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IDENTIFICATION OF BIOMARKERS INVOLVED IN THE RESOLUTION PHASE OF INFLAMMATION: A TRANSLATIONAL STUDY OF SPECIALIZED PRO-RESOLVING MEDIATORS ROLE IN RHEUMATOID ARTHRITIS

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PhD thesis

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SOMMARIO

SCOPO DELLO STUDIO: l'Artrite Reumatoide (AR) è una patologia cronica autoimmune in cui la disregolazione del sistema immunitario innato e adattativo comportano un danno a livello articolare ed extra-articolare, conducendo alla disabilità. Ad oggi, grazie all'implementazione dell'armamentario farmacologico, sempre più pazienti riescono a controllare la patologia e a raggiungere lo stato di remissione sostenuta. Tuttavia, ancora poco si conosce dei meccanismi sottesi alla risoluzione dell'infiammazione e, in particolare, al ruolo degli Specialized Pro-Resolving Mediator (SPM), mediatori lipidici che potrebbero avere un ruolo nella patogenesi e/o come futuri bersagli di terapie dell'AR. Lo scopo di questo studio è stato quello di identificare gli SPM e i loro recettori ERV1, ALX/FPR2 e BLT1 come possibili biomarkers solubili e tissutali nell'AR, utili per la stratificazione dei pazienti e il miglioramento della gestione della strategia terapeutica. Scopi secondari, inoltre, sono stati quelli rivelare nuovi possibili aspetti patofisiologici dell'AR in remissione.

METODI: 68 pazienti affetti da AR (27 naïve-ai-trattamenti, 23 non-responsivi-ai-DMARDs e 18 in remissione sostenuta) sono stati arruolati nello studio e sono stati sottoposti al prelievo di sangue periferico (PB) e biopsia della membrana sinoviale (ST) eco-guidata (n = 48). 13 pazienti con artrite infiammatoria periferica indifferenziata (UPIA) e 9 affetti da osteoartrosi (OA) sono stati arruolati come gruppi di confronto. Per ogni paziente arruolato sono state raccolte le caratteristiche demografiche, cliniche, immunologiche ed ecografiche. La determinazione delle concentrazioni sieriche di citochine e chemochine (IL-1beta, TNF-alfa, IL-6, IFN-gamma, IL-12p70, IL-10, IL-4, IL-2, Chemerina e GAS6) è stata eseguita mediante metodica ELISA. Inoltre, le determinazioni di molecole pro-infiammatorie derivate dal metabolismo dell'acido arachidonico (AA) e degli SPM sono state eseguite mediante metodica LC-MS/MS su campioni di ST congelata nelle due fasi di malattia, AR in alta attività di malattia e in remissione sostenuta. L'espressione di ERV1, ALX/FPR2 e BLT1 nei linfociti CD45+CD3+ e CD45+CD19+ è stata valutata mediante metodica FACS su PB e sospensione cellulare derivata dalla ST digerita meccanicamente ed enzimaticamente. Inoltre, l'espressione di ERV1, ALX/FPR2 e BLT1 è stata valutata mediante FACS solo su cellule NK (CD45+CD3-CD19-CD56+), neutrofili e monociti CD45+CD14+ su PB e macrofagi (CD45+CD11b+CD64+) su ST. Il grado di sinovite è stato valutato attraverso uno score semiquantitativo, determinato dalla colorazione eosina-ematossilina della ST. Alcuni campioni di ST sono stati utilizzati per quantificare l'espressione genica di ERV1, ALX/FPR2 e BLT1 mediante RT-PCR.

RISULTATI: l'infiammazione della ST in termini di score semiquantitativo e dei livelli sierici di citochine e chemochine rispecchiava lo stato di attività di malattia nell'AR. L'analisi RT-PCR su ST ha rivelato che nell'AR ad alta attività di malattia i recettori degli SPM erano maggiormente espressi rispetto al quadro di remissione sostenuta e rispetto all'OA (ERV1: 4,4 vs 1,1 (p= 0,012) e 1,2 (p= 0,005); ALX/ FPR2: 4,9 vs 1,5 (p= 0,0006) e 0,8 (p= 0,003); BLT1: 5,9 vs 1,6 (p= 0,016) e 1,1 (p= 0,002) rispettivamente). Inoltre, all'analisi FACS, i livelli sierici di proteina C-reattiva (PCR), correlavano direttamente con l'espressione di BLT1 nelle cellule CD45+CD14+ nel PB (r=0.27; p=0.023) di RA indipendentemente dalla fase di malattia. Al contrario, nel ST in corso di remissione sostenuta, l'espressione di BLT1 risultava estremamente bassa nelle cellule CD45+CD3+ rispetto alle altre condizioni (OA p=0,017; UPIA p=0,002; RA naïve-ai-trattamenti p=0,01). L'analisi LC-MS/MS nella ST ha mostrato che in stato di remissione il rapporto tra SPM e molecole pro-infiammatorie derivate da AA è aumentato se confrontato allo stato di elevata attività di malattia (101,3 vs 2153,00 (84.06-3333.00) rispettivamente).

CONCLUSIONI: l'espressione dei recettori SPM nei compartimenti PB e ST è reciprocamente correlata all'attività della malattia per tutte le fasi dell'AR. Questo suggerisce un ruolo di attiva modulazione nel mantenimento dello stato di remissione.

ABSTRACT

OBJECTIVES: Rheumatoid Arthritis (RA) is a chronic autoimmune disease in which uncontrolled inflammation lead by cells from innate and adaptive immune system leads to tissue damage and disability. To date, the wider pharmacological armamentarium significantly increased the chance of disease control and sustained clinical remission achievement in RA. However, little is known about the mechanisms involved in the resolution phase of inflammation in rheumatic diseases as well as the possible role of Specialized Pro-resolving Mediators (SPMs) as putative pathogenetic and/or therapeutic targets. The aim of this translation study was to dissect whether SPMs and their receptors ERV1, ALX/FPR2 and BLT1 might act as soluble or tissue biomarkers in RA useful for patient stratification across disease phases in clinical practice, improving the therapy management. Moreover, the secondary outcome was wider aiming to increase our knowledge about RA pathophysiology of remission status in.

METHODS: 68 patients with RA (27 naïve-to-treatment, 23 DMARDs-not-responder and 18 in sustained clinical and ultrasound remission respectively) were enrolled in the study and underwent PB drawing and ultrasound-guided ST biopsy (n=48). 13 patients with undifferentiated peripheral inflammatory arthritis (UPIA) and 9 with osteoarthritis (OA) were enrolled as comparison groups. Demographic, clinical, immunological and ultrasonographic features were collected for each patient. Determination of serum cytokines and chemokines concentrations (IL-1beta, TNF-alpha, IL-6, IFN-gamma, IL-12p70, IL-10, IL-4, IL-2, Chemerin and GAS6) were performed by ELISA. Furthermore, SPMs and Arachidonic Acid (AA) derived pro-inflammatory molecules determinations in snap frozen synovial tissue biopsies from RA patients in different disease phases (active and remission respectively) were performed by LC-MS/MS. Expression of ERV1, ALX/FPR2 and BLT1 in $CD45^+CD3^+$ and $CD45^+CD19^+$ was assessed by FACS on PB and on synovial tissue-derived cell suspensions. Moreover, ERV1, ALX/FPR2 and BLT1 expression was assessed by FACS on NK cells ($CD45^+CD3^-CD19^-CD56^+$), neutrophils and monocytes ($CD45^+CD14^+$) from PB and macrophages ($CD45^+CD11b^+CD64^+$) from ST only respectively. Synovitis degree was determined using a H&E based semiquantitative score. Some ST samples were used for quantification of ERV1, ALX/FPR2 and BLT1 genes expression by RT-PCR.

RESULTS: Synovial tissue inflammation in terms of semiquantitive score and the cytokine milieu in peripheral blood directly mirror the disease Activity status in RA. RT-PCR on ST samples revealed that ST from RA in high disease activity was enriched of SPM receptors when compared to RA in sustained remission and OA (ERV1: 4.4 vs 1.1 ($p= 0.012$) and 1.2 ($p= 0.005$); ALX/FPR2: 4.9 vs 1.5 ($p= 0.0006$) and 0.8 ($p= 0.003$); BLT1: 5.9 vs 1.6 ($p= 0.016$) and 1.1 ($p= 0.002$) respectively). In particular, C-Reactive Protein (CRP) serum levels, directly correlated with BLT1 expression on PB-derived $CD45^+CD14^+$ cells ($r=0.27$; $p=0.023$) of RA regardless to the disease phase. Conversely, ST of RA in sustained remission was depleted of BLT1 in $CD45^+CD3^+$ cells compared to other conditions (OA $p=0.017$; UPIA $p=0.002$; naïve-to-treatment RA $p=0.01$). LC-MS/MS analysis revealed that synovial tissue of RA in sustained remission the ratio between SPM and AA-derived pro-inflammatory molecules is significantly increased when compared to synovial tissue of RA patients with high disease activity (101.3 vs 2153.00 (84.06-3333.00) respectively)

CONCLUSIONS: SPM receptors expression in PB and ST compartments are reciprocally related to disease activity across disease phases in RA suggesting a putative active modulatory role in maintaining the remission phase.

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1. INTRODUCTION

Rheumatoid Arthritis is a chronic autoimmune disease where an uncontrolled innate and adaptive immune system causes damage to joint and extra-joint tissues. In this scenario, the interaction between genetic and environmental factors leads to the abnormal activation of the immune system contributing to the loss of immunological tolerance. It is conceivable that an impairment of inflammation/resolution balance may contribute to the persistence of tissue inflammation and might play a key role in chronic inflammatory diseases, as in RA in which, despite effective pharmacological treatments, some patients may present subclinical residual synovial tissue inflammation.
To date, little is known about the mechanisms involved in the resolution phase of inflammation in rheumatic diseases as well as the possible role of Specialized Pro-resolvin Mediators as pathogenetic and/or therapeutic targets.

The translation study presented in this thesis is the result of the work within the SYNGem Biopsy Unit at the Division of Rheumatology at Fondazione Policlinico Universitario Agostino Gemelli IRCCS (Rome)

The first part of this work (Chapter 2 and 3) is dedicated to the introduction of Rheumatoid Arthritis and the resolution mechanism involved in acute and chronic inflammation. The second part (Chapter 4, 5 and 6) describes the study project and its results.

2. RHEUMATOID ARTHRITIS

2.1 EPIDEMIOLOGY

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects nearly 0.5%–1% of the world population. It is characterized by synovial tissue inflammation with generally symmetrical polyarticular involvement. This inflammation leads to pain and stiffness and can result in progressive joint damage, deformities, and loss of function. RA is associated to organ damage having extra-articular manifestations whose development contributes to severe disability.

RA occurs twice as often in women compared with men, with a prevalence of 1.06% in women (as a percentage of the total population) compared to 0.61% in men (1). The incidence of RA increases with increasing age in most populations until about the eighth decade of life, when it declines. The incidence peaked earlier for women than men at about ages 55–64 years for women compared with 75–84 years for men (2).

2.2 PATHOGENESIS

The few last decades of research have dramatically increased our knowledge of RA pathogenesis and disease course (**Fig. 1**). Nowadays, it is possible to identify a preclinical phase of the disease preceding any clinical symptom, during which the loss of immunological tolerance is promoted by different trigger factors. Among them, the complex interplay between the innate and adaptive immune system has a critical role in the onset and perpetuation of synovial tissue inflammation in RA (3). The presence of autoantibodies in the sera of asymptomatic individuals up to 10 years before the onset of clinical disease clearly supports the notion that the clinical manifestation of the disease represents an already advanced step in the disease course. In this context, the genetic background characterized by markers able to predict the onset or severity of RA suggests that these biological processes might be operational throughout an individual's lifetime (4). The association with the human leukocyte antigen (HLA)-DRB1 locus, called the "shared epitope", was demonstrated in multiple populations, mainly in RA patients seropositive for rheumatoid factor (RF) or anticitrullinated peptide antibodies (ACPA) (5). To date other risk alleles have been identified in ACPA-positive RA patients, functionally linked with the immune regulation including T cell stimulation, activation and functional differentiation (i.e., PTPN22 and CTLA4) (6-8). Studies of gene-environment interaction showed that smoking and other forms of bronchial stress are linked to increased risk of RA development in HLA-DR4 carriers (9).

Moreover, smoking and HLA-DRB1 alleles synergistically increase the risk to have ACPA (10).

Among the environmental factors, a high body mass index has been studied as having a potential role in RA development and outcomes (11, 12). The association between an excess of white adipose tissue (WAT) and RA onset and progression may be explained by the fact that WAT is an active endocrine organ, playing a role not only on metabolism but also on immune and inflammatory processes by releasing several adipokines and proinflammatory mediators (13-15). Recently, Qin et al. reviewed the published data, concluding that being overweight or obese is associated with a significantly increased risk (1.15 and 1.31, respectively) of RA development (13). This association was even stronger among women diagnosed at younger ages. Finally, data from three different cohorts of patients seem to indicate that obesity is associated with a likelihood of developing a seronegative RA, with a relative risk between 1.6 and 3.5 (16-18). Obesity itself seems to influence disease phenotype also in terms of activity and severity, even if few data are available at the earliest stage of the disease. Controversial data provided by randomized controlled trials and observational studies were found on the association between obesity and inflammatory parameters or disease activity in early RA at the time of diagnosis, despite obese patients characterized by more severe disease in terms of Health Assessment Questionnaire (HAQ), pain, and global health assessment (14, 19, 20). The association between obesity and worse disease activity and disability was also observed in cohorts of patients with established RA (21).

Other factors as epigenetic changes can contribute significantly. Epigenetic mechanisms are potentially heritable and regulate gene expression without DNA sequence changes, able to remodel chromatin leading to activation or silencing gene transcription. Additional epigenetic mechanisms are microRNA (miRNA), which are posttranscriptional regulators of gene expression by binding to complementary target mRNA leading to their degradation. Among them, increasing evidences have been produced demonstrating the crucial role of some miRNA species as miR-155 whose expression is aberrantly regulated in inflammatory cells of the innate (22) and the adaptive (23) immune system in RA patients mirroring the disease activity (24). In particular, miR-155 knockout mice are resistant to collagen-induced arthritis, bone damage, and autoantibody production by B cells (22). This is because of the repressive action by miR-155 on anti-inflammatory molecular as phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP-1) in macrophages and PU.1 in B lymphocytes, which is a crucial transcription factor for B cell maturation and antibody production, whose expression was confirmed to be significantly repressed in RA patients.

RA is characterized by inflammation of the synovial tissue that displays as synovial lining layer hyperplasia, sublining infiltration with mononuclear cells, increased vascularity, and fibrin deposition. These features can be very heterogeneous among RA patients from pauci-immune or diffuse infiltrate till the formation of lymphoid structures within the synovium (follicular synovitis) (23). Synovial tissue inflammation (synovitis) occurs when leukocytes infiltrate the synovial compartment because of an increase of their migration within the tissue. Inflammatory cells migration is enabled by endothelial cells activation in synovial vessels which are increased in number and show an overexpression of adhesion molecules and lead the release of cytokines and chemokines (25).

Several inflammatory cells of the innate immune system are found in the synovial membrane of RA patients, including macrophages, mast cells, and natural killer cells. Among them, macrophages play a central role in promoting synovial tissue inflammation through the release of inflammatory cytokines (tumor necrosis factor alpha (TNF-alpha) and interleukin (IL) 1, IL-6, IL-12, IL-15, IL-18 and IL-23), reactive oxygen products, and matrix-degrading enzymes (26). Moreover, the synovial molecular microenvironment is responsible for macrophages reprogramming that can be divided into classical and alternative activation leading to pro-inflammatory and anti-inflammatory phenotypes, respectively (27). Pro-inflammatory macrophages play a crucial role in the initiation and development of inflammation by producing a large number of proinflammatory factors (i.e. IL-6, TNF-alpha, IL-12 and IL-23), by promoting the recruitment of Th1 and natural killer (NK) cells within the inflamed tissue. Conversely anti-inflammatory macrophages are usually induced by Th2 cytokines (i.e. IL-4 and IL-10) and show high surface expression of CD206 and CD163. In particular, recently Alivernini et al. characterized macrophages phenotypes in synovial membrane, defining two major groups based on MERTK expression. $MerTK^{pos}$ synovial tissue macrophages were described as a sources of inflammation-resolving lipid mediators and repair response inducer of synovial fibroblasts in vitro. Moreover, a low proportion of $MerTK^{pos}$ synovial tissue macrophages in remission was associated with increased risk of disease flare after treatment cessation (28). Similarly, Croft et al. identified two distinct fibroblast-like synoviocytes (FLSs) subsets within the FAPα+ population implying in the pathogenesis of RA: FAPα+ THY1+ immune effector FLSs located in the synovial sub-lining, and FAPα+ THY1- destructive subgroup restricted to the synovial lining layer. In particular, FAPα+ THY1- FLSs selectively mediate bone and cartilage damage with little effect on inflammation, whereas FAPα+ THY1+ FLSs were associated to a more severe and persistent inflammatory arthritis, with minimal effect on bone and cartilage (29).

Even neutrophils, which are abundantly found in the synovial fluid of RA patients, act as crucial cells in the promotion of synovial tissue inflammation, releasing IL-8, which enhances tissue homing of macrophages already in the preclinical phases of the disease, promoting the development of pain and

subclinical bone damage (30).

RA is characterized by the positivity of various antibodies as autoantibodies against citrullinated antigens. Citrulline derives from arginine by post-translational modification induced by peptidylarginine deiminases (PAD) activity. ACPA are present in nearly two-third of RA patients and are more specific than the RF for RA (31-34). In this contest, Brink M et al. analysed a wide cohort of individuals at risk of developing RA showing that the development of an immune response toward citrullinated peptides is initially restricted but expands over time to induce a more specific response, with autoantibodies levels increasing during the predating time period closer to the onset of clinical symptoms (35). In addition, years before RA onset, antibodies positivity is associated with inflammatory cytokines deregulation (36, 37) (including TNF-alpha, IL-6, IL-12p70, and IFN-gamma), suggesting that the autoimmune processes leading to clinical manifestation of arthritis is tightly related to the proinflammatory status. Therefore, ACPA role in the preclinical phase of RA seems to be limited not only to their high specificity for RA but also by their well-established association with a severe erosive phenotype (38-41). Despite autoantibodies positivity in this early period, ACPA do not cause apparent pathology because individuals at risk of RA development (ACPA and/or RF positive) do not show evidence of histologically proven synovitis in the preclinical phase (42). However, ACPA positivity fosters a wide range of systemic and local inflammatory processes as enhancement of osteoclastogenesis, osteoclasts differentiation, and bone resorption, respectively (43, 44). Indeed, Krishnamurty et al. found that ACPA, binding to osteoclast precursors, induce osteoclastogenesis (45). These findings provide the biological basis for the detection of erosive damage in asymptomatic individuals at risk of developing RA before any clinical manifestation.

