecular pattern and CV risk.

To assess whether these CV-risk related molecular signatures are modulated by b-DMARDs and ts-DMARDs, a clinical-molecular analysis was carried out before and after 6 months of treatments in a new cohort of RA patients starting TNFi, IL6Ri and JAKinib drugs.

Firstly, we demonstrated in this RA cohort a direct relationship among increased basal levels of various inflammatory, NETotic and oxidative stress-related biomolecules and the emergence of elevated CV risk factors, including cardiovascular score, hypertension, and dyslipidemia.

Accordingly, all therapies, in parallel to the clinical improvement, fostered a significant reversion in the expression levels of inflammatory molecules -although in a specific way depending on the therapy administered-, lipoperoxides, and NETosis bioproducts.

Regarding inflammatory profiles evaluated, our data support previously reported effects of b-DMARDs (particularly TNFi and IL6Ri) on the downregulation of several cytokines and chemokines that are central for both, the activity of the disease and the increased CV risk observed in RA patients (i.e. IL-6, IL-8, IL1RA, TNFa, MIP1a, etc) [31–34].

More than 50 cytokines use the JAK/STAT pathway to orchestrate haematopoiesis, induce inflammation and control the immune response. These cytokines act primarily as regulators of the differentiation, proliferation and activation of T and B cells, macrophages, NK cells and ECs [35]. Among them, in the present study we have identified eight (IL-2, IL-5, IL-6, IL-10, IL-12, IL-15, IFNg and GM-CSF), significantly altered in relation to healthy donors, particularly in RA patients with increased CV risk.

Although the precise alterations in the secretion of cytokines and other inflammatory mediators due to JAKinibs have not yet been precisely identified in the field of RA, in a recent study, knock-out murine models for the expression of JAK1 and JAK2, the main targets of JAKinibs, demonstrated specific dysregulation of the expression of IL-2, various cytokines belonging to the IL-6 family, IFNs, IL-3, IL-5, GM-CSF and IFNgamma [36]. In line with these findings, in our study we observed a significant modulation in serum levels of several of these pro- and anti-inflammatory cytokines following JAKinib therapy.

Many reports documented that TNFi and IL6Ri have positive effects on the oxidant damage of RA, promoting reduction in serum and urinary levels of oxidative DNA damage, myeloperoxidase activity, and lipid

peroxidation, in parallel to an equivalent decrease in DAS28 [37–39]. Our results also support the reduction of oxidative stress in the serum of RA patients by effect of these b-DMARDs. Accordingly, as previously reported by some groups, including ours, NETosis was found downregulated after six months of *in vivo* treatment with both b-DMARDs and by ts-DMARDs [27].

Remarkably, this is, to the best of our knowledge, the first study that demonstrates the antioxidant effects of JAKinibs in RA patients, identified by a significant reduction, after six months of *in vivo* therapy, in serum lipoperoxides. In the same way, to date, no study has deeply evaluated *in vivo* the effects of JAKinibs on NETosis production.

Intriguingly, our findings not only demonstrated the inhibition of NETosis after *in vivo* treatment with JAKinibs, but also provided valuable insights from *in vitro* experiments using HD neutrophils treated with serum samples obtained from RA patients belonging to cluster 3. These *in vitro* investigations conducted both, in the presence and absence of TNFi, IL6Ri, or JAKinibs, further demonstrated the ability of inflammatory mediators present in the serum of these patients to promote the activation of neutrophils, in parallel to a concomitant increase in NETosis bioproducts. Importantly, all three inhibitors effectively prevented the expression of these biomolecules, thus highlighting their potential in controlling the activity of neutrophils.

These results highlight the essential role of neutrophils in the enhanced CV risk present in RA patients and support the development of therapies targeting neutrophil-mediated inflammation.

It is well known the influence of monocytes in the increased risk of CVD, being involved in chronic inflammation, a key factor in the development and progression of CVD, through the induction of atherosclerosis, plaque rupture and thrombosis [12,13].