The presence of autoantibodies clearly places the adaptive immune cells at the center of RA pathogenesis. The synovial tissue of RA patients contains abundant myeloid cells and plasmacytoid dendritic cells that express cytokines as IL-12, IL-15, and IL-23, HLA class II molecules and co-stimulatory molecules that are crucial for antigen presentation, loss of immunological tolerance, and T cell activation (46, 47). Moreover, there are increasing evidences about the presence of synovial T cell oligoclonality and B cell hypermutation already in the preclinical phases with increasing rate close to the disease onset suggesting the aberrant activation of the adaptive immune system as early phenomenon contributing to the disease (48-51). Despite RA being conventionally considered a type 1 helper-mediated disease, there are increasing evidences about the role of type 17 helper T cells (the so-called Th17), which represent a T cell subset that produces IL-17A and IL-22 as well as TNF-alpha (52, 53).

Macrophage and dendritic cell–derived inflammatory molecules (as IL-1beta, IL-21, and IL-23) create an inflammatory setting able to support Th17 differentiation and suppress the development of regulatory T cells (Treg). In particular, Treg cells have been detected in tissue of RA patients with impaired regulatory properties (54).

Synovial B cells are localized mainly within the synovial aggregates with the formation of ectopic lymphoid follicles that have been shown to support the production of autoantibodies (49). Moreover, it has been demonstrated that B cells undergo clonal expansion within the synovial tissue which harbours populations of expanded B cell clones mirrored in the draining lymphoid organs (lymphnodes) (51).

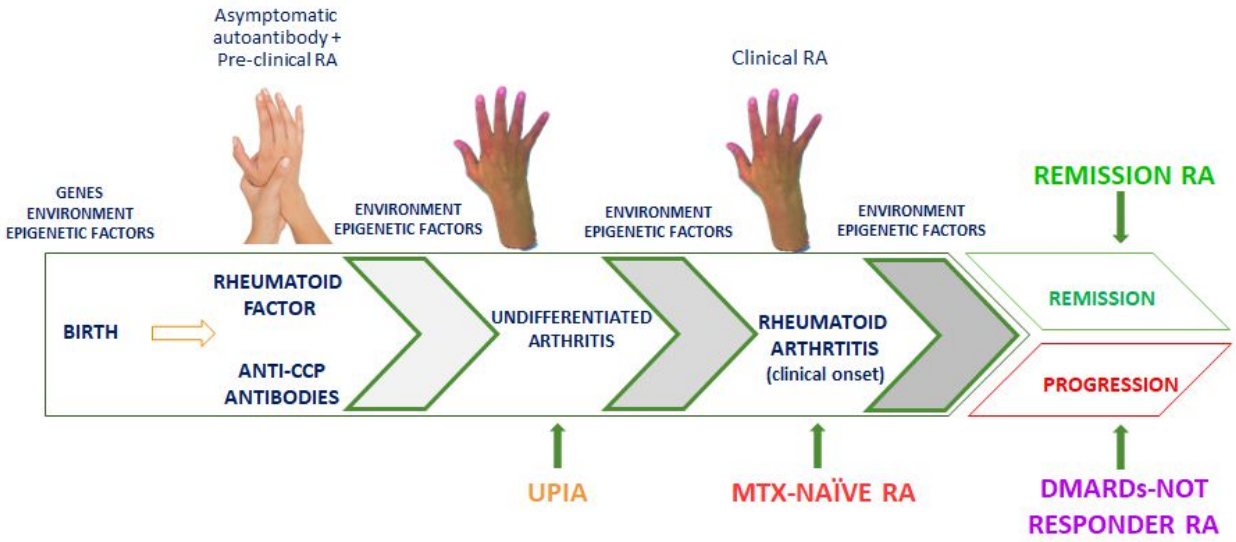


Figure 1. Schematic disease course of seropositive RA. Seropositive RA is a chronic inflammatory disease characterized by the presence of autoantibodies (as ACPA and/or RF). In genetically susceptible individual, environmental challenges (such as infection) could lead to local inflammatory changes and immune activation resulting in the loss of immunological tolerance and the generation of autoantibodies. This aberrant mechanism is under epigenetic control because of DNA and histone modifications, as well as microRNA network effect. This process can occur several years before the onset of joint disease during which the titer of autoantibodies gradually increases undergoing to epitope spreading able to result in bone loss and pain before the clinical onset of the disease. Therefore, based on the current theories, in the presence of autoantibodies and antibodies induced bone damage, minor joint challenges, which normally resolve without consequences, will instead lead to chronic inflammation. Once the disease is clinically evident, multiple immunological, genetic, and epigenetic factors play a crucial role in the determinism of disease prognosis toward clinical remission or disease progression. Modified from (55). RA: Rheumatoid Arthritis; UPIA: undifferentiated peripheral inflammatory arthritis; MTX: methotrexate; DMARDs: Disease-Modifying Anti-Rheumatic Drugs.

2.3 CLINICAL MANIFESTATIONS

RA is a chronic, systemic, inflammatory disorder that primarily involves synovial joints with symmetrical synovitis of diarthrodial joints that leads to their destruction due to bone and cartilage erosion. This process causes joint deformities, resulting in significant disability for patients. Most patients have an insidious disease, whereas 20% of patients have an intermediate onset and 10% have a sudden acute onset.

Different patterns of activity have been described for RA, with some patients having a course referred as “palindromic rheumatism” that may last for several years before the chronic, persistent RA becomes clear. Palindromic rheumatism is characterized by acute, recurrent “palindromic” oligoarthritis without bone or cartilage destruction development and generally with a favourable long-term prognosis.

One of the most common complaints of RA patients at disease onset is morning stiffness together with pain and swelling of peripheral joints. Joint stiffness is considered to be relevant if lasts more than 30 min and is markedly worse in the morning or after long periods of inactivity and requires a few hours of movement to loosen it up. The physical examination of the joints reveals tenderness of the joint sites, synovial thickening, and/or joint effusion. Generally, RA begins in one or a few joints and evolves in an additive pattern. Articular symptoms are symmetric at disease onset in 70% of cases and become symmetric within 1 year after disease onset in 85% of cases. The most commonly affected joints are the proximal interphalangeal (PIP) and metacarpophalangeal (MCP) joints of the hands and wrists, followed by the metatarsophalangeal (MTP) joints of the feet, ankles, and shoulders. RA patients can also experience tendon ruptures because of tendons rubbing against bony prominences from eroded bone, resulting in loss of extension or flexion. The most frequent site for extensor tendon rupture is at the distal end of the ulna with loss of movement of the third, fourth, and/or fifth fingers, respectively. Late manifestations of RA are anatomic disruptions of the integrity of the joint surfaces causing the visible joint deformities that subsequently lead to loss of function and disability. Moreover, ongoing inflammation can lead to nerve compression and erosions promoting carpal tunnel syndrome (median nerve entrapment) and ulnar nerve entrapment development, which can lead to motor loss and thenar muscle atrophy if long-standing.

Figure 2. Joint involvement in RA patients. (A) Ulnar subluxation of the fingers; (B) swan neck deformities; (C) limitation in extension of the elbow; (D) Z deformity of the thumb and Boutonniere deformity. Property of the Biopsy Unit of the Institute of Rheumatology – IRCCS – Fondazione Policlinico Universitario A. Gemelli – Catholic University of the Sacred Heart, Rome – Italy (55).

Polyarticular severe involvement (especially of the hands), presence of rheumatoid nodules, high-titer autoantibody, high-dose steroid treatment, longer disease duration, presence of radiographic erosions in hands and feet, and the presence of extra-articular manifestations are risk factors for cervical spine abnormalities The most frequently involved cervical segments are the atlanto-occipital and atlanto-axial (C1–C2) joints causing cervical instability due to atlantoaxial subluxation. Synovial tissue thickening can cause spinal canal stenosis resulting in cord compression (**Fig. 2**).

RA is a systemic, inflammatory disease, so systemic symptoms may also be present in these patients. The extra-articular manifestations can occur in about 40% of patients, either at the disease onset or during the disease course. Generally, extra-articular manifestations of RA are more frequently seen in patients with severe and active disease and are associated with increased mortality: in particular, RA patients are at increased risk of developing cardiovascular disease (CVD), malignancy (non-Hodgkin's lymphoma), or severe infections (56, 57). Predictors of developing severe extra-articular manifestations include clinical, serologic (autoantibody positivity), and genetic markers (double copies of HLA-DRB1*04 shared epitope alleles). Even environmental factors, such as smoking habit, are associated with early rheumatoid nodules onset and development of severe extra-articular manifestation.

In general, in up to one-third of RA patients, the acute onset of arthritis is associated with

constitutional symptoms such as prominent myalgia, fatigue, low-grade fever, weight loss and depression. Therefore, uncontrolled systemic inflammation in RA is associated with several long-term complications. Persistent elevation of inflammatory markers (erythrocyte sedimentation rate-ESR and C-reactive protein-CRP) levels and high disease activity are associated with tissue amyloidosis and organ damage. Rheumatoid nodules are the most frequent extra-articular skin manifestations (with an incidence of around 20%) and are characterized by painless subcutaneous lumps that develop most commonly on pressure areas, including the elbows, finger joints, ischial and sacral prominences, occipital scalp and Achilles tendon. At physical examination, these nodules may move easily when touched, or they may be fixed to deeper tissues. They occur mainly in autoantibody (ACPA or RF) positive RA patients and their presence may reflect the level of disease activity but can occur in cases of relatively quiescent joint disease. Moreover, RA patients with early diagnosis having rheumatoid nodules at the time of diagnosis are at an increased risk of developing more severe extra-articular manifestations. The aetiology of rheumatoid nodules is uncertain but it is believed to occur as a result of small vessel vasculitis: histologically there is a central focal fibrinoid necrosis with surrounding fibroblasts and granulomatous inflammation (58). Other manifestations of RA small vessel vasculitis may present with involvement of small- and medium-sized vessels of the skin (causing nail fold infarcts, leg ulcers, purpura, and digital gangrene) together with a progressive sensory motor neuropathy. This may lead to mononeuritis multiplex being involved in the development of severe vasculitis. Vasculitis during RA may involve mesenteric, coronary and cerebral arteries and be associated with severe eye involvement. RA patients with vasculitis are usually characterized by high serum titres of autoantibodies, cryoglobulins and low levels of complement because of its degradation. Digital gangrene and sharply demarcated ulcerations appear mostly at the lower extremities or in sites of skin pressure. Lower extremities ulcers are clinical manifestation of severe, generally in patients with long-standing disease.

Skin manifestations are frequently associated with episcleritis, pleural, and pericardial effusion. Episcleritis is a common ocular involvement of RA which causes an inflammation of the superficial layer to the sclera and it occurs in less than 1% of patients with RA and is generally a self-limiting condition. Scleritis represents a more aggressive process characterized by an intensely painful inflammation of the sclera. The most frequent ocular manifestation is keratoconjunctivitis sicca which is frequently associated with xerostomia (oral dryness) in a secondary Sjögren's syndrome with salivary gland swelling more rarely found.

RA patients may present with haematological abnormalities either at the time of diagnosis or during the course of the disease. In particular, it is possible to find anaemia, neutropenia, thrombocytopenia, thrombocytosis, eosinophilia, and haematological malignancies. Anaemia is one of the most common

extra-articular symptoms of RA and its cause is multifactorial: disease activity drug-induced, nutritional, gastrointestinal bleeding, bone marrow suppression may be due to immunosuppressive therapy and ineffective erythropoiesis. Anaemia of chronic disease observed in RA patients usually correlates with disease activity (particular the degree of articular inflammation) and is normochromic/normocytic. Active RA is associated with lymphadenopathy and also with an increased risk of non-Hodgkin lymphoma compared with the general population whose most frequent subtype is large-cell, B cell lymphoma (59).

Pulmonary involvement in RA is one of the most frequent extraarticular manifestations, although not always clinically recognized at the first clinical examination. It includes pleuritis, RA-associated interstitial pneumonitis, cryptogenic organizing pneumonia, obliterative bronchiolitis, and intrapulmonary rheumatoid nodules. Pleural disease is common but usually asymptomatic and it is detected only at routine chest X-ray assessment. Pleural effusions are usually exudates with mixed cell counts and high protein concentration with the presence of multinucleated giant cells which are highly specific for RA. The lung involvement in RA patients is frequently associated with exudative pericarditis and with interstitial lung disease. Parenchymal lung nodules generally are asymptomatic and found in autoantibody-positive patients with concomitant rheumatoid nodules elsewhere. During the disease course they can cavitate and cause pleural effusions. The clinical presentation and evolution of RA-associated interstitial lung disease seems to be similar to that of idiopathic pulmonary fibrosis, but with a better response to immunosuppression. The possibility of a superimposed respiratory tract infection must be considered in the setting of rapid progression of pulmonary symptoms in patients with suspected RA-associated or treatment-related lung involvement.

Pericarditis is one of the most common cardiac manifestations in RA, and although symptomatic pericarditis is relatively uncommon, autoptic studies revealed the presence of pericardial inflammation in nearly 50% of RA patients. It usually occurs in autoantibody-positive RA patients with nodules, and analysis of pericardial fluid reveals changes similar to those found in RA-related pleural effusions. Myocarditis (even with the presence of rheumatoid nodules within the myocardium) has been observed in autoptic studies, and myocardial fibrosis can lead to conduction abnormalities. In addition, myocarditis and endocarditis have been observed in autoptic studies with the formation of rheumatoid nodules in the aortic or mitral valves edges that can lead to valvular dysfunction. Arterial stiffness is an important factor in cardiovascular comorbidity in patients with RA and it has been observed that the decreased arterial elasticity correlates with disease severity. RA patients are also more prone to heart conditions such as the thickening of the artery walls (atherosclerosis) and heart attacks (60). Among the different factors associated with CVD development in RA patients, the

seropositivity for ACPA arose to be associated with the development of ischemic heart disease in RA. Interestingly, ACPA positivity is associated with subclinical atherosclerosis in RA patients measured as the carotid intima-media thickness (61), suggesting the pathological role of ACPA antibodies in the disruption of atherosclerotic plaques causing the release of peroxides and peroxynitrites products by neutrophils secondary to the increase of mitochondria depolarization.
Kidney involvement in RA (developing as glomerulonephritis and interstitial renal disease) is a rare extra-articular manifestation and may represent the expression of an underlying vasculitis. However, kidney function abnormalities are frequently iatrogenic in RA patients. The most common histological finding is represented by mesangial glomerulonephritis associated with variable levels of interstitial inflammation. Amyloidosis is the most common finding among RA patients with nephritic/nephrotic syndrome.

2.4 CLINICAL ASSESSMENT

Disease activity is a central aspect in the evaluation of RA patients because it evaluates, at the time of clinical assessment, signs and symptoms of the disease (i.e., inflammatory pain, swelling, and stiffness). Based on that, the reduction of disease activity is the major target of therapeutic interventions. The overall assessment of the disease activity consists of a complete set of parameters that include counts of swollen and tender joints, patient assessment of pain, patient and evaluator global assessment of disease activity, and measure of the acute-phase response.

Joint involvement is typically evaluated for soft tissue swelling and effusion (swollen joint count, SJC) and tenderness on pressure or motion (tender joint count, TJC). Several joint indices and counts have been developed differing mainly for the number of the assessed joints. The comprehensive 66/68-joint count, as suggested by the American College of Rheumatology (ACR) is time-consuming, with limited usage in clinical practice. Another index, the Ritchie Articular Index (RAI), assesses graded tenderness in 26 joint areas. Simplifications of the extensive ACR joint count have been developed over time, reducing the number of assessed joints to 28 (62), excluding the assessment of the foot and ankle joints because swelling and tenderness in these joints are frequently present in disorders other than RA.
Pain is clearly the predominant symptom in RA patients and its assessment is important in the understanding of the disease impact on patients' life. Most commonly pain is measured on 100-mm horizontal visual analog scales (VAS) on which patients are asked to estimate their feeling based on

the previous week because of the fluctuating nature of pain. As pain, the global level of disease activity can be directly rated by the patient. In addition to pain evaluation, it is often valuable to use the rating of the physician and of the evaluator on the global assessment of disease activity, both presented using 100-mm VASs.

General health/global health (GH) is a patient self-report "global measure" and it is part of the ACR core data set and a component of multiple composite indices used for RA activity assessment and treatment response. Patients are asked to estimate their health condition from 0 "very well" to 10 "very poor."

RA is a prototype of a multifaceted disease in which the evaluation of any of the available measures does not allow the clinician to reliably identify a patient's disease activity or the response to therapy. Pooled indices were developed to be used for standardized clinical assessment in clinical trials; however, they have been shown to be very useful in following patients in clinical practice in outpatient settings. The disease activity score (DAS) is derived by a complex formula which takes into account the RAI, SJC-44 (count over 44 joints), ESR, and GH. As the complex RAI for tenderness and SJC-44 are rarely used in clinical practice and trials, the joint assessment was simplified to include the 28-joint counts for both tenderness and swelling (TJC-28, SJC-28). This index, the DAS28, is calculated with a complex formula using the following parameters: TJC-28, SJC-28, ESR, and GH. Currently, there are several modifications of the DAS and the DAS28 available: one modification is the use of CRP instead of ESR (DAS-CRP and DAS28-CRP, respectively); another modification is the exclusion of GH.

To overcome the major practical limitations of the DAS-based indices, simpler indices have been recently developed. The simplified disease activity index (SDAI) was the first to include a sum of variables that were untransformed and unweighted. The SDAI, DAS, and DAS28 all require laboratory measures (CRP or ESR), which might constitute a limitation in clinical practice because it frequently prevents the immediate assessment because of waiting time for lab results (if available at all). To overcome this issue, the CDAI (clinical disease activity index), which is a simplified index, based solely on clinical measures (CDAI = SJC-28 + TJC-28 + PGA + EGA), has been validated. The CDAI allows making prompt assessment of disease activity, facilitating immediate treatment decisions.

The DAS, DAS28, SDAI, and CDAI not only allow us to determine the level of a patient's disease activity but also to categorize disease activity states as high, moderate, and low disease activity and remission. Although they are more similar in patients with severe disease activity, the remission criteria of the SDAI and CDAI are more stringent than those of the DAS28 (63, 64).

Recently an additional more stringent remission definition was created to be used in the assessment of RA patients: the Boolean remission. Based on this definition, an RA patient is classified as having reached the Boolean remission if, at any time, the following parameters are satisfied: TJC-28 and SJC-28 ≤ 1 and CRP ≤ 1 mg/dL and PGA ≤ 1 (on a 0–10 scale).

2.5 FOCUS ON REMISSION

Remission is the main goal of the RA therapy and still today it remains the real target of the RA management, preventing joint damage and disability (65). To date, the wider pharmacological armamentarium significantly increased the chance of disease control and clinical remission achievement in RA, despite the presence of many definition of remission and the different grade of remission they describe (66). In particular, Boolean remission stratifies RA patients into a deeper grade of remission than SDAI or CDAI, which in turn give a more stringent grade of remission than DAS or DAS28. The different grade of remission gives the opportunity to sort RA patients and to adequately treat them, reducing the risk of joint damage and damage progression (67).