The present study further underlined their relevance in these processes in the setting of RA. Thus, in vitro treatment of monocytes with serum from RA-C3 patients promoted a significant increase in the levels of CV-related proteins, which were prevented by effect of b- and ts-DMARDs.

All in all, overall data demonstrated that the pharmacological therapy with b-DMARDs and ts-DMARDs promotes an improvement in the inflammatory, NETotic, and redox state of RA patients, that is further related to the success of the therapy administered. Moreover, by reducing systemic inflammation, these treatments not only manage RA symptoms but also potentially mitigate CV risk. Therefore, the measurement of these biomolecules may be helpful to evaluate CV risk and therapy effectiveness.

In RA, the chronic systemic inflammatory burden is largely suspected and widely assumed to contribute to endothelial dysfunction. Existing evidence supports the direct influence of inflammation on the vascular endothelium and highlights the interplay between systemic inflammation and classic CV risk factors. This interplay further contributes to an elevated CV risk in RA patients [40–42]. Notably, primary mediators of endothelial dysfunction, including TNFα, IL-17, and various IL-1 family cytokines derived from activated leukocytes, have been identified. In response to these stimuli, endothelial cells (ECs) enhance the production of adhesion molecules, accumulate reactive oxygen species (ROS), and release chemokines, cytokines, and other factors. This cascade ultimately leads to diminished vasodilation, a proinflammatory state, and the acquisition of proliferative and prothrombotic properties.

Besides, endothelial dysfunction is an early preclinical marker of atherosclerosis, and is commonly found in patients with RA [43,44]. As such, assessments of endothelial function could prove to be a useful tool in the identification and monitoring of CV risk in RA patients [45].

To evaluate the influence of endothelial dysfunction in the increased CV-risk observed in RA, we developed *in vitro* studies on which ECs were incubated with the serum of RA patients belonging to the cluster showing the most significantly increased CV-risk (C3), either in the presence or in the absence of b-DMARDs and ts-DMARDs, and the changes occurred in a wide panel of CV-related proteins were assessed.

Through this approach, we have identified numerous biomolecules serving as mediators of inflammation, which are closely associated with an augmented CV risk. These include ligands of certain chemokines, growth factors, and coagulation-related proteins. We observed alterations in these biomolecules within endothelial cells cultured in the presence of inflammatory mediators present in the serum of RA patients. Importantly, these alterations were successfully reversed by inhibitors targeting these inflammatory biomolecules and/or those regulating the related intracellular pathways.

In summary, these *in vitro* studies demonstrated that serum from RA patients exhibiting a high CV risk, induce endothelial damage and leukocytes activation by altering the expression of key molecules associated with CVD in RA. Interestingly, these effects were recapitulated by the treatment with serum from RA-CVD patients, underscoring the imperative need for vigilant monitoring of RA patients at heightened CV risk. Such monitoring could potentially be enhanced by assessing a panel of biomolecules present in the serum, offering a valuable avenue for comprehensive evaluation and proactive management of CV health in this patient population.

The existing evidence confirms that various antirheumatic drugs can influence CV involvement in RA. This effect is especially clear for b-DMARDs, although some differences can occur across b-DMARDs with different therapeutic targets. Thus, effects of abatacept (a recombinant fusion protein selectively modulating the CD89/CD86-CD28 co-stimulatory signal required for T cell activation) have been proven to be rather neutral, so that therapy with this drug in RA is safe and not likely to increase the incidence of CV events [46]. Similarly, rituximab-induced B cell depletion seem to be associated with a strong anti-inflammatory effect that could explain the vascular protective actions of this therapy [47]. However, some B cells also have atheroprotective functions, including the production of protective antibodies [48]. Therefore, the potential beneficial effect of RTX on CV outcomes must be interpreted with caution.

Some other therapeutic options, such as glucocorticoids and other conventional synthetic DMARDS (csDMARDs), are associated with poorer CV outcomes, despite also having strong anti-inflammatory effects [30]. The off-target effects and widespread actions of these drugs, as well as their potential detrimental effects on vascular repair mechanisms, might be responsible for such outcomes.