In RA disease, remission is defined also at other levels than clinical one. In particular, US examination can give information about the presence or not of synovitis, defining by Gray-Scale analysis for the synovial membrane hypertrophy (SMH) and by Power Doppler (PD) signal expressed as PD grade for synovial vascularity. Different studies demonstrated that SMH and PD grade mirror disease activity and predict disease relapse within a short term after therapy change (68, 69). In conclusion, thanks to US examination, it can be possible to define subclinical synovitis as a condition fulfilled by remission clinical criteria but in which synovitis is present as SMH and PD signal, influencing therapeutic strategy (70, 71). Considering how many information US examination can give, to avoid any misdiagnosis or overestimation, EULAR-Outcome Measure in Rheumatology (OMERACT) defined the criteria to use for the diagnosis of synovitis (72, 73).

The definition of subclinical synovitis opened a breach in the concept of remission, pushing the rheumatologists over the clinical assessment to find a new and deeper grade of remission. In that scenario, synovial tissue evaluation acquired great importance. Different studies demonstrated that, despite clinical and US remission, some RA patients present elements of histological synovitis as the presence of lymphocytes aggregates (74-76). Furthermore, through synovial tissue and cellular analysis, Alivernini et al. demonstrated that synovial tissue macrophages (STM) are distinct in nine

discrete phenotypic clusters within four distinct subpopulations with diverse homeostatic, regulatory and inflammatory functions. In particular, two STM subpopulations ($MerTK^{pos}TREM2^{high}$ and $MerTK^{pos}LYVE1^{pos}$) present unique remission transcriptomic signatures enriched in negative regulators of inflammation. These STMs were potent producers of inflammatory resolving lipid mediators and induced the repair response of synovial fibroblasts *in vitro*. Moreover, a low proportion of $MerTK^{pos}$ STMs in remission was associated with increased risk of disease flare after treatment changing.

2.6 DIAGNOSIS

Classification criteria for RA was updated by a collaborative initiative between ACR and the European League Against Rheumatism (EULAR) in 2010 (77). The 2010 classification criteria require the presence of an active clinical synovitis (i.e., swelling) in at least 1 joint as determined by an expert assessor and the exclusion that the observed synovitis is not better explained by another diagnosis. Additional criteria (autoimmunity, acute-phase reactants, joint involvement, and symptoms duration) are then applied to eligible patients providing a score of 0–10, with a score ≥6 being indicative of the presence of definite RA.

In clinical practice, to maximize sensitivity, most rheumatologists recommend measuring ACPA together with RF because ACPA have moderate sensitivity, especially for early RA. The simultaneous evaluation of ACPA and RF provides a trade-off between overall sensitivity and specificity. Indeed, in rheumatology outpatient setting, in which the probability of RA development is relatively high, measuring ACPA and/or IgM-RF seems to be a reasonable strategy that avoids missing potentially treatable patients.

2.7 TREATMENT

Over the past several decades the treatment of RA has been revolutionized by the development of powerful biologic disease modifying antirheumatic drugs and by better understanding of how to effectively use conventional synthetic DMARDs (csDMARDs).

Several lines of evidences have been produced showing that a treatment “window of opportunity” exists in early RA, during which optimal treatment provides a better long-term outcome. A delay in the administration of DMARD therapy reduces the likelihood of achieving disease remission in RA

patients and is associated with more rapid radiological progression and worse functional outcomes.

To date, different csDMARDs are available for the treatment of RA such:

- Methotrexate (MTX): MTX is widely considered the cornerstone and the drug of first choice in the treatment of RA and may be used as monotherapy as well as in combination with other agents. MTX has demonstrated to be efficacious in the management of RA in multiple studies including both in radiographic and clinical outcomes. It is the first DMARD prescribed following the diagnosis of RA because significant percentage of patients responds favourably to it. MTX inhibits dihydrofolate reductase, an important enzyme for the synthesis of DNA mainly in actively dividing cells. MTX can be given orally or parentally in a once-a-week regimen. MTX should be started early in the disease course and rapidly increased to maximal tolerated dose during the first 3–6 months to exert its effects. Furthermore, folate supplementation is recommended with MTX therapy and reduces the frequency and severity of side effects without affecting efficacy. MTX has a favourable long-term safety profile, and withdrawal of treatment for toxicity is less common than for other DMARDs. Almost 20% of RA patients develop transient elevation of hepatic transaminases but it rarely necessitates treatment discontinuation. Myelosuppression and oral ulcers may occur, generally responding to folate supplementation. MTX pulmonary toxicity may include a hypersensitivity pneumonitis, interstitial fibrosis, pleuritis, pleural effusions, and lung nodules. Hypersensitivity pneumonitis is a rare but life-threatening complication, occurring in less than 0.5% of RA patients. Moreover, being teratogenic, MTX should be discontinued in women who are considering conception. Despite this, the most common reason for discontinuation has been shown to be lack of efficacy rather than toxicity, and retention rates for MTX are higher than those of other DMARDs.
- Sulfasalazine (SSZ): after oral administration this drug undergoes reduction by colonic bacteria into sulfapyridine and 5-aminosalicylic acid. Both derivatives act by inhibiting T cell proliferation, reducing IL-2 production, and enhancing T cell apoptosis. Moreover, sulfasalazine reduces the production of IL-1, IL-6, and TNF-alpha by monocytes and macrophages. Multiple features make it an acceptable drug to both patient and physician such as being effective in different age groups and in both RF-positive and negative RA patients. Sustained improvement data have been shown over 5–10 years for both clinical and laboratory variables of disease activity. It has been shown to be superior to hydroxychloroquine but equivalent to gold and leflunomide. The ability of sulfasalazine in reducing bone damage in RA patients was confirmed as well. It’s thought that nearly 30% of RA patients discontinue

sulfasalazine treatment because of side effect. Most of them occur within the first few months of treatment, although not all of them are serious requiring withdrawal. Gastrointestinal manifestations are common with nausea, loss of appetite, and more rarely diarrhoea. Even mucocutaneous reactions can occur such as maculopapular rashes, urticaria, and photosensitivity. Moreover, up to 3% of patients can develop leukopenia because of either lymphopenia or neutropenia. Occasionally treated patients may develop minor rises in transaminases level whereas rare is an eosinophilic pneumonia. Although sulfasalazine can be safely continued by women who wish to conceive, a reversible decline in sperm number and morphology can lead in infertility in men even if no association with teratogenicity has been described.

- Leflunomide (LFN): LFN is an antimetabolite with antiproliferative effects on T lymphocytes through the inhibition of pyrimidine synthesis. Leflunomide effectively blocks the proliferation of human lymphocytes, which are highly dependent on this pathway for nucleotide synthesis leading to the arrest in the G1/S phase of the cell cycle, interrupting the T lymphocytes clonal expansion. Moreover, LFN has been shown to inhibit the activation of NF-kB being able to block TNF-mediated NF-kB activation in a dose- and time-dependent manner with an inhibitory effect not limited to cell type, as NF-kB activation was inhibited in myeloid and epithelial cells as well as in T lymphocytes. Leflunomide also inhibits the expression of cell adhesion molecules, which facilitate cellular interactions and diapedesis within the inflamed synovial tissue. Studies on synovial tissues in RA patients receiving leflunomide showed a reduction in synovial macrophages as well as intracellular adhesion molecule and vascular cell adhesion molecule expression. The ease of administration, its efficacy, and mechanism of action make it a useful alternative to MTX in monotherapy and in combination therapy even with anti–TNF-alpha agents.

Biologic and target synthetic agents (bDMARDs and tsDMARDs respectively) represented efficacious options for patients who did not respond to or are intolerant to cDMARDs, allowing not only to improve signs and symptoms of the disease but also to prevent joint damage and future disease severity. To date, different b/tsDMARDs are available for the treatment of RA such:

- Tumor Necrosis Factor Alpha Inhibitors (TNF-in): TNF-alpha is a proinflammatory cytokine mainly produced by monocytes and macrophages involved in the normal inflammatory response. It has been found elevated in different rheumatic pathological conditions and in inflamed joints and blood of RA patients. Among its functions, TNF-alpha recruits inflammatory cells, increases vasodilation and bone damage, and stimulates the production of

prostaglandins and collagenases (78). Among them, infliximab, etanercept, adalimumab, certolizumab, and golimumab are currently available for the treatment of RA. Infliximab is a chimeric monoclonal antibody against TNF-alpha and is used to treat not only RA but also other autoimmune conditions as Crohn's disease, ulcerative colitis, psoriasis, psoriatic arthritis, and ankylosing spondylitis. Infliximab is an artificial antibody. It was originally developed in mice as a mouse antibody. To avoid immune reactions to mouse proteins, the mouse common domains have been replaced with similar human antibody domains. They are monoclonal antibodies and have identical structures and affinities to the target. Etanercept is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kDa (p75) TNF receptor (TNFR) linked to the Fc portion of human IgG1. The Fc component of etanercept contains the CH2 domain, the CH3 domain, and hinge region, but not the CH1 domain of IgG1. Etanercept is produced by recombinant DNA technology in a Chinese hamster ovary mammalian cell expression system and has indication for the treatment of RA, juvenile RA, psoriatic arthritis, plaque psoriasis, and ankylosing spondylitis. Adalimumab is a fully human monoclonal antibody against TNF. It binds specifically to TNF-alpha and blocks its interaction with the p55 and p75 cell surface TNFRs. Adalimumab also lyses surface TNF expressing cells in vitro in the presence of complement. However, it does not bind or inactivate lymphotoxin (TNF-beta). It had been approved by the FDA for the treatment of RA, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, moderate to severe chronic psoriasis, and juvenile idiopathic arthritis. Certolizumab pegol is a recombinant, humanized antibody Fab fragment conjugated to polyethylene glycol (PEG), specific for human TNF-alpha. The PEG portion is an inert molecule that increases the plasma half-life of the drug. Certolizumab is unable to activate complement or initiate antibody-dependent cellular cytotoxicity (ADCC) because it is structurally different from other anti–TNF-alpha. Golimumab is a human immunoglobulin G1-kappa monoclonal antibody that is specific for TNF-α and binds to both the soluble and transmembrane forms of human TNF-alpha. It is a fully human monoclonal antibody with the amino acid sequences of the light and heavy chains identical to those of infliximab.

- Non-antiTNF agents (Rituximab, Abatacept, Tocilizumab): both B and T cells are known to play an important role in the pathogenesis of RA. B cells role in the pathogenesis of RA is shown by the presence of RF and ACPA in blood and synovial fluid of RA patients. CD20 is a surface antigen on B cells except for stem cells, early pre-B cells, or plasmacells. Rituximab, a genetically engineered chimeric anti-CD20 monoclonal antibody, can deplete CD20 positive cells. Rituximab is able to induce depletion of B cells by different mechanisms. Fc domain of rituximab is recognized by phagocytes, as macrophages, through Fcγ receptor causing the

ADCC. Rituximab bound to CD20 on B cells can also cause the activation of complement, which eventually leads to the formation of membrane attack complex and complement-dependent lysis of B cells. To date, rituximab is approved as a therapeutic choice for RA patients who failed a course of TNF-α blockade therapy (in Italy; in other countries even approved to be used as a first-line biologic). The proliferation and the full activation of an antigen-specific T cell need a signal mediated by the specific T cell receptor and at least one co-stimulatory signal by an antigen-presenting cell. Among co-stimulatory molecules, one of the most important involves the interactions between the CD28 and CTLA4 (CD152) molecules and their ligands CD80 (B7-1) and CD86 (B7-2) on the T cell and antigen-presenting cell membrane, respectively. Abatacept is a fusion protein made by combining the external domain of human CTLA4 to the heavy chain constant region of the human IgG1. This molecule is able to bind both CD80 and CD86 with high avidity preventing these molecules from linking CD28 on T cells. IL-6 is a pleiotropic proinflammatory cytokine produced by T cells, B cells, lymphocytes, and monocytes in areas affected by inflammation, with a role in T cell activation and immunoglobulin secretion. Its signal transduction is mediated by membrane-bound and soluble IL-6 receptors. Tocilizumab is a humanized antihuman IL-6 receptor IgG1 monoclonal antibody against the IL-6 receptor. The efficacy and safety of tocilizumab monotherapy compared with MTX was demonstrated in patients with active RA. Tocilizumab is generally well tolerated and the most commonly reported adverse events were upper respiratory tract infections, nasopharyngitis, headache, hypertension, and increased transaminases.

- Janus kinase (JAK) inhibitors (JAK-in): JAK family of tyrosine kinases includes JAK1, JAK2, JAK3, and TYK2 and is required for signalling through type I and type II cytokine receptors. Once activated, JAKs phosphorylate the signal transducers and activators of transcription (STAT) that subsequently induce the expression of many genes. This pathway is involved in the pathogenesis of RA. Among them, JAK3 is predominantly expressed in cells of the immune system and plays a crucial role for signal transduction from receptors for different cytokines involved in lymphocyte activation, function, and proliferation. The first oral tsDMARDs was tofacitinib, a pan-JAK inhibitor that primarily inhibits JAK1 and JAK3. In addition to tofacitinib, other JAK inhibitor molecules have also been studied in RCTs. These molecules show different degrees of specificity toward the four JAKs. Among these new drugs as Baricitinib (an oral, selective and reversible inhibitor of JAK1 and JAK2), Upadacitinib (an oral, selective and reversible inhibitor of JAK1 and less JAK3) and Filgotinib (an oral, selective and reversible inhibitor of JAK1) were approved for the treatment of RA (55).

In 2019, EULAR published the updated recommendations for the management of RA therapy (**Fig. 3**). Briefly, in phase I they recommend to use csDMARDs (in particular MTX) as first line therapy. In case of contraindication or evidence of not-responding to csDMARDs, phase II recommend to use another csDMARDs or to pass to b/tsDMARDs (better if it is combined with csDMARDs). In case of not-responding to the first b/tsDMARDs, it is suggesting to use b/tsDMARDs with different mechanism of action (i.e. passage from b- to ts-DMARDs or vice versa or passage from TNF-in to non-antiTNF agents).

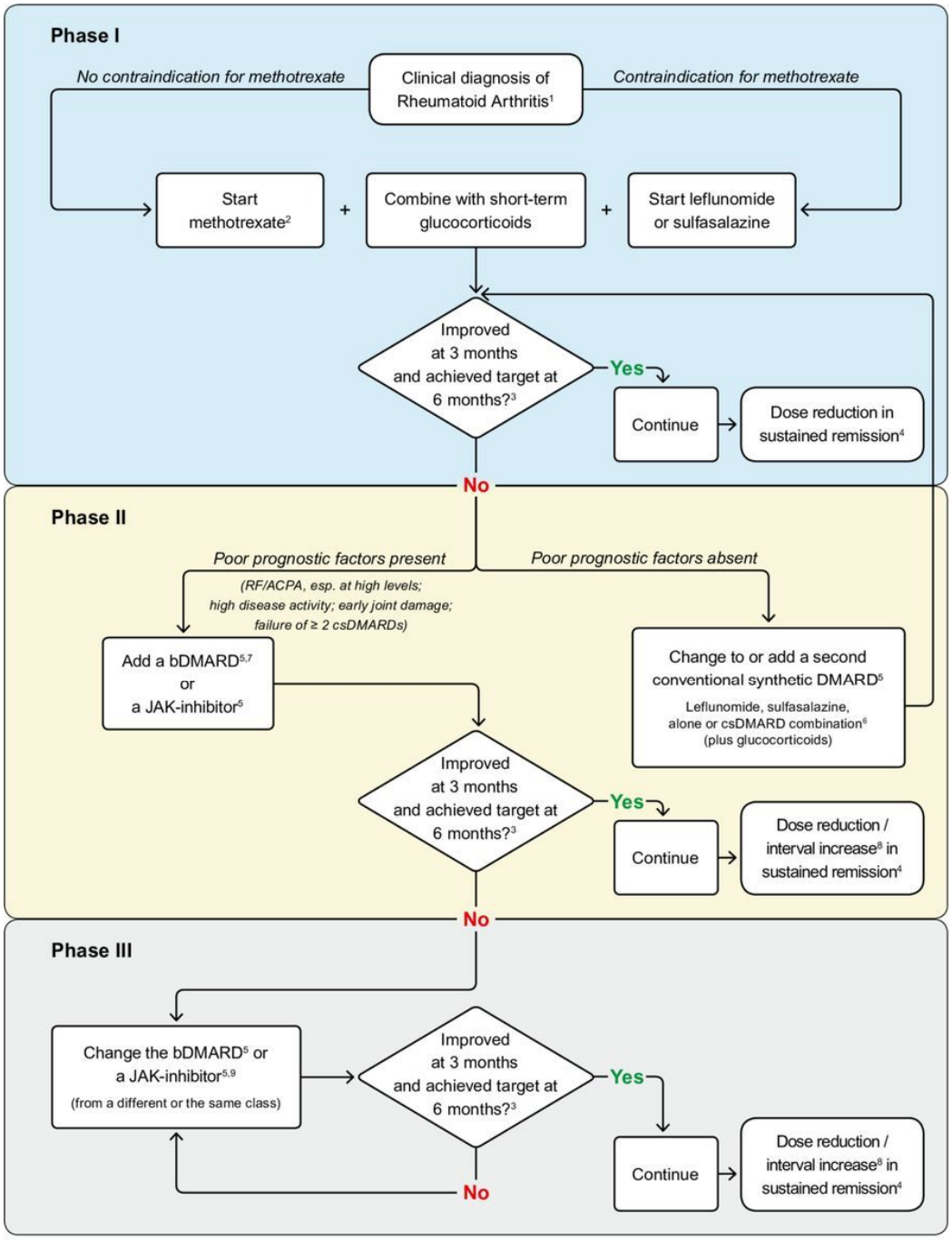


Figure 3. 2019 update of the EULAR RA management recommendations in form of an algorithm. This is an abbreviated version aiming to provide a general overview of the treatment strategy. ACPA: anti-citrullinated protein antibody; ACR: American College of Rheumatology; bDMARDs: biological DMARDs; bsDMARD: biosimilar DMARDs; csDMARDs: conventional synthetic DMARDs; Disease-Modifying Anti-Rheumatic Drugs; EMA: European Medicines Agency; EULAR: European League Against Rheumatism; FDA: Food and Drug Administration; IL-6R: interleukin 6 receptor; JAK: Janus kinase; MTX: methotrexate; RA: rheumatoid arthritis; RF: rheumatoid factor; TNF: tumour necrosis factor; tsDMARDs: targeted synthetic DMARDs (79).

3. RESOLUTION OF INFLAMMATION

3.1 RESOLUTION IN ACUTE INFLAMMATION

Acute inflammation is a physiologic defensive response against noxious stimuli and conditions, such as infection and tissue injury. Regardless of the cause, the initial phase of inflammation is characterized by an increase of pro-inflammatory molecules, with consequent leakage of blood components (plasma and leukocytes) towards the site of infection or injury (80). In the meanwhile, the resolution of inflammation starts, by which tissue repair is promoted through the clearance of apoptotic cells and debris to avoid the activation of adaptive immune system (81), and in this process Specialized Pro-resolving Mediators (SPMs) play a pivotal role.

Nowadays, it is known that the resolution phase is an active and tightly regulated program, driven by several molecules as SPMs, which are bioactive metabolites derived from omega-3 essential fatty acids (EFAs) such as Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA). Among them, Resolvin D- and E-series, Protectins, Maresins and Lipoxins (belonging to Arachidonic Acid (AA) metabolites) have been identified so far (82, 83).
The mechanisms of SPMs biosynthesis have been studied and have been summarized in details (84-87). In addition to the most studied SPMs, DHA and AA can be substrates of Cyclooxygenase (COX) 2-acetylsalicylic acid complex first then of LOX system, producing the Aspirin-Triggered (AT) epimers Resolvin D- and Lipoxin-series respectively (88, 89) (**Fig. 4**).
SPMs act on multiple immune and stromal cells by binding to specific surface receptors as, for example, E-Resolvin Receptor 1 (ERV1, also known as ChemR23 or CMKLR1) and Leukotriene B4 Receptor 1 (BLT1, also known as LTB4R) for Resolvin E1, Formyl Peptide Receptor 2 (ALX/FPR2) for Resolvin D1 and Lipoxin A4 and G Protein-coupled Receptor 32 (GPR32 also known as DRV1) for Resolvin D1. Moreover, SPMs are antagonized by many pro-inflammatory molecules at receptor level: Leukotriene B4 (LTB4) binds BLT1, Chemerin its receptor CMKLR1 and acute phase protein Serum Amyloid A (SAA) binds ALX/FPR2 respectively (85, 90-93).

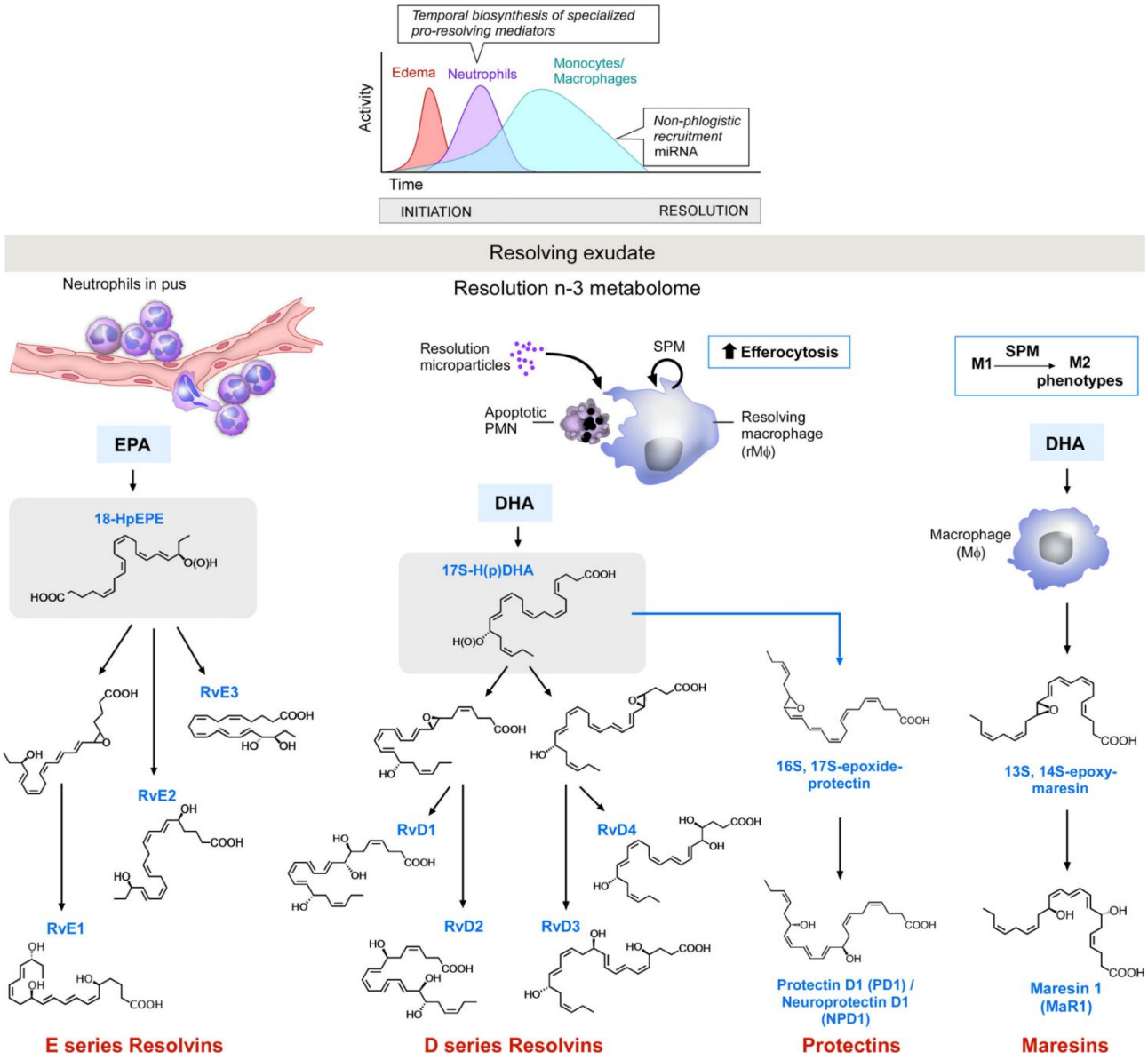


Figure 4. SPM production in resolving inflammatory exudates.
UPPER PANEL: depicts a typical self-limited acute inflammatory response time course encountered in experimental settings from initiation to resolution: oedema, neutrophilic infiltration and non-inflammatory recruitment of monocytes/macrophages. Biosynthesis of SPM occurs temporally in resolving exudates. Non-inflammatory recruitment of monocytes and macrophages is required for homeostasis, repair and regeneration of injured tissues. **LOWER PANEL:** biosynthesis of Resolvins, Protectins and Maresins from EPA and DHA with the main bioactive structures from each family (modified from (82))
EPA: Eicosapentaenoic Acid; DHA: Docosahexaenoic Acid; SPM: Specialized Pro-resolvin Mediator; Rv: Resolvin; PD: Protectin; MaR: Maresin

In particular, SPMs pro-resolving actions are dependent upon which cell type they bind, as Resolvin E1 (5S, 18R-trihydroxy-6E, 8Z, 11Z, 14Z, 16E-eicosapentaenoic acid; RvE1) binds ERV1 on monocytes/macrophages (94) and BLT1 on neutrophils (95). In monocyte-derived dendritic cells and macrophages, via releasing of intracellular Ca2+ and by inhibition of cAMP accumulation (96), RvE1/ERV1 regulates Akt phosphorylation (97), enhancing FCγR-mediated phagocytosis and

ribosomal protein S6 (directly and indirectly by ribosomal protein S6 kinase (p70S6K), activated by phosphorylated Akt), promoting the translation of ribosomal proteins mRNAs, elongation factors and growth factors (98, 99). Conversely, RvE1/BLT1 regulates the migration of leukocytes reducing LTB4-induced calcium response (RvE1-BLT1 interaction increased intracellular Ca2+, but less than that of LTB4) and attenuates LTB4-induced proinflammatory signals (90). The final effect is to enhance efferocytosis and to limit PMNs accumulation in the inflammatory site.

Resolvin D1 (7S, 8R, 17S-trihydroxy-4Z, 9E, 11E, 13Z, 15E, 19Z-docosahexaenoic acid; RvD1) binds ALX/FPR2 and DRV1 on neutrophils and monocytes/macrophages (91). The interaction of RvD1 with its receptors activating the cAMP/Activated Protein Kinase (PKA) signalling with consequent blocking of efferocytosis-induced ROS generation by inhibition of NOX2 activation in macrophages with engulfed apoptotic cells (100) and promoting cellular switching from pro-inflammatory towards anti-inflammatory macrophage phenotype (101). Therefore, these mechanisms prevent macrophage apoptosis after efferocytosis and promote inflammatory resolution. In neutrophils, RvD1 is an antagonist of LTB4, blocking LTB4-upregulated cell-surface expression of CD11b affecting the cellular migration into the inflammatory site (neutrophil recruitment) (91, 102).

Lipoxin A4 (5S, 14R, 15S-Trihydroxy-6E, 8Z, 10E, 12E-eicosatetraenoic acid; LXA4) binds ALX/FPR2 and DRV1 as well as RvD1. Although data about how LXA4 acts through its interaction with its receptors are not reported, it is known the final effect: LXA4 reduces PMNs and eosinophil infiltration (103, 104), stimulates efferocytosis (105) and regulates cytokines/chemokines production (106-108). Probably, LXA4 has a key role in the switch from arachidonic acid metabolism (with production of pro-inflammatory molecules such as prostaglandins and leukotrienes) to EPA and DHA ones. As suggested by Serhan and colleagues, SPMs act limiting PMNs recruitment to the inflammatory locus and stimulating the non-flogistic recruitment of monocytes with consequent synthesis and secretion of the SPMs (109).

Maresin-1 (7R, 14S-dihydroxy-4Z, 8E, 10E, 12Z, 16Z, 19Z-docosahexaenoic acid; MaR-1) binds BLT1 receptor (110). It is produced by resident and anti-inflammatory macrophages in the early phase of inflammation resolution (111, 112) and stimulates the clearance of apoptotic debris by enhancing macrophagic non-flogistic phagocytosis in the inflammatory site (113). On neutrophils, MaR-1 inhibits the activation of survival signal pathway while promotes the caspase-dependent apoptosis through attenuation of AKT, ERK and p38 phosphorylation (enhanced by LPS) (114).

Finally, Protectin D1 ((4Z, 7Z, 10R, 11E, 13E, 15Z, 17S, 19Z)-10, 17-Dihydroxy-4, 7, 11, 13, 15, 19-docosahexaenoic acid, PD1; previously known as Neuroprotectin D1) acts on macrophages reducing PMN infiltration as shown in a spontaneous resolving zymosan-initiated peritonitis mouse model (115). Moreover, it has neuroprotective bioactivity in human and mice brain and retinal cells against

oxidative injury, ischemia-reperfusion, with anti-amyloidogenic (by PPARγ and PPARγ-independent pathways) and anti-apoptotic potential (116-119). GPR37 has been recently identified as the PD1 receptor (120).

3.2 RESOLUTION IN CHRONIC INFLAMMATION

Chronic inflammation is the physiological answer when noxious stimuli are not removed by the innate immune cells, bringing to eliminate or isolate the cause of immune system activation. During that phase, neoangiogenesis and activation of adaptive immune cells induce the rise in the level of pro-inflammatory molecules leading to the modification of micro-environmental homeostasis and to the increase of the risk for cancer, metabolic and cardiovascular diseases development (121).

In this biological setting, the aberrant activity of the innate immune system may be consequence of synergistic effect among genetic factors promoting the exasperation of immune responses (i.e. HLA-DRB1 haplotype in RA), biochemical mechanisms that lead to the development of autoantigens and autoantibodies (like ACPA in RA) and epithelial surfaces abnormalities with the alteration of microbiota and prolonged exposure to harmful microorganisms (122).

With the discovery of SPMs, a new view about the biological mechanisms of inflammation steps was achieved. Chilton et al. reviewed how omega-3 EFAs play an important biological role and how a deficiency in their assumption leads to chronic inflammatory diseases (123). Furthermore, new evidence has been produced about the importance of a proper intake of omega-3 EFAs: in mice, an AA-rich and omega-3-poor EFA diet leads to the reduction of EPA and DHA levels in plasma, kidney and liver (124). Likewise, in knock-out Elovl2 (a key enzyme involved in the synthesis of DHA) mice, the generation of pro-inflammatory and anti-inflammatory macrophages is still permitted but the former became hyperactive and more pro-inflammatory while the latter less protective, with a reduced ability to recruit T-helper 2 and regulatory T cells increasing risk of autoimmune disease development (125). Conversely, the supplementation of DHA or EPA is able to improve the functions of the immune system with a protective role in many chronic inflammatory diseases as exhaustively described by Yates et al. (126).

The influence of omega-3 EFA and SPMs on the human adaptive immune cells still remains poorly explored. In particular, RvD1 and its precursor 17-HDHA, but not PD1, increase IgG and IgM production and the antibody-secreting phenotype B cells (CD27+CD38+) (127) but reduce IgE production by suppressing class switching (without interfering with IgG and IgM synthesis) via stabilizing the transcriptional repressor B-cell lymphoma 6 (128). Moreover, in T Lymphocytes, EPA

and DHA inhibit the activation of Protein Kinases C (PKC), with decrease in nuclear translocation of NF-κB and inhibition of IL-2 production. Moreover, the impairment of IL-2 synthesis by EPA and DHA is also associated with the reduction of other cytokines as TNF-alpha or IL-4 but not IFN-gamma in T helper cells (129, 130). In particular, RvD1 and MaR-1 influence the activity of the existing and activated Th1 and Th17 cells, preventing their generation from naïve CD4+ T cells and promoting the differentiation of CD4+ T cells into Treg cells; nevertheless, SPMs seem not to modulate Th2 cells (131).

In summary, there is growing evidence about the effects of omega-3 EFAs metabolites on the modulation of the innate and adaptive immune cells supporting the concept of their putative role in immune-mediated rheumatologic conditions which are characterized by a relapsing-remitting inflammatory course.

In **Fig. 5** is shown the resolution pathway involved in acute and chronic inflammation.

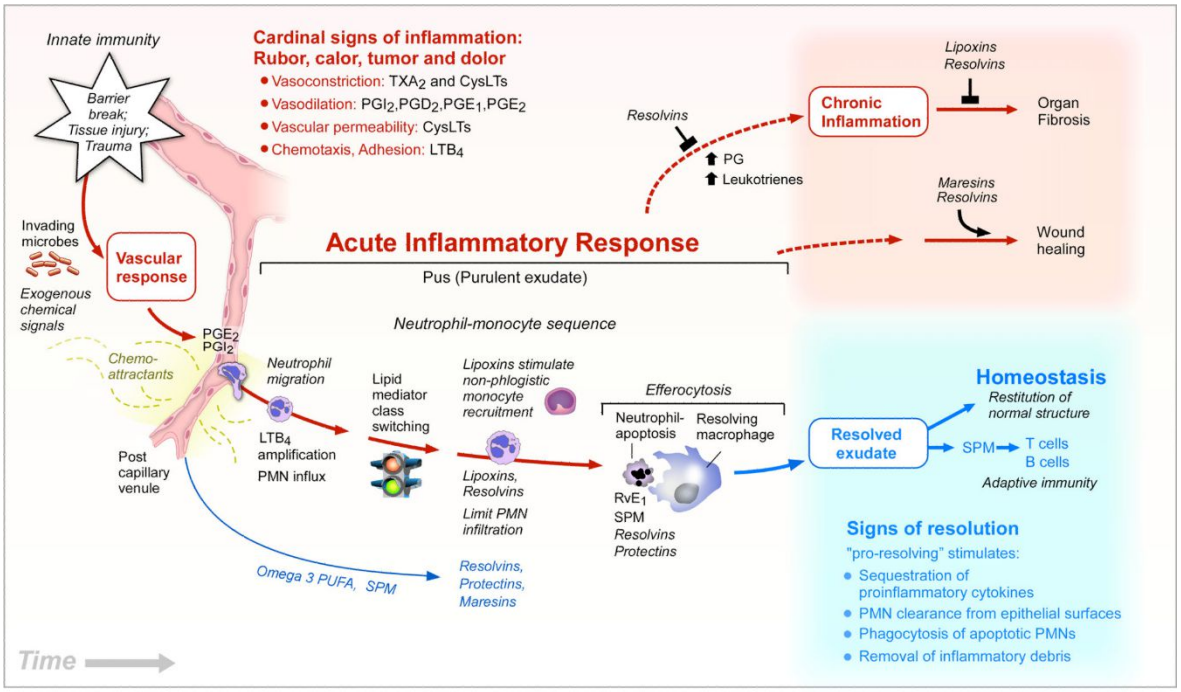


Figure 5. Lipid mediators in the acute inflammatory response, resolution and other outcomes
LM play pivotal roles in the vascular response and leukocyte trafficking, from initiation to resolution. Eicosanoids are critical in initiating the cardinal signs of inflammation (upper left). The lipoxins, resolvins, protectins and maresins, Specialized Pro-resolving Mediators (SPM), are produced in self-limited responses (Fig. 2). SPM stimulate cellular events that counter-regulate pro-inflammatory mediators and regulate PMN, monocyte and macrophage response, leading to resolution. Depicted are some pro-resolving actions in leukocyte trafficking (neutrophil-monocyte sequence), lipid mediator class switching and efferocytosis of apoptotic PMN that must occur in resolving exudates for restoration of normal structure and homeostasis. In addition to the release of n-3 substrate from phospholipid stores95, omega-3 substrates can enter mouse exudates via oedema from peripheral blood57. SPM enhance efferocytosis, stimulate signs of resolution (lower right) and signal to adaptive immunity via lymphocytes. Failed resolution may lead to enhanced prostaglandins and leukotrienes, chronic inflammation and fibrosis. SPM counterregulate pro-inflammatory chemical mediators, reducing magnitude and duration of inflammation, and stimulate reepithelialization, wound healing, and tissue regeneration in model organisms. (modified from (82)). SPM: Specialized Pro-resolvin Mediator; Rv: Resolvin; LT: Leukotriene; TX: Thromboxane; PG: Prostaglandin.

3.3 RESOLUTION IN RHEUMATOID ARTHRITIS

RA is a chronic inflammatory autoimmune disease characterized by a complex interaction among genotype and environmental factors that leads to the abnormal activation of innate and adaptive immune cells and synoviocytes, releasing inflammatory cytokines, degradative mediators and autoantibodies that cause joint damage and systemic symptoms (3, 132-134).

Although there are not clear data about the levels of lipid metabolites in plasma of RA patients (135, 136), the importance of omega-6 EFA metabolites within pathophysiology at disease onset of RA have been showed (3, 137). On the other hand, defining a role for SPMs in RA pathogenesis can be tricky, since when arthritis is well-stabilized as a chronic inflammation, the balance between omega-6 and omega-3 EFA is already skewed to the former. Giera et al. (138) evaluated LXA4 and DHA derived mediators (MaR-1 and Resolvin D5 (RvD5)) levels in synovial fluid of a small cohort of RA patients showing that omega-3 were significantly lower than omega-6-metabolites. Moreover, Barden et al. (139) showed that the analysis of plasma derived from different types of chronic arthritis (including RA) revealed similar EPA and DHA levels between patients and controls under supplemented diet with fish oil (10–15 mL daily), whereas SPMs plasma levels were significantly increased in arthritic patients. Of note, the authors demonstrated E- and D-series Resolvin precursors concentrations were higher in synovial fluid than in plasma, suggesting a possible site-effect of their synthesis and release.