Except for lipid changes, IL-6 inhibitors are thought to prompt similar mechanisms to TNF inhibition, including broad inhibition of inflammation and downregulation of adhesion molecules [49]. On the other hand, JAKs and some JAK-dependent cytokines are implicated in the pathogenesis of atherosclerosis, owing to their pivotal role in inflammation. Therefore, broad inhibition of various inflammatory circuits might largely account for protective CV effects of these JAK inhibitors [30].

In alignment with these findings, our current study corroborates earlier reports affirming the efficacy of certain b-DMARDs in mitigating NETosis, inflammation, and oxidative stress within the context of RA [17,19–21,50]. Notably, this investigation stands out as the inaugural study showcasing the effectiveness of Janus kinase inhibitors (JAKinibs) in reducing NETosis, oxidative stress, and a plethora of proinflammatory and prothrombotic mediators.

On the whole, despite certain specificities and likely distinct underlying mechanisms, our data suggests that key biomolecules associated with RA severity and heightened CV risk in these patients are collectively modulated by TNFi, IL6Ri, and JAKinibs. This global regulation appears to offer beneficial effects in preventing CV events.

The present study has several limitations:

– We have demonstrated a clear relationship between an increased cardiovascular risk and the presence of elevated inflammatory status in these patients, and even proven that TNFi, IL6Ri, and JAKi therapies reduce inflammation alongside improving clinical status., Future longitudinal follow-up studies conducted over 5–10 years

may provide insights into the potential of these drugs to prevent CV events in RA patients.
- The present study has conducted a screening of the inflammatory, netotic, and oxidative molecules most significantly associated in previous studies with cardiovascular risk in these patients. Unsupervised multiomic studies could offer additional valuable insights to further define the cardiovascular risk in RA and how it is influenced by b-DMARDs and ts-DMARDs.
- In this study, we utilized SOM clustering to categorize patients, which is a widely accepted un-supervising clustering method for patients' stratification using molecular data. However, we cannot guarantee that patient distribution would remain exactly the same using other clustering approaches.
- Lastly, further studies are required to assess the underlying mechanisms through which DMARDs impact CV risk in these patients, beyond their proinflammatory effects. These mechanisms are likely to be complex and influenced by the patients' clinical status, presence of comorbidities, and concurrent pharmacological treatments. Therefore, personalized studies may be necessary to fully understand these mechanisms.

Thus, although new studies and broader cohorts should evaluate the effects of b-DMARDs and ts-DMARDS on the CV risk present in RA patients, this study validates previous reports and adds a mechanistic characterization of the effects on atherosclerosis mediators involving inflammatory, prothrombotic, oxidative and NETotic molecules in some of the most largely used b-DMARDS in the treatment of RA patients (TNFi and IL6Ri), as well as of relatively recent new therapeutic approaches, still not accurately evaluated, such as JAK inhibitors.

Taken together, our overall data suggest that: 1. The circulating inflammatory, oxidative and NETotic profiles of RA identified patients' subgroups with enhanced CV-risk, not associated with their disease activity status. 2. The molecular profile exhibited by patients' subgroups with heightened CV-risk closely mirrored that of RA patients that have suffered CV events. 3. *In vivo*, TNFi, IL6Ri and JAKinibs restored normal levels of circulating pro-inflammatory proteins, mitigate NETosis, and alleviate oxidative stress biomolecules, reducing CV-risk in RA. 4. *In vitro* studies unveiled that RA-serum inflammatory mediators induced secretion of numerous proteins involved in atherothrombosis in monocytes and endothelial cells, along with NETosis in neutrophils, which were prevented by effect of both, b-DMARDs and ts-DMARDs' therapy.

Based on these findings, it is evident that analysing the circulating molecular profiles of RA patients can contribute significantly to enhancing personalized clinical management and addressing their CV risk. This knowledge can guide healthcare professionals in tailoring appropriate treatment strategies and interventions to optimize the overall care of RA patients in relation to their CV health.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116357.

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