Since in humans the study of the pathophysiologic passage from acute to chronic arthritis may be difficult in terms of lipid mediators modulation, Arnardottir et al. (140) used the neutrophilic mouse model of arthritis triggered by arthritogenic serum transfer (eliciting only innate immune cells) to study the kinetics of SPMs expression. The authors showed that Resolvin D3 (RvD3) was repressed in both delayed-resolving murine arthritis as well as in RA sera and when administered to mice, RvD3 ameliorated arthritis severity. Norling et al. (141) used the same mouse model to investigate the effects of omega-3 EFAs dietary supplementation on arthritis onset, showing that enhanced omega-3 EFAs uptake increased SPMs tissue concentration in arthritic mice than standard diet and reduce arthritis severity. Further, the same research group tested the effect of 17R-epimer RvD1 (17R-RvD1) in the pre-clinical phase of arthritis showing that 17R-RvD1 treatment limited leukocyte recruitment, synovitis score and pannus invasiveness giving protection from damage for the cartilage.

Among SPMs, immunomodulatory properties are not limited to Resolvins but also to other metabolites. In particular, Jin et al. (142) showed that MaR-1 treatment reduced the clinical scores and paw swelling as well as the pro-inflammatory cytokines and Th17 cell-related cytokine (IL-17) release and increased Treg cell-derived cytokines (i.e. IL-10 and TGF-beta) in the collagen-induced

arthritis (CIA) mouse model. Furthermore, the authors demonstrated that MaR-1 levels were significantly repressed in active RA whereas increased, at similar level, in RA patients in clinical remission as well as in controls. Finally, MaR-1 was demonstrated to promote in vitro Treg cells differentiation while inhibiting Th17 cells differentiation, via miRNA-21 upregulation, using RA-derived naive CD4+ T-cells. In this context, not only SPMs showed immune-resolving function since BML-11 (5S, 6R, 7-Trihydroxyheptanoic acid methylester), a LXA4 analog and potent ALXR receptor agonist, was demonstrated to markedly reduce the clinical severity and the histological arthritic lesions in a CIA mouse model (143). Based on these data, the balance between omega-6 and omega-3 EFA metabolites arose a crucial player in driving the onset and perpetuation of arthritis. Moreover, it is clear that SPMs can inhibit the pro-inflammatory pathways without an extreme immunosuppression, identifying SPMs and their analogue as "immunoresolvent" drugs (144, 145).

To date, limited evidences were provided on the beneficial effects of omega-3 EFAs dietary supplementation in RA in vivo. In particular, a systematic literature review including 20 trials (16 double-blind clinical trials, 11 of which were also randomized) and 3 meta-analyses (the first on 19 RCTs, the second on 10 RCTs and the third on four case-controls and three prospective cohorts) showed that, despite omega-3 EFAs integration has a protective role for RA development in subjects at risk, there are no information about the effectiveness of such supplementation in combination with DMARDs, its influence on radiographic progression or on synovial histopathology (146).

4. STUDY PROJECT

4.1 AIMS OF THE STUDY

RA is a complex disease where an uncontrolled innate and adaptive immune system causes damage to cells and tissues. In this scenario, the interaction between genetic and environmental factors leads to the abnormal activation of the immune system contributing to the loss of immunological tolerance. To date, the wider pharmacological armamentarium, due to the development of targeted biological therapies, significantly increased the chance of disease control and sustained clinical remission achievement in RA (147). However, the analysis of synovial tissue of RA patients in sustained clinical and US remission revealed that some inflammatory cells still remain within the synovium. In this scenario, the mechanisms regulating the homeostasis of synovial tissue towards healthy condition remain unknown and, in particular, little is known about the mechanisms involved in the resolution phase of inflammation in rheumatic diseases as well as the possible role of SPMs as pathogenetic and/or therapeutic targets.

This is a translational study with the aim of identifying soluble or tissue biomarkers of inflammation resolution useful for RA patient stratification in clinical practice, improving the management of therapy, with benefits also for the healthcare system. In particular, the levels of the Specialized Pro-Resolving Mediators (SPMs) (like Resolvin, Protectins, Maresins and Lipoxin A4, bioactive metabolites of omega-3 fatty acids) and their receptors (ERV1, ALX/FPR and BLT1) were evaluated in synovial membrane and in peripheral blood derived leukocytes of RA patients and correlated with the inflammatory status and sustained clinical remission phases.
Moreover, the secondary outcome is to identify among studied molecules possible soluble or tissue biomarkers of RA outcomes, allowing us to reach the tailored medicine for the RA patients and increasing the knowledge about the pathophysiology of remission status in RA.

4.2 PATIENTS AND METHODS

4.2.1 PATIENTS

Sixty-eight patients with RA were enrolled in this study at the Rheumatology Unit of the Fondazione Policlinico Universitario Agostino Gemelli-IRCCS (Rome). At study entry, demographic and clinical characteristics were recorded for each patient. In particular, the following parameters are being collected: body mass index (BMI); disease activity score on 28 joints (DAS28-CRP); clinical disease activity index (CDAI); tender joint count (TJC) and swollen joint count (SJC) on 28 sites; anti-CCP antibodies and rheumatoid factor (RF) status; erosive status by X-Ray; use of corticosteroids, cDMARDs, bDMARDs, tsDMARDs and concomitant therapy; ESR and CRP plasma levels. Moreover, peripheral venous blood sample was collected for each subject at study entry. Nine osteoarthritis (OA), 13 undifferentiated peripheral inflammatory arthritis (UPIA) patients and 10 healthy controls (HC) were enrolled as control groups. Patients gave their written informed consent to take part to the study. This study conformed to the ethical guidelines of the 1975 Helsinki's Declaration and was approved by the Ethical Committee of Fondazione Policlinico Universitario Agostino Gemelli-IRCCS (Rome) (ID2667, GR-201812366992).
Once enrolled, each RA patient was adequately treated and followed every 3 months for at least 12 months in an outpatient setting following the RA "treat-to-target" strategy (T2T) (67). To assess clinical response rate, the ACR/EULAR core set criteria was recorded at each study visit (148). Remission is defined as Boolean (the rule of 1) or DAS28-CRP remission (DAS28-CRP < 2.6) and the sustained remission (REM) will be persistent for 6 months without any treatment changing.

4.2.2 INCLUSION AND EXCLUSION CRITERIA IN THE STUDY

Patients classified as having RA according to ACR/EULAR 2010 classification criteria (77) were enrolled in the study. Stable low doses of equivalent prednisone (<5 mg/daily) for at least three months, if necessary, are allowed at the time of study enrolment for RA patients in sustained remission.

Patients were being excluded by enrolment if:

- RA patients treated with more than one bDMARD.
- Severe and uncontrolled infections such as sepsis and opportunistic infections.

- Subjects with evidence (as assessed by the investigator) of active or latent bacterial or viral infections at the time of potential enrollment, including subjects with evidence of human immunodeficiency virus (HIV) detected during screening.
- Subjects with herpes zoster or cytomegalovirus (CMV) that resolved less than 2 months before the informed consent document was signed.
- Subjects with any serious bacterial infection within the last 3 months, unless treated and resolved with antibiotics, or any chronic bacterial infection (e.g., chronic pyelonephritis, osteomyelitis, or bronchiectasis).
- Patients who are currently included in any interventional clinical trial in RA.
- Subjects who are impaired, incapacitated, or incapable of completing study-related assessments
- Subjects with active vasculitis of a major organ system, with the exception of rheumatoid nodules.
- Subjects with current symptoms of severe, progressive, or uncontrolled renal, hepatic, hematologic, gastrointestinal, pulmonary, cardiac, neurologic, or cerebral disease, whether or not related to RA and which, in the opinion of the investigator, might place a subject at unacceptable risk for participation in the study.
- Female subjects who have had a breast cancer screening that is suspicious for malignancy and in whom the possibility of malignancy cannot be reasonably excluded by additional clinical, laboratory, or other diagnostic evaluations.
- Subjects with a history of cancer in the last 5 years, other than non-melanoma skin cell cancers cured by local resection or carcinoma in situ.
- Subjects who currently abuse drugs or alcohol.
- Subjects who have received any live vaccines within 3 months of the anticipated first dose of study medication.

4.2.3 JOINTS ULTRASOUND AND SYNOVIAL TISSUE BIOPSY PROCEDURES

At baseline, each enrolled patient underwent bilateral ultrasound examination for assessment of synovitis using Gray-Scale for the determination of the SMH and PD sign to assess vascularity, according to the OMERACT definition and EULAR-OMERACT scoring system of severity (72, 73) of wrists, MCP (1-5), PIP (1-5), knee, ankles and MTP (1-5) performed by an expert rheumatologist blinded to clinical findings. In particular, SMH was measured in centimetres (cm) and a semi-

quantitative scoring method, which consists of a 0–3 scale, was used to grade the PD grade where 0=no PD, 1=minimal PD, 2=moderate PD, and 3=severe PD. After the ultrasound evaluation, patients underwent to the ultrasound-guided mini-invasive synovial tissue (ST) biopsy following the published protocol (42, 76). In particular, each H&E stained synovial tissue specimen is being scored by using the Krenn guidelines for synovitis score assessment (KSS) (149): synovitis severity was graded according to three ST features (synovial lining cell layer, stromal cell density and inflammatory infiltrate, respectively), each ranked on a scale from none (0), slight (1) and moderate (2) to strong (3). The values of the parameters were summed and interpreted as follows: 0–1=no synovitis; 2–4=low-grade synovitis; and 5–9=high-grade synovitis (**Fig. 6**).

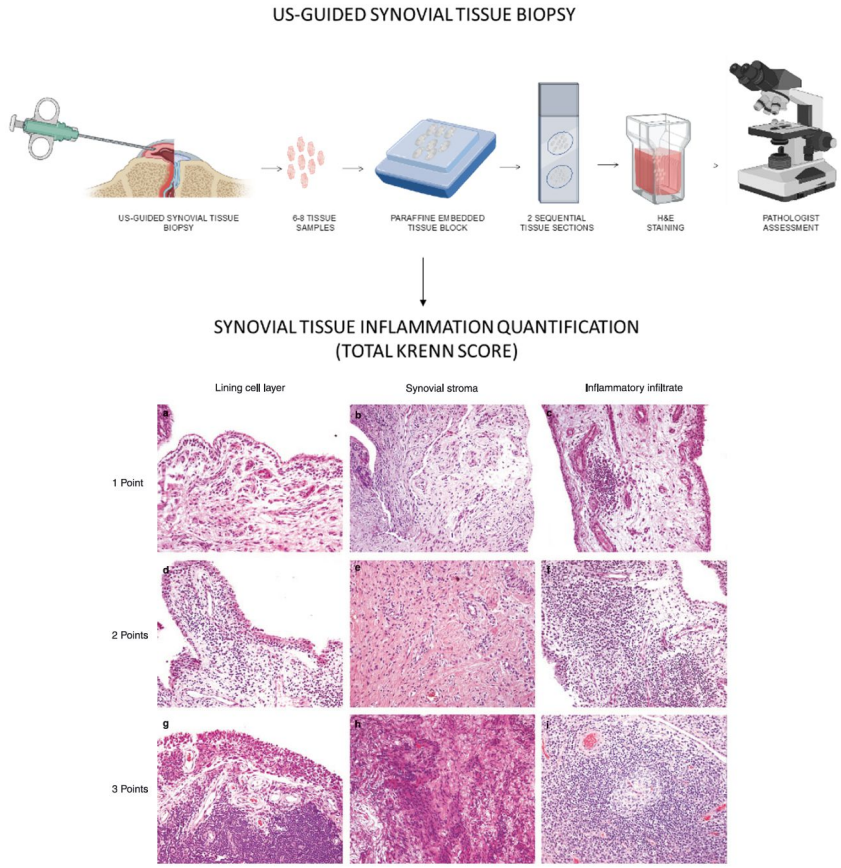


Figure 6. US-guided synovial tissue biopsy procedure and synovial tissue inflammation quantification (Total Krenn Score).
Representation of synovial tissue biopsy procedure: paraffined synovial tissue samples were sectioned and undergone to H&E staining, then evaluated by expert pathologist. Synovitis severity was graded evaluating synovial lining cell layer, stromal cell density and inflammatory infiltrate. Each feature was ranked on a scale from none (0), slight (1) and moderate (2) to strong (3). The values of the parameters were summed and interpreted as follows: 0–1=no synovitis; 2–4=low-grade synovitis; and 5–9=high-grade synovitis. (modified from (76, 149)). US: ultrasound; H&E: hematoxylin and eosin stain; KSS: Krenn Score.

4.2.4 SYNOVIAL TISSUE DIGESTION AND SYNOVIAL CELL ISOLATION

For each patient enrolled for determination SPM receptors expression in ST, ten synovial tissue fragments were mechanically digested and incubated with 300 µl of Liberase TM (Roche, 05401127001) in RPMI 1640 (Corning, 15-040-CV) plus Penicillin/Streptomycin (Corning, 30-002-CI), L-glutamin (Corning, 30-002-CI) and Fetal Bovine Serum (Corning, 35-079-CV) for 15 minutes at 37°C under continuous rolling. The digested tissue was filtered through a nylon Cell Strainer (Falcon, 352360; pore size: 100 µm) and the remaining tissue was mashed and washed through the same nylon strainer. The cell suspension was washed three times in RPMI 1640. For the exclusion of dead cells and debris, Dead Cell Removal MicroBeads (Miltenyi Biotec, 130-090-101) was performed according to the manufacturer's instructions.

4.2.5 RT-PCR EVALUATION FOR ERV1, ALX/FPR AND BLT1 ON SYNOVIAL TISSUE

Total RNA was isolated from synovial tissue of 24 RA (9 from RA MTX-naïve and DMARDs-not responder and 15 from RA in sustained remission) and 7 OA patients using the miRneasy kit (Qiagen). RNA was reverse transcribed using a cDNA conversion kit (Qiagen). The cDNA was used on the real-time RT2 Profiler PCR Array (QIAGEN, Cat. no. PAHS-077Z) in combination with RT2 SYBR® Green qPCR Mastermix (Cat. no. 330529). A set of controls was included on each plate which enabled data analysis using ΔΔCt method of relative quantification, assessment of reverse transcription performance and assessment of PCR performance. The RT² Profiler PCR Array enables SYBR Green-based real-time PCR analysis using Biorad iQ5 real-time PCR system as follows: 95 °C for 15 min; 40 cycles of 94 °C for 15 s; 55 °C for 30 s; and 70 °C for 30 s. The relative expression was calculated using the ΔΔCt method (relative gene expression = 2(ΔCt test − ΔCt control)] and is presented in fold increase relative to control. The Web-based GeneGlobe Data Analysis Center was used to analyse the real-time PCR data (Qiagen).

Primers for human ERV1 (PPHO2349A-200), ALX/FPR2 (PPHO2564A-200), BLT1 (PPHO1341B-200) and GAPDH (housekeeping gene; PPHO0150F-200) were used (Qiagen).

4.2.6 FACS EVALUATION FOR ERV1, ALX/FPR AND BLT1 ON PERIPHERAL BLOOD AND SYNOVIAL TISSUE DERIVED CELLS

For each patient enrolled for determination of SPM receptors expression on PB and ST, peripheral blood cells (PBCs) were stained by specific surface antibodies (Beckman Coulter unless otherwise specified): Krome Orange (KO)-conjugated CD45, Pacific Blue (PB)-conjugated CD3, Allophycocyanin-A700 (APC-A700)-conjugated CD19, Phycoerythrin-Cyan7 (PE-Cy7)-conjugated CD14, Energy Coupled Dye (ECD)-conjugated CD56, APC-conjugated ERV1 (MACS, 130-107-643), PE-conjugated ALX/FPR2 (R&D Systems, FAB3479P), Fluorescein Isothiocyanate (FITC)-conjugated BLT1 (Biorad, MCA2108F). From digested synovial tissue, the suspended synovial cells were stained by specific surface antibodies (Beckman Coulter unless otherwise specified): KO-conjugated CD45, PB-conjugated CD3, ECD-conjugated CD19, APC-conjugated ERV1 (MACS, 130-107-643), PE-conjugated ALX/FPR (R&D Systems, FAB3479P), FITC-conjugated BLT1 (Biorad, MCA2108F). **Table 1** shows the surface antibodies used and their features. **Fig. 7** shows the emission fluorescence spectra of each panel.

All incubations were performed for 30 minutes, at room temperature and a negative sample was used properly for every test. Red cells were lysed by BD lysis buffer 10x (BD; 349202) according to the manufacturer's instructions. The samples were immediately acquired with a Beckman Coulter Navios flow cytometer. Every acquisition was settled for 100,000 events. **Fig. 8** depict gating strategy used to evaluate SPM receptors expression on PB- and ST-derived cell suspensions. Briefly, doublets and multiplets were excluded from FSC vs FSC-h plot. Then, debris were excluded from FSC vs SSC plot and the events were gated by KO-conjugated CD45. Within CD45+ vs SSC gate, lymphocytes, CD14+ monocytes (only in PB) and neutrophils (only in PB) were gated based on FSC values. Finally, CD3+ vs CD19+ plot was set on lymphocytes gate and NK cells (only in PB) on CD45+CD3-CD19-CD56+ gate. A second plate was created for gating ST macrophages. Briefly, doublets and multiplets were excluded from FSC vs FSC-h plot. Then, debris were excluded from FSC vs SSC plot and the events were gated by KO-conjugated CD45. Within CD45+ gate, CD11b+CD64+ cells were selected as ST macrophages. The assessment of the ERV1, ALX/FRP2 and BLT1 receptors were expressed as percentage of positive cells and as mean fluorescence intensity (MFI). The analysis was performed by Kaluza software (v 2.1, Beckman Coulter).

Table 1. Surface antibodies used in FACS staining, their features and where they were used.

Antibody	Brand	Reference code	Clone	Isotype	Compartment
CD45-KO	Beckman Coulter	B36294	J.33	msIgG1	PB, ST
CD3-PB	Beckman Coulter	B49204	UCHT1	msIgG1	PB, ST
CD19-APC-A700	Beckman Coulter	B49212	J3.119	msIgG1	PB
CD14-PE-Cy7	Beckman Coulter	A22331	RMO52	msIgG2a	PB
CD56-ECD	Beckman Coulter	B49214	N901	msIgG1	PB
CD19-ECD	Beckman Coulter	A07770	J3.119	msIgG1	ST
CD11b-APC-A750	Beckman Coulter	B36295	Bear1	msIgG1	ST
CD64-PB	Beckman Coulter	B19718	22	msIgG1	ST
ERV1-APC	MACS	130-107-643	REA455	rhIgG1	PB, ST
ALX/FPR2-PE	R&D Systems	FAB3479P	304405	msIgG2b	PB, ST
BLT1-FITC	Bio Rad	MCA2108F	220/7B1	msIgG2a	PB, ST

Brand, reference code, clone, isotype and compartment where used are shown for each antibody. KO: Krome Orange, PB: Pacific Blue, APC-A700: Allophycocyanin-A700, PE-Cy7: Phycoerythrin-Cyanine7, ECD: Energy Coupled Dye, APC: Allophycocyanin, PE: Phycoerythrin, FITC: Fluorescein Isothiocyanate. PB: peripheral blood derived cells; ST: synovial tissue derived cells.

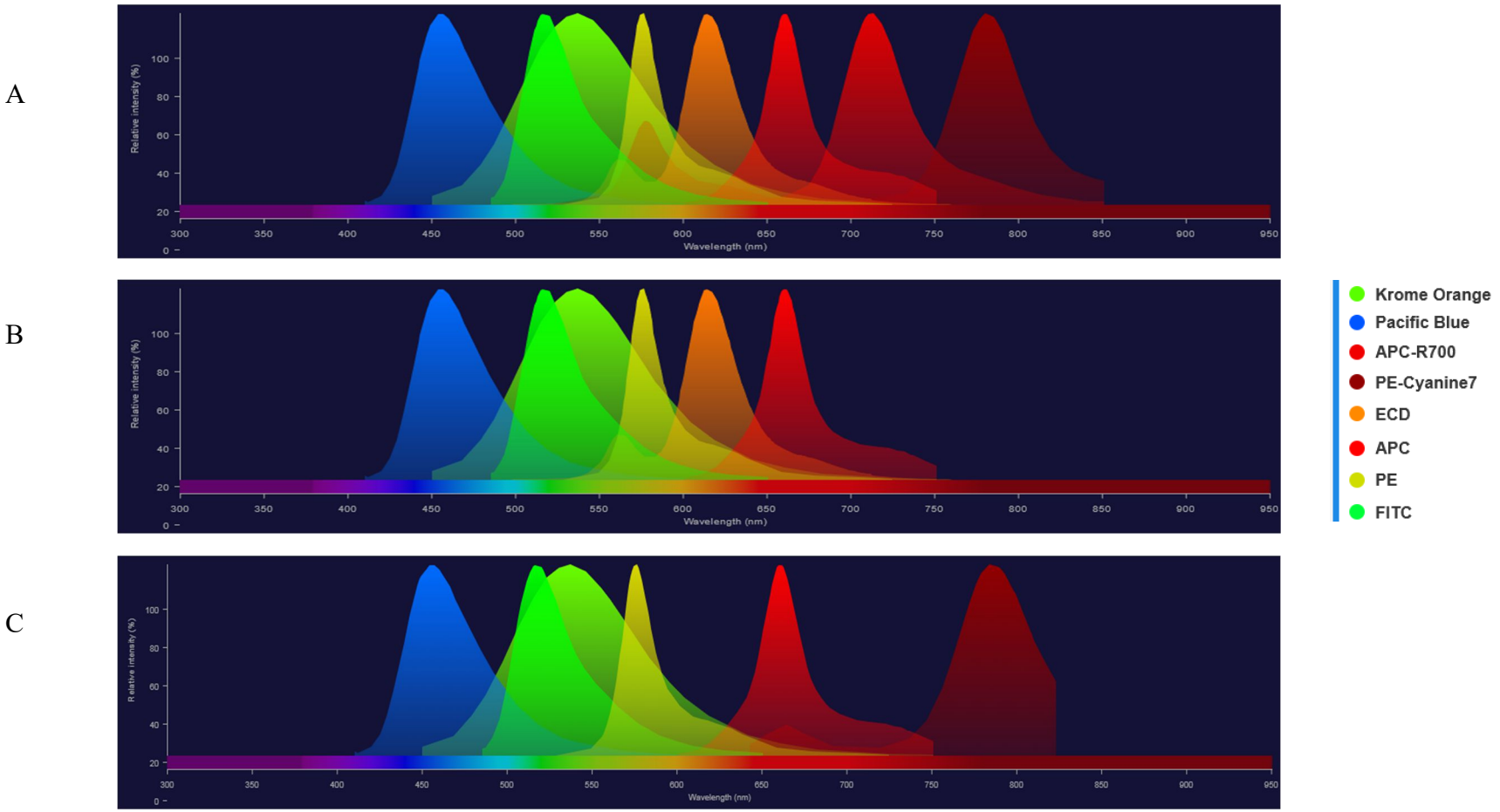


Figure 7. Emission fluorescence spectra of FACS staining antibodies used for PB and ST compartment.
A: emission fluorescence spectra of FACS staining antibodies used for PB compartment. B: emission fluorescence spectra of FACS staining antibodies used for ST compartment to gate CD3+ and CD19+ cells. C: emission fluorescence spectra of FACS staining antibodies used for ST compartment to gate macrophages. KO: Krome Orange, PB: Pacific Blue, APC-A700: Allophycocyanin-A700, PE-Cy7: Phycoerythrin-Cyanine7, ECD: Energy Coupled Dye, APC: Allophycocyanin, PE: Phycoerythrin, FITC: Fluorescein Isothiocyanate. PB: peripheral blood derived cells; ST: synovial tissue derived cells.

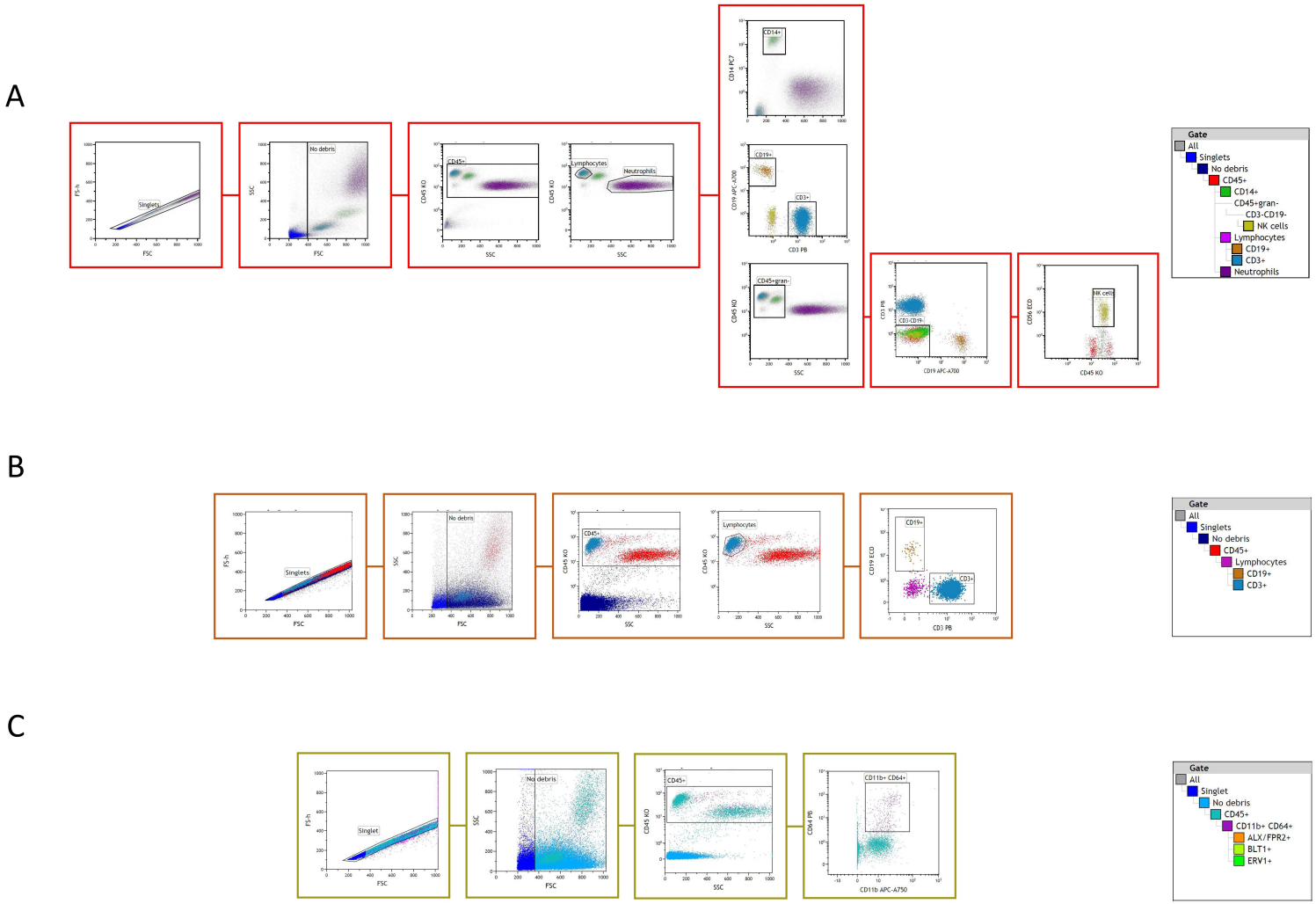


Figure 8. Gating strategies and hierarchy charts adopted to evaluate SPM receptors in PB and ST compartment.
A: gating strategy adopted to evaluate ERV1, ALX/FPR2 and BLT1 expression in PB neutrophils, CD14+ monocytes, NK cells, CD3+ and CD19+ cells. For each cells, SPM receptors were assessed as percentage of positive cells and MFI value. B: gating strategy adopted to evaluate ERV1, ALX/FPR2 and BLT1 expression in ST lymphocytes, CD3+ and CD19+ cells. For each cells, SPM receptors were assessed as percentage of positive cells and MFI value. C: gating strategy adopted to evaluate ERV1, ALX/FPR2 and BLT1 expression in ST macrophages (CD45+CD11b+CD64+ cells). For each cells, SPM receptors were assessed as percentage of positive cells and MFI value. KO: Krome Orange, PB: Pacific Blue, APC-A700: Allophycocyanin-A700, PE-Cy7: Phycoerythrin-Cyanine7, ECD: Energy Coupled Dye, APC: Allophycocyanin, PE: Phycoerythrin, FITC: Fluorescein Isothiocyanate. PB: peripheral blood derived cells; ST: synovial tissue derived cells.

4.2.7 CYTOKINES AND CHEMOKINES LEVELS DETERMINATION

For each subject enrolled for determination of cytokines and chemokines serum level, peripheral venous blood was collected and centrifuged on 3500 rpm for 30 min on 4 °C and stored on -80 °C until analysis. Serum levels of 8 cytokines were measured by using ELLA rapid detection ELISA microfluidics platform run in duplicate following manufacturer's instructions. As shown in **Table 2**, the measured cytokines panel included IL-1beta, tumor necrosis factor-alpha (TNF-alpha), IL-6, interferon-gamma (IFN-gamma), IL-12p70, IL-10, IL-4 and IL-2 (proteinsimple, SPCKE-PS-002797). Serum levels of Chemerin (R&D Systems, DCHM00) and GAS6 (R&D Systems, DY885B) were measured by using ELISA following manufacturer's instructions.

Table 2. Measured cytokines and chemokines panel by ELLA rapid detection ELISA microfluidics platform and by ELISA

Molecules	Brand	Sensitivity (pg/ml)	Intra-assey CV%	Inter-assey CV%
IL-1beta, pg/ml	proteinsimple	0.064	3.7 - 4.9	4.0 - 5.7
TNF-alpha, pg/ml	proteinsimple	1.05	4.9 - 5.4	8.5 - 9.0
IL-6, pg/ml	proteinsimple	0.26	2.4 - 3.9	7.1 - 8.3
IFN-gamma, pg/ml	proteinsimple	0.05	2.8 - 7.0	8.3 - 10.8
IL-12p70, pg/ml	proteinsimple	0.39	3.4 - 5.4	5.9 - 8.6
IL-10, pg/ml	proteinsimple	0.14	4.6 - 6.0	7.1 - 7.1
IL-4, pg/ml	proteinsimple	0.05	6.7 - 8.3	9.7 - 12.3
IL-2, pg/ml	proteinsimple	0.18	5.1 - 5.3	5.6 - 8.7
Chemerin, ng/ml	R&D Systems	7.80	2.8 - 4.5	6.4 - 7.9
GAS6, ng/ml	R&D Systems	16.00	na	na

Brand, sensitivity (expressed in pg/ml), intra-assay coefficient of variability (CV) % and inter-assay CV% are shown for each molecule.

4.2.8 SPM AND AA-DERIVED PRO-INFLAMMATORY MOLECULES SYNOVIAL TISSUE DETERMINATION BY LC-MS/MS ANALYSIS

LC-MS/MS analysis was performed at Queen Mary University of London - Lipid Mediator Unit (William Harvey Research Institute, Charterhouse Square, London, UK) on 10 synovial tissue fragments. Step-by-step description of the extraction, analysis and quantitation procedures are detailed in the following protocol found in Protocol Exchange (150-152). Liquid chromatography (LC)-grade solvents were purchased from Fisher Scientific; Poroshell 120 EC-C18 column (100 mm × 4.6 mm × 2.7 μm) was obtained from Agilent (Cheshire, UK); C18 SPE columns were from Biotage; synthetic standards for LC-tandem mass spectrometry (MS-MS) quantitation and deuterated (d) internal standards (d8-5S-HETE (CAY334230); d5-RvD2 (CAY11184); d5-LXA4 (CAY10007737); d4-PGE2 (CAY314010); d4-LTB4 (CAY320110); d5-LTC4 (CAY10006198); d5-LTD4 (CAY10006199); d5-LTE4 (CAY10007858)) and synthetic lipid mediator standards (RvD1, CAY10012554; 17R-RvD1 (CAY13060); RvD2 (CAY10007279); RvD3 (CAY13834); 17R-RvD3 (CAY9002880); RvD4 (CAY13835); RvD5 (CAY10007280); MaR1 (CAY10878); MaR2 (CAY16369); MCTR1 (CAY17007); MCTR2 (CAY17008); MCTR3 (CAY19067); PDX (CAY10008128); PCTR1 (CAY19064); PCTR2 (CAY19065); PCTR3 (CAY19066); 4-HDHA (CAY33200); 7-HDHA (CAY33300); 14-HDHA (CAY33550); 17-HDHA (CAY33650); RvE1 (CAY10007848); 5-HEPE (CAY32210); 12-HEPE (CAY32540); 15-HEPE (CAY32700); 18-HEPE (CAY32840); RvD5n-3 DPA, CAY10546; LXA4 (CAY90410); 15-epi-LXA4 (CAY90415); LXB4 (CAY90420); 5S,15S-diHETE (CAY35280); PGD2 (CAY12010); PGE2 (CAY14010); PGF2α (CAY16010); TXB2 (CAY19030); LTB4 (CAY20110); 6-trans-LTB4 (CAY35250); 6-trans,12-epi-LTB4 (CAY35265); 20-OH-LTB4 (CAY20190); 20-COOH-LTB4 (CAY20180); LTC4 (CAY20210); LTD4 (CAY20310); LTE4 (CAY20410); 5-HETE (CAY34210); 12-HETE (CAY34550); 15-HETE (CAY34700)) were purchased from Cambridge Bioscience or provided by Charles N. Serhan (Harvard Medical School, Boston, Massachusetts, USA; supported by NIH-funded P01GM095467); Dulbecco's phosphate-buffered saline (DPBS, without calcium and magnesium, Sigma (D8537)).

4.2.9 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS v. 26.0 (SPSS. Chicago, Illinois, USA) and Prism software (GraphPad-8, San Diego, California, USA). Shapiro-Wilk-test was used for assessing normality of the variables. For continuous variables, normally distributed data were presented as the mean ± standard deviation (SD), while those non-normally distributed were given as median with interquartile range (IQR). Clinical characteristics and measurements were compared by ANOVA, Mann-Whitney U-test for medians, Pearson Chi-square for proportions, Students t-test if data followed an approximatively normality. Spearman rank test was used to perform the correlation analysis.

Exploratory univariate data analysis was first conducted to assess adequate event frequency between the outcome and the candidate prognostic factors. A receiver operating characteristics (ROC) curve analysis of ST $BLT1^{+}CD3^{+}$ MFI value related to DAS28-CRP remission achievement after 12 months of therapy was performed to obtain relevant thresholds allowing the prediction therapy response at baseline. The non-parametric ROC plot uses all the data, makes no parametric assumption and provides unbiased estimates of sensitivity and specificity. The optimal cut-off point was determined to yield the maximum corresponding sensitivity and specificity. For all the analyses, a $p<0.05$ was considered as statistically significant and all tests were 2-tailed, unless otherwise indicated.

Since in RA patients treated according to the treat-to-target strategy the disease remission achievement rate ranges from 40% to 70% (133), we planned to enrol in the study 80 consecutive RA patients assuming that the expression of SPM's receptors is significantly different in RA patients achieving the highest rate of remission (power: 81.3%, α: 0.03).

4.3 RESULTS

4.3.1 DEMOGRAPHIC, CLINICAL, IMMUNOLOGICAL, ULTRASOUND AND HISTOLOGICAL CHARACTERISTICS

This study enrolled 90 patients, including 68 patients affected by RA, 13 UPIA and 9 OA patients. **Table 3** summarizes demographic, clinical (disease duration, TJC-28, SJC-28, DAS28-CRP, CDAI), immunological (RF/ACPA positivity, ESR, CRP), ultrasound (SMH and PD grade) and histological (Krenn Score) findings of patients along with 10 healthy control (HC) subjects. Furthermore, within the RA group, patients were divided in different clinical status as MTX-naïve (n= 27), DMARDs-not responder (n= 23) and patients in sustained remission (n= 18). RA subgroups demographic, clinical, immunological, ultrasound and histological findings are shown in **Table 4**.

RA, UPIA and OA patients and healthy controls did not differ in age (ANOVA test, p= 0.07), gender (p= 0.98), BMI (p= 0.30) and smoking status (p= 0.29). Similarly, within the RA groups, no differences in age (ANOVA test, p= 0.57), gender (p= 0.88), BMI (p= 0.43) and smoking status (p= 0.69) were found. In contrast, between those groups different clinical (**Fig. 9A**), immunological, ultrasound (**Fig. 9B-9D**) and histological aspects (**Fig. 9F-9G**) were expected due to the DAS28-CRP-based selection. In particular, RA patient in sustained remission (REM) presented lower DAS28-CRP (1.62 ± 0.70) compared to UPIA (3.28 ± 1.14, p= 0.0001), RA MTX-naïve (5.28 ± 1.41, p= 0.0001) and RA DMARDs-not responder (5.37 ± 1.36, p= 0.0001) patients, as well as lower SMH (0.83 ± 0.23) compared to RA MTX-naïve (1.03 ± 0.26, p= 0.02) and RA DMARDs-not responder (1.01 ± 0.26, p= 0.02). Similarly, REM subjects showed lower PD-grade (0.10 ± 0.23) compared to OA (0.67 ± 0.58, p= 0.020), UPIA (± 0.92, p= 0.001), RA MTX-naïve (1.95 ± 0.82, p= 0.0001) and RA DMARDs-not responder (1.30 ± 0.80, p= 0.0001) patients, respectively. Finally, also the KSS was lower in REM (1.89 ± 1.53) when compared to MTX-naïve (5.04 ± 2.35, p= 0.0001) and DMARDS-not responder (4.04 ± 2.42, p= 0.001) RA patients. SHM and KSS comparable values were found between REM, OA and UPIA groups.

Moreover, considering concomitant pharmacological treatments, none of REM patients were using steroids and 13 (72%) were using csDMARDs, comparing to 4 (15%, p= 0.09) MTX-naïve RA patients that were on treatment with steroids (mean dosage: 5.06 ± 0.92) and 1 (4%, p< 0.0001) with csDMARDs, 11 (48%, p= 0.0001) (mean dosage: 5.07 ± 2.32) and 19 (83%, p= 0.425) DMARDs-

non responder RA patients, respectively. Furthermore, 12 (67%; 58.3% TNFi, 33.3% IL-6Ri, 8.4% other mechanism of action) REM and 8 (35%; 25.0% TNFi, 25.0% IL-6Ri, 37.5% JAKi, 12.5% other mechanism of action; p= 0.02) DMARDs-non responder RA patients were using bDMARDs.

Table 3. Demographic, immunological, clinical, ultrasound and histological findings of enrolled subjects.

	HC (N: 10)	OA (N: 9)	UPIA (N: 13)	RA (N: 68)	p-value
Age	51 (10.77)	53.46 (28.59)	57.53 (9.52)	57.93 (13.31)	0.07
Female (n, %)	8 (80%)	4 (44%)	9 (69%)	52 (76%)	0.98
BMI (Kg/mq)	26.43 (5.27)	27.13 (2.48)	27.5 (5.05)	24.71 (4.58)	0.30
Smoking Status (n, %)	4 (40%)	3 (33%)	5 (38%)	16 (23%)	0.29
Disease Duration (mo)		78.00 (1.41)	45.83 (44.98)	81.98 (102.59)	**0.02**
RF/ACPA positivity (n, %)		0 (0%)	5 (38%)	37 (54%)	**0.01**
ESR (mm/h)		11.25 (10.53)	30.46 (21.17)	36.22 (28.09)	**0.0001**
CRP (mg/l)		0.8 (0.81)	6.14 (13.79)	23.01 (34.68)	**0.003**
TJC-28		2.00 (2.45)	1.75 (1.66)	5.09 (4.81)	**0.0001**
SJC-28		2.80 (4.14)	2.25 (0.965)	4.75 (4.53)	**0.0001**
DAS28-CRP			3.28 (1.14)	4.34 (2.05)	**0.0001**
CDAI			13.80 (4.14)	19.63 (15.34)	**0.0001**
SMH (cm)		0.97 (0.20)	0.92 (0.17)	0.98 (0.26)	0.93
Power-doppler Grade		0.67 (0.58)	1.10 (0.99)	1.24 (1.03)	**0.0001**
Krenn Score		2.50 (1.00)	3.00 (2.04)	3.83 (2.50)	**0.0001**
Steroids (n, %)			0	15 (22%)	
csDMARDs (n, %)			0	33 (48%)	
b/tsDMARDs (n, %)			0	20 (29%)	

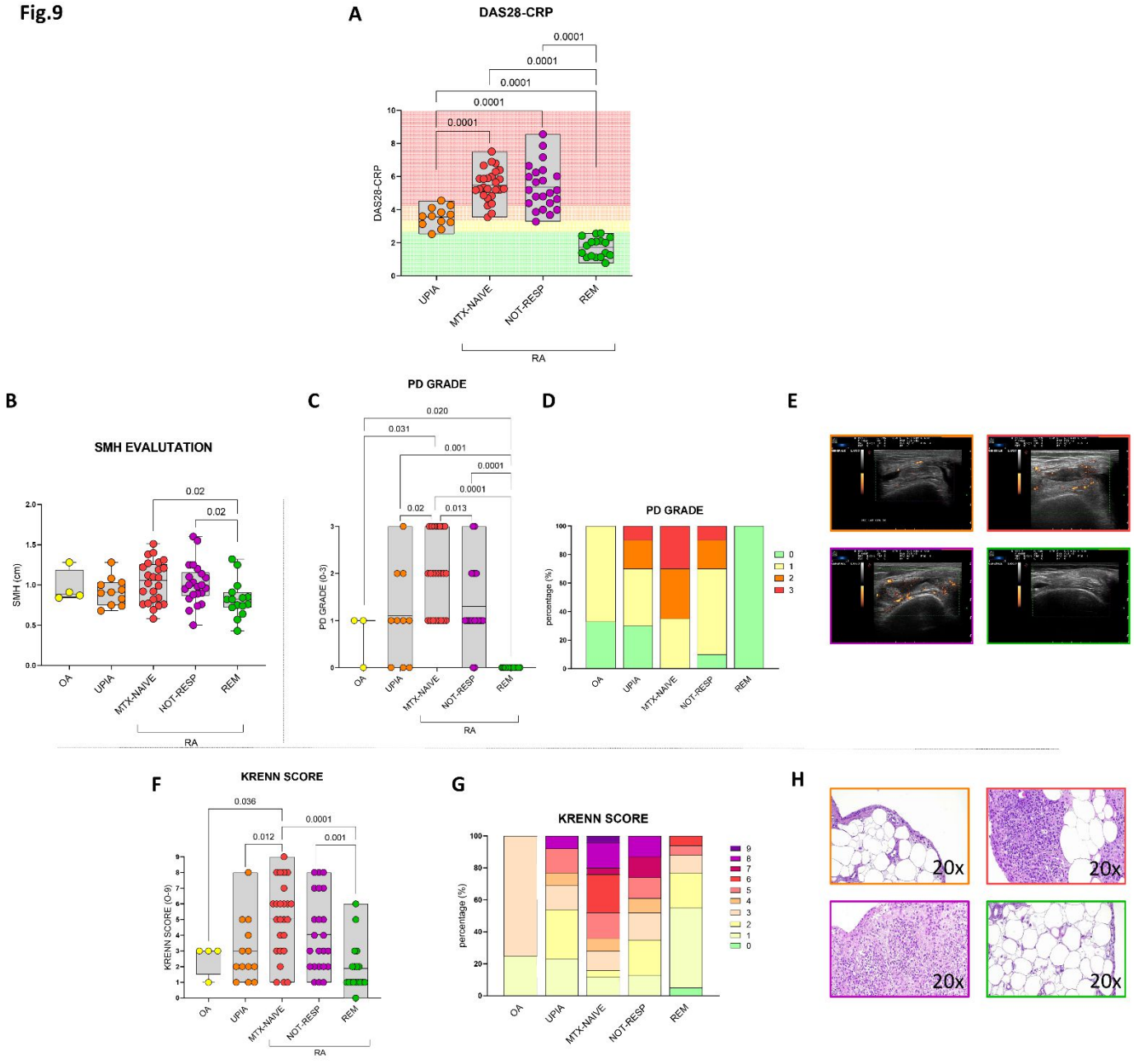
HC: healthy controls; OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic Disease Modifying Anti-Rheumatic Drugs; b/tsDMARDs: biological / target synthetic Disease Modifying Anti-Rheumatic Drugs. Mean ± SEM or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

Table 4. Demographic, immunological, clinical, ultrasound and histological findings of enrolled RA patients divided by clinical phases.

	MTX-NAÏVE (a) (N: 27)	DMARDs-NOT RESPONDER (b) (N: 23)	REMISSION (c) (N: 18)	p-value a vs b	p-value a vs c	p-value b vs c
Age	59.14 (13.76)	58.74 (13.47)	55.06 (12.74)		0.57	
Female (n, %)	21 (78%)	18 (78%)	13 (72%)		0.88	
BMI (Kg/mq)	24.10 (4.22)	25.71 (5.28)	24.26 (4.10)		0.43	
Smoking Status (n, %)	7 (26%)	4 (17%)	5 (28%)		0.69	
Disease Duration (mo)	26.44 (46.50)	113.27 (118.38)	123.18 (108.80)	**0.0001**	**0.0001**	0.301
RF/ACPA positivity (n, %)	11 (41%)	14 (61%)	12 (67%)	**0.046**	0.053	0.970
ESR (mm/h)	46.04 (30.63)	41.91 (24.70)	12.94 (10.29)	0.93	**0.0001**	**0.0001**
CRP (mg/l)	33.50 (38.77)	26.79 (36.24)	1.22 (1.27)	0.45	**0.0001**	**0.0001**
TJC-28	6.92 (3.35)	6.96 (5.31)	0.10 (0.24)	0.64	**0.0001**	**0.0001**
SJC-28	6.62 (3.43)	6.17 (4.99)	0.22 (0.55)	0.26	**0.0001**	**0.0001**
DAS28-CRP	5.28 (1.41)	5.37 (1.36)	1.62 (0.70)	0.59	**0.0001**	**0.0001**
CDAI	27.71 (9.34)	26.82 (13.86)	1.37 (2.02)	0.46	**0.0001**	**0.0001**
SMH (cm)	1.03 (0.26)	1.01 (0.26)	0.83 (0.23)	0.65	**0.01**	**0.02**
Power-doppler Grade	1.95 (0.82)	1.30 (0.80)	0.1 (0.23)	**0.01**	**0.0001**	**0.0001**
Krenn Score	5.04 (2.35)	4.04 (2.42)	1.89 (1.53)	0.14	**0.0001**	**0.001**
Steroids (n, %)	4 (15%)	11 (48%)	0	**0.001**	0.09	**0.0006**
csDMARDs (n, %)	1 (4%)	19 (83%)	13 (72%)	**<0.0001**	**<0.0001**	0.42
b/tsDMARDs (n, %)	0	8 (35%)	12 (67%)	**0.002**	**<0.0001**	**0.02**

MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic DMARDs; b/tsDMARDs: biological / target synthetic DMARDs. Mean ± SEM or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

Fig.9


A
DAS28-CRP
0.0001
0.0001
0.0001
0.0001
0.0001
DAS28-CRP
UPIA
MTX-NAIVE
NOT-RESP
REM
RA
B
SMH EVALUTATION
0.02
0.02
SMH (cm)
OA
UPIA
MTX-NAIVE
NOT-RESP
REM
RA
C
PD GRADE
0.020
0.031
0.001
0.0001
0.0001
0.02
0.013
PD GRADE (0-3)
OA
UPIA
MTX-NAIVE
NOT-RESP
REM
RA
D
PD GRADE
percentage (%)
0
1
2
3
OA
UPIA
MTX-NAIVE
NOT-RESP
REM
E
F
KRENN SCORE
0.036
0.0001
0.012
0.001
KRENN SCORE (0-9)
OA
UPIA
MTX-NAIVE
NOT-RESP
REM
RA
G
KRENN SCORE
percentage (%)
9
8
7
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OA
UPIA
MTX-NAIVE
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20x
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20x

Figure 9. Representation of clinical, ultrasound and histological findings of enrolled subjects.
A: DAS28-CRP values across diseases phases: orange for UPIA, red for MTX-naïve RA, violet for DMARDs-not responder RA, green for sustained remission RA. Background colours defined the disease activity category: green for remission (DAS28-CRP ≤ 2.3), yellow for low (DAS28-CRP ≤ 2.7), orange for moderate (2.7 < DAS28-CRP ≤ 4.1), red for high (DAS28-CRP ≤ 2.3). Sustained remission RA presented the lowest DAS28-CRP value (1.62 ± 0.70) compared to UPIA (3.28 ± 1.14, p= 0.0001), MTX-naïve (5.28 ± 1.41, p= 0.0001) and DMARDs-not responder (5.37 ± 1.36, p= 0.0001) patients **B:** SMH values across diseases phases: yellow for OA, orange for UPIA, red for MTX-naïve RA, violet for DMARDs-not responder RA, green for sustained remission RA. Sustained remission RA presented the lowest SMH value (0.83 ± 0.23) compared to MTX-naïve (1.03 ± 0.26, p= 0.02) and DMARDs-not responder (1.01 ± 0.26, p= 0.02) but comparable to UPIA (0.92 ± 0.17) and OA (0.97 ± 0.20) patients. **C:** PD Grade values across diseases phases: yellow for OA, orange for UPIA, red for MTX-naïve RA, violet for DMARDs-not responder RA, green for sustained remission RA. Sustained remission RA presented the lowest PD Grade (0.10 ± 0.23) compared to OA (0.67 ± 0.58, p= 0.020), UPIA (± 0.92, p= 0.001), MTX-naïve (1.95 ± 0.82, p= 0.0001) and DMARDs-not responder (1.30 ± 0.80, p= 0.0001) patients. **D:** distribution of PD Grade (0: green, 1: yellow, 2: orange, 3: red) among different patients' categories. All sustained remission RA patients did not present PD signal compared to other conditions. **E:** example photos of ultrasound assessment with PD Grade of the knee used for ST biopsy of each patients' category (border colour identifies each category: orange for UPIA, red for MTX-naïve, violet for DMARDs-not responder and green for remission RA patients). **F:** Krenn Score (KSS) values across diseases phases: yellow for OA, orange for UPIA, red for MTX-naïve RA, violet for DMARDs-not responder RA, green for sustained remission RA. Sustained remission RA presented lower KSS (1.89 ± 1.53) comparing to MTX-naïve (5.04 ± 2.35, p= 0.0001) and DMARDS-not responder (4.04 ± 2.42, p= 0.001) but comparable to OA and UPIA (2.50 ± 1.00, p= 0.166 and 3.00 ± 2.04, p= 0.066 respectively). **G**: distribution of Krenn Score (from 0: green to 9: violet) among different patients' categories. All sustained remission RA patients did not present PD signal compared to other conditions. Despite clinical and ultrasound remission, almost 10% of sustained remission RA patients presented a high synovitis score (KSS ≥ 5). **H**: example photos of H&E staining of ST obtained by minimal invasive US-guided biopsy of each patients' category (border colour identifies each category: orange for UPIA, red for MTX-naïve, violet for DMARDs-not responder and green for remission RA patients). The values are expressed as mean ± SEM. Each dot represents a patient. P-value <0.05 was considered statistically significant. OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs; REM: sustained remission RA; CRP: C-reactive protein (in mg/l: milligram per litre); DAS28-CRP: disease activity score CRP-based of 28 joints; SMH: synovial membrane hyperplasia (in cm: centimetre); KSS: Krenn Score; US: ultrasound; H&E: hematoxylin and eosin stain.

4.3.2 CORRELATIONS OF DEMOGRAPHICS, CLINICAL, IMMUNOLOGICAL, ULTRASOUND AND HISTOLOGICAL FEATURES

Considering UPIA as the early phase of specific arthritis as RA, as shown in **Fig. 10A**, in the former cohort, CDAI directly correlated with age (r= 0.819, p= 0.013) and SMH with BMI (r= 0.667, p= 0.050). Finally, KSS directly correlated with CRP and PD-grade (r= 0.598, p= 0.031 and r= 0.754, p= 0.012 respectively).

Considering the whole RA cohort, age directly correlated with CRP (r= 0.402, p= 0.001). Furthermore, the disease activity evaluated in terms of DAS28-CRP and CDAI directly correlated with PD grade (r= 0.63, p= 0.0001; r= 0.75, p= 0.0001 respectively), KSS (r= 0.61, p= 0.0001; r= 0.74, p= 0.0001 respectively) and SMH (only CDAI: r= 0.41, p= 0.004). Moreover, DAS28-CRP and CDAI clinical indexes inversely correlated also with disease duration (r= -0.356, p= 0.004 and r= -0.423, p= 0.002 respectively) (**Fig. 10B**).
When considering the RA sub-groups, also in MTX-naïve RA cohort the disease activity evaluated in terms of DAS28-CRP and CDAI directly correlated with PD grade (only DAS28-CRP: r= 0.57, p= 0.002) and KSS (r= 0.68, p= 0.0001; r= 0.62, p= 0.005 respectively). Furthermore, disease duration inversely correlated with CRP (r= -0.475, p= 0.017) (**Fig. 10C**). Similarly, in DMARDs-not responder RA cohort, DAS28-CRP directly correlated with KSS (r= 0.44, p= 0.036) and CDAI with PD grade and KSS (r= 0.57, p= 0.032; r= 0.55, p= 0.021 respectively). Moreover, age directly correlated with CRP (r= 0.42, p= 0.046) but inversely correlated with SMH and PD grade (r= -0.479, p= 0.024; r= -0.475, p= 0.034 respectively), BMI directly correlated with CRP (r= 0.42, p= 0.045) while disease duration inversely correlated with CRP (r= -0.44, p= 0.038) (**FIG. 10D**). Finally, in REM cohort DAS28-CRP directly correlated with BMI (r= 0.57, p= 0.014) and age directly correlated with ESR, CRP and IgM RF level (r= 0.52, p= 0.033; r= 0.66, p= 0.004; r= 0.51, p= 0.048 respectively) (**Fig. 10E**).

These data showed that disease activity was contingent on ultrasound and histological features in patients with RA. In particular, ST assessment directly mirrors the disease activity status across RA phases.

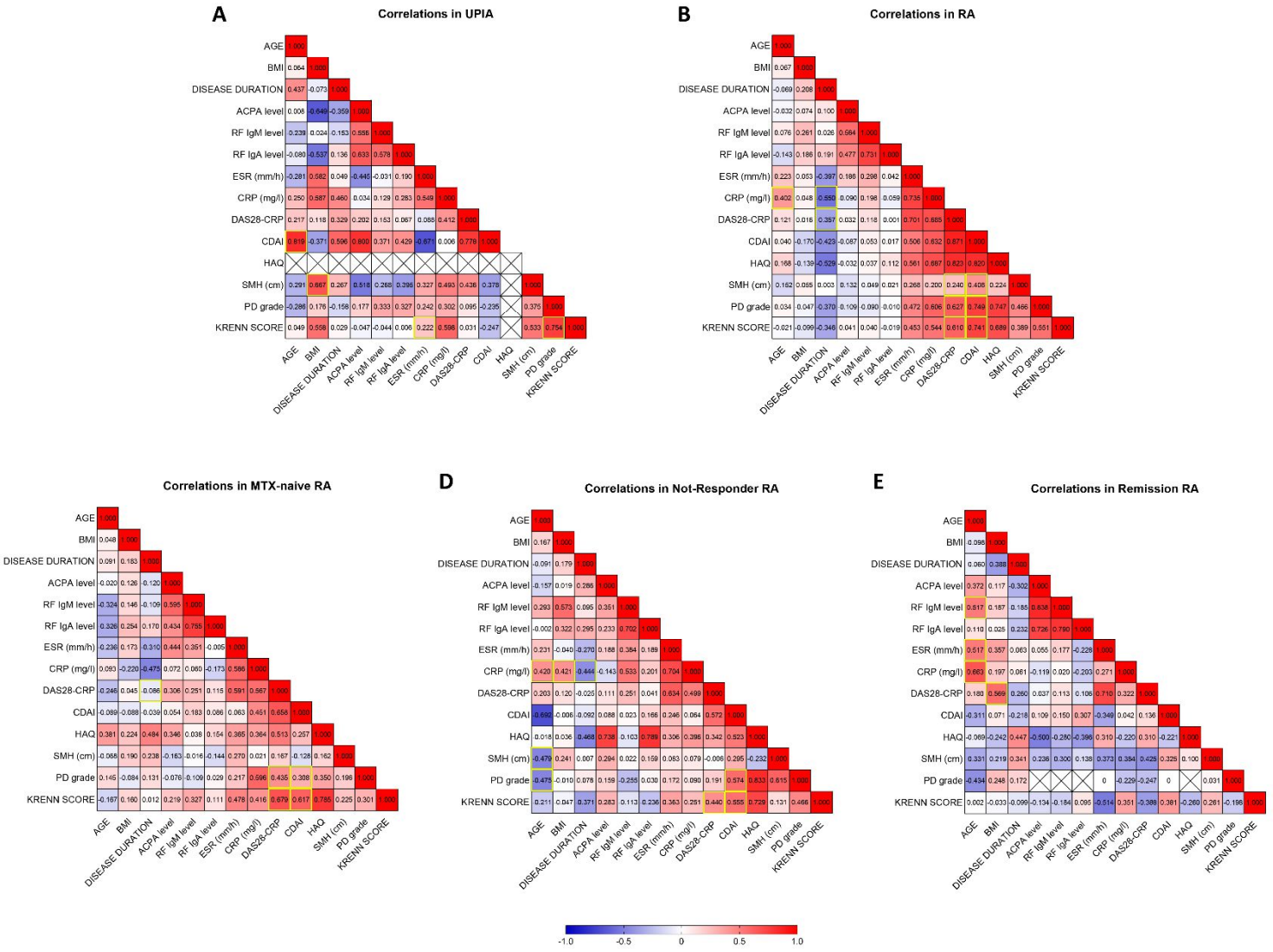


Figure 10. Correlation heatmaps of demographics, clinical, immunological, ultrasound and histological features of UPIA (A) and RA groups (B-C). UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index; RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre). Two-tailed Spearman's correlation are shown in every cell. Yellow square: p-value <0.05, considered statistically significant.

4.3.3 SERUM CYTOKINES AND CHEMOKINES PLASMA LEVELS DETERMINATION

Table 5 and **Table 6** summarize the demographic, immunological, clinical, ultrasound and histological findings of subjects enrolling to assess the inflammatory burden in terms of cytokines and chemokines levels. For this purpose, IL-1beta, TNF-alpha, IL-6, IFN-gamma, IL-12p70, IL-10, IL-4, IL-2, Chemerin and GAS6 serum concentrations were determined (in pg/ml, except for Chemerin and GAS6 in ng/ml). Within the pro-inflammatory cytokines, IL-1beta serum levels did not change between the different patient groups (**Fig. 11A**). Moreover, TNF-alpha serum levels were higher in sustained remission and DMARDs-non responder RA patients (median and IQR are shown: 10.01 (6.18-22.55) and 8.86 (8.15-11.95) respectively) compared to other conditions as HC (6.57 (5.66-7.18), p= 0.018 and p= 0.001) and OA (5.54 (5.06-7.92), p= 0.049 and p= 0.002), being comparable between UPIA (7.76 (6.46-8.82) and MTX-naïve (10.02 (8.10-10.83) (**Fig. 11B**). IL-6 levels in REM were comparable with HC, OA and UPIA (3.39 (1.99-18.45), 2.39 (1.94-2.81), 2.94 (2.3-3.29), 3.18 (2.23-3.74), respectively) but lower than MTX-naïve and DMARDs-not responder RA (12.70 (4.84-34.25), p= 0.022 and 27.20 (5.65-127.00), p= 0.005 respectively) (**Fig. 11C**). IFN-gamma concentration was slightly higher in REM than in HC (1.39 (1.17-2.35) and 1.21 (0.86-1.30), respectively, p= 0.021) but comparable with other conditions (OA: 1.37 (1.25-3.68); UPIA 1.31 (1.01-1.69); MTX-naïve RA (1.36 (1.02-2.12); DMARDs-not responder RA (1.55 (1.39-4.26)) (**Fig. 11D**). Similarly, IL-12p70 levels was higher in RA sustained remission (1.60 (1.28-2.34)) compared to HC and UPIA (1.16 (0.92-1.40), p= 0.035 and 1.04 (0.88-1.24), respectively, p= 0.002) but comparable with OA, MTX-naïve and DMARDs-not responder RA (1.02 (0.75-1.48), 1.38 (1.10-1.94), 2.03 (1.33-2.89) respectively) (**Fig. 11E**). Finally, within the pro-inflammatory chemokines, comparable Chemerin concentration was detected between REM, HC, OA and UPIA (50.72 (37.00-64.08), 48.49 (46.02-59.00), 48.37 (33.54-67.53), 62.59 (48.31-95.73) respectively). In contrast higher Chemerin level was found in MTX-naïve (82.75 (71.12-97.17) comparing to REM and DMARDs-not responder RA (50.72 (37.00-64.08), p= 0.0001 and 66.43 (43.30-89.79), p= 0.049, respectively) (**Fig. 11F**). Within anti-inflammatory cytokines and chemokines, IL-10 significantly higher levels were found in REM than in UPIA patients (2.57 (2.00-3.26) and 2.16 (1.95-2.36), respectively, p= 0.031) but comparable with other groups (HC: 2.28 (2.02-2.38); OA: 1.67 (1.50-2.78); MTX-naïve RA (2.85 (2.11-4.01); DMARDs-non responder RA (2.73 (2.31-6.50)) (**Fig. 11G**). Considering IL-4 levels, it presented the highest level in REM (0.84 (0.66-1.33)) comparing to other condition as HC (0.58 (0.30-0.84), p= 0.035), UPIA (0.51 (0.39-0.72), p= 0.006) and MTX-naïve RA (0.63 (0.35-0.84), p= 0.021) (**Fig. 11H**). Finally, comparable levels of IL-2 and GAS6 were found between REM and other

conditions (**Fig. 11I-11J**).

Considering the UPIA cohort, only Chemerin showed a directly correlation with BMI (r= 0.66, p= 0.038) and CRP (r= 0.71, p= 0.009) (**Fig.12A**). When considered the next phases, in whole RA cohort, IL-6 directly correlated with ESR (r= 0.47, p= 0.0001) and CRP (r= 0.59, p= 0.0001) and also with PD grade (r= 0.35, p= 0.01), KSS (r= 0.43, p= 0.0001), DAS28-CRP (r= 0.47, p= 0.0001) and CDAI (r= 0.62, p= 0.0001) while the pro-inflammatory cytokine inversely correlated with disease duration (r= -0.41, p= 0.001). Furthermore, IL-12p70 weakly correlated with BMI (r= 0.261, p= 0.043). In contrast, within the anti-inflammatory molecules, IL-4 inversely correlated with PD grade, KSS and CDAI (r= -0.39, p= 0.004; r= -0.35, p= 0.005; r= 0.47, p= 0.0001; r= -0.33, p= 0.019 respectively) (**Fig. 12B**).

In particular, considering RA subgroups (**Fig. 12C**), in MTX-naïve patients IL-6 directly correlated with ESR (r= 0.55, p= 0.004), CRP (r= 0.75, p= 0.0001), KSS (r= 0.42, p= 0.042), DAS28-CRP (r= 0.68, p= 0.0001) and CDAI (r= 0.50, p= 0.025). Moreover, in DMARDs-non responder patients IL-6 still presented direct correlation with CDAI (r= 0.64, p= 0.010) and KSS (r= 0.53, p= 0.013) and inverse correlation with disease duration (r= -0.59, p= 0.007). Differently to other conditions, in that subgroup IL-1beta directly correlated to ESR (r= 0.62, p= 0.003), CRP (r= 0.57, p= 0.007) and DAS28-CRP (r= 0.43, p= 0.050). Finally, in RA patients in sustained remissions, TNF-alpha directly correlated to disease duration (r= 0.55, p= 0.026), while IL-12p70 to CRP (r= 0.54, p= 0.032). Moreover, age directly correlated with anti-inflammatory cytokines as IL-2, IL-4 and IL-10 (r= 0.53, p= 0.029; r= 0.55, p= 0.023; r= 0.75, p= 0.001 respectively).

In conclusion IL-6, Chemerin, IL-10 and IL-4 directly correlated with the patients' clinical status in terms of DAS28-CRP (or CDAI), PD grade and KSS respectively in RA. Moreover, IL-6 directly correlated with the patients' clinical status in terms of DAS28-CRP (or CDAI) and KSS respectively in MTX-naïve and DMARDs-not Responder RA patients in RA subgroups with the exception of sustained remission RA, where no correlations were found, due to the lowest level of inflammatory status.

Table 5. Demographic, immunological, clinical, ultrasound and histological findings of enrolled subjects for ELISA analysis

ELISA ANALYSIS	HC (N: 10)	OA (N: 7)	UPIA (N: 13)	RA (N: 64)	p-value
Age	50.90 (10.77)	32.00 (30.63)	57.54 (9.53)	57.55 (13.29)	0.061
Female (n, %)	8 (80%)	3 (43%)	9 (69%)	48 (75%)	0.168
BMI (Kg/mq)	26.43 (5.27)	27.80 (3.11)	27.52 (5.05)	24.61 (4.61)	0.303
Smoking Status (n, %)	4 (40%)	0 (0%)	5 (38%)	16 (25%)	0.145
Disease Duration (mo)		78.00 (1.41)	45.83 (44.98)	78.43 (99.73)	**0.022**
RF/ACPA positivity (n, %)		0 (0%)	5 (42%)	35 (58%)	**0.016**
ESR (mm/h)		11.25 (10.53)	30.46 (21.17)	36.41 (28.64)	**0.004**
CRP (mg/l)		0.8 (0.81)	6.14 (13.79)	23.85 (35.56)	**0.003**
TJC-28		2.50 (2.51)	1.75 (1.66)	5.13 (4.86)	**0.018**
SJC-28		3.50 (4.43)	2.25 (0.96)	4.78 (4.59)	**0.022**
DAS28-CRP			3.28 (1.14)	4.35 (2.08)	**0.038**
CDAI			13.81 (4.14)	19.59 (15.68)	**0.041**
SMH (cm)		0.97 (0.20)	0.92 (0.17)	0.98 (0.26)	0.938
Power-doppler Grade		0.67 (0.58)	1.10 (0.99)	1.25 (1.04)	0.051
Krenn Score		2.50 (1.00)	3.00 (2.04)	3.85 (2.57)	**0.038**
Steroids (n, %)		0 (0%)	0 (0%)	14 (22%)	0.072
csDMARDs (n, %)		0 (0%)	0 (0%)	30 (47%)	**<0.0001**
b/tsDMARDs (n, %)		0 (0%)	0 (0%)	19 (30%)	**0.022**

HC: healthy controls; OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic Disease Modifying Anti-Rheumatic Drugs; b/tsDMARDs: biological / target synthetic Disease Modifying Anti-Rheumatic Drugs. Mean ± SD or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

Table 6. Demographic, immunological, clinical, ultrasound and histological findings of enrolled RA patients divided by clinical phases for ELISA analysis

ELISA ANALYSIS	MTX-NAÏVE (a) (N: 26)	DMARDs-NOT RESPONDER (b) (N: 21)	REMISSION (c) (N: 17)	p-value a vs b	p-value a vs c	p-value b vs c
Age	58.38 (13.44)	59.33 (13.91)	54.06 (12.39)		0.573	
Female (n, %)	20 (77%)	16 (76%)	12 (71%)		0.885	
BMI (Kg/mq)	23.95 (4.25)	25.66 (5.27)	24.22 (4.23)		0.439	
Smoking Status (n, %)	7 (27%)	4 (19%)	5 (29%)		0.732	
Disease Duration (mo)	26.44 (46.50)	102.95 (113.36)	129.00 (109.60)	**0.0001**	**0.0001**	0.101
RF/ACPA positivity (n, %)	11 (42%)	13 (72%)	11 (69%)	0.181	0.083	0.666
ESR (mm/h)	46.92 (30.88)	41.24 (25.71)	13.00 (10.62)	0.971	**0.0001**	**0.0001**
CRP (mg/l)	34.27 (39.33)	28.31 (37.53)	1.06 (1.12)	0.542	**0.0001**	**0.0001**
TJC-28	7.12 (3.26)	6.86 (5.52)	0.06 (0.24)	0.636	**0.0001**	**0.0001**
SJC-28	6.80 (3.37)	6.14 (5.12)	0.12 (0.33)	0.614	**0.0001**	**0.0001**
DAS28-CRP	5.32 (1.43)	5.40 (1.35)	1.58 (0.69)	0.598	**0.0001**	**0.0001**
CDAI	28.25 (9.25)	26.55 (14.67)	1.07 (1.67)	0.469	**0.0001**	**0.0001**
SMH (cm)	1.05 (0.25)	1.01 (0.26)	0.84 (0.23)	0.654	0.113	0.320
Power-doppler Grade	2.00 (0.82)	1.26 (0.80)	0.07 (0.27)	**0.01**	**0.0001**	**0.0001**
Krenn Score	5.04 (2.40)	4.14 (2.51)	1.82 (1.55)	0.053	**0.0001**	**0.001**
Steroids (n, %)	3 (11%)	11 (52%)	0 (0%)	**0.002**	0.146	**<0.0001**
csDMARDs (n, %)	0 (0%)	18 (86%)	12 (71%)	**<0.0001**	**<0.0001**	0.255
b/tsDMARDs (n, %)	0 (0%)	8 (38%)	11 (65%)	**<0.0001**	**<0.0001**	0.103

MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic DMARDs; b/tsDMARDs: biological / target synthetic DMARDs. Mean ± SD or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

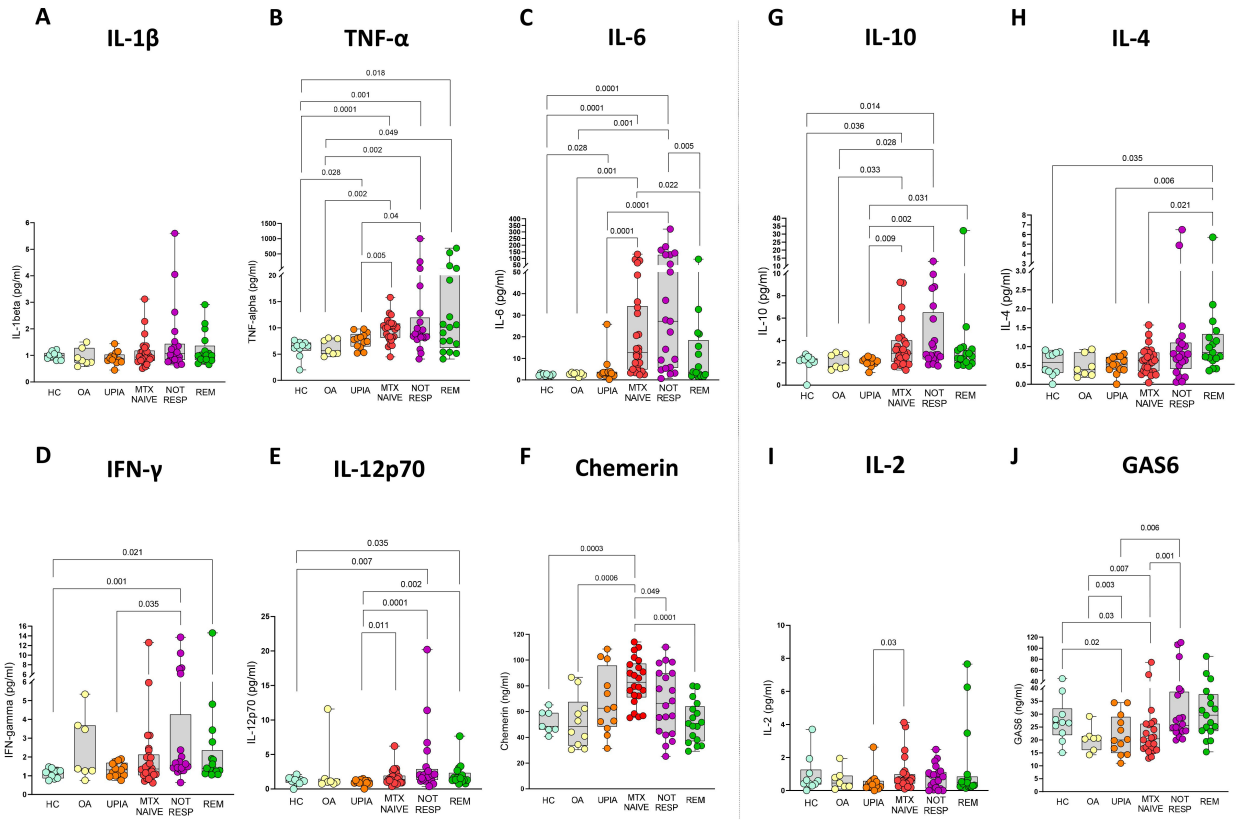


Figure 11. Serum cytokines and chemokines plasma levels determination across diseases phases.
A: IL-1beta concentration did not change between different condition. **B:** TNF-alpha concentration was higher in sustained remission RA patients (10.01 (6.18-22.55)) compared to HC (6.57 (5.66-7.18), p= 0.018) and OA (5.54 (5.06-7.92), p= 0.049) but comparable to UPIA (7.76 (6.46-8.82) and MTX-naïve (10.02 (8.10-10.83). **C:** IL-6 levels were comparable between RA in sustained remission (3.39 (1.99-18.45)) and HC, OA and UPIA but lower than MTX-naïve and DMARDs-not responder RA (12.70 (4.84-34.25), p= 0.022 and 27.20 (5.65-127.00), p= 0.005 respectively). **D:** IFN-gamma concentration was slightly higher in RA sustained remission than HC (1.39 (1.17-2.35) vs 1.21 (0.86-1.30) respectively; p= 0.021) but comparable with other conditions. **E:** IL-12p70 level was higher in RA sustained remission (1.60 (1.28-2.34)) rather than HC and UPIA (1.16 (0.92-1.40), p= 0.035 and 1.04 (0.88-1.24), p= 0.002) but comparable to other conditions. **F:** Chemerin concentration was comparable between RA sustained remission (50.72 (37.00-64.08)) and other conditions, with the exception to MTX-naïve (82.75 (71.12-97.17), p= 0.0001). **G:** significantly IL-10 higher levels were found in sustained remission RA than UPIA patients (2.57 (2.00-3.26) and 2.16 (1.95-2.36) respectively, p= 0.031) but comparable to other conditions. **H:** IL-4 presented the highest level in RA sustained remission (0.84 (0.66-1.33)) comparing to HC (0.58 (0.30-0.84), p= 0.035), UPIA (0.51 (0.39-0.72), p= 0.006) and MTX-naïve RA (0.63 (0.35-0.84), p= 0.021). **I:** comparable IL-2 levels were found between sustained remission RA and other conditions. **J:** comparable GAS6 levels were found between sustained remission RA and other conditions.
The values are expressed as median and IQR (grey bar). Minimal and maximal values are also shown. Each dot represents a patient. P-value <0.05 was considered statistically significant. HC: healthy controls; OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs; REM: sustained remission RA.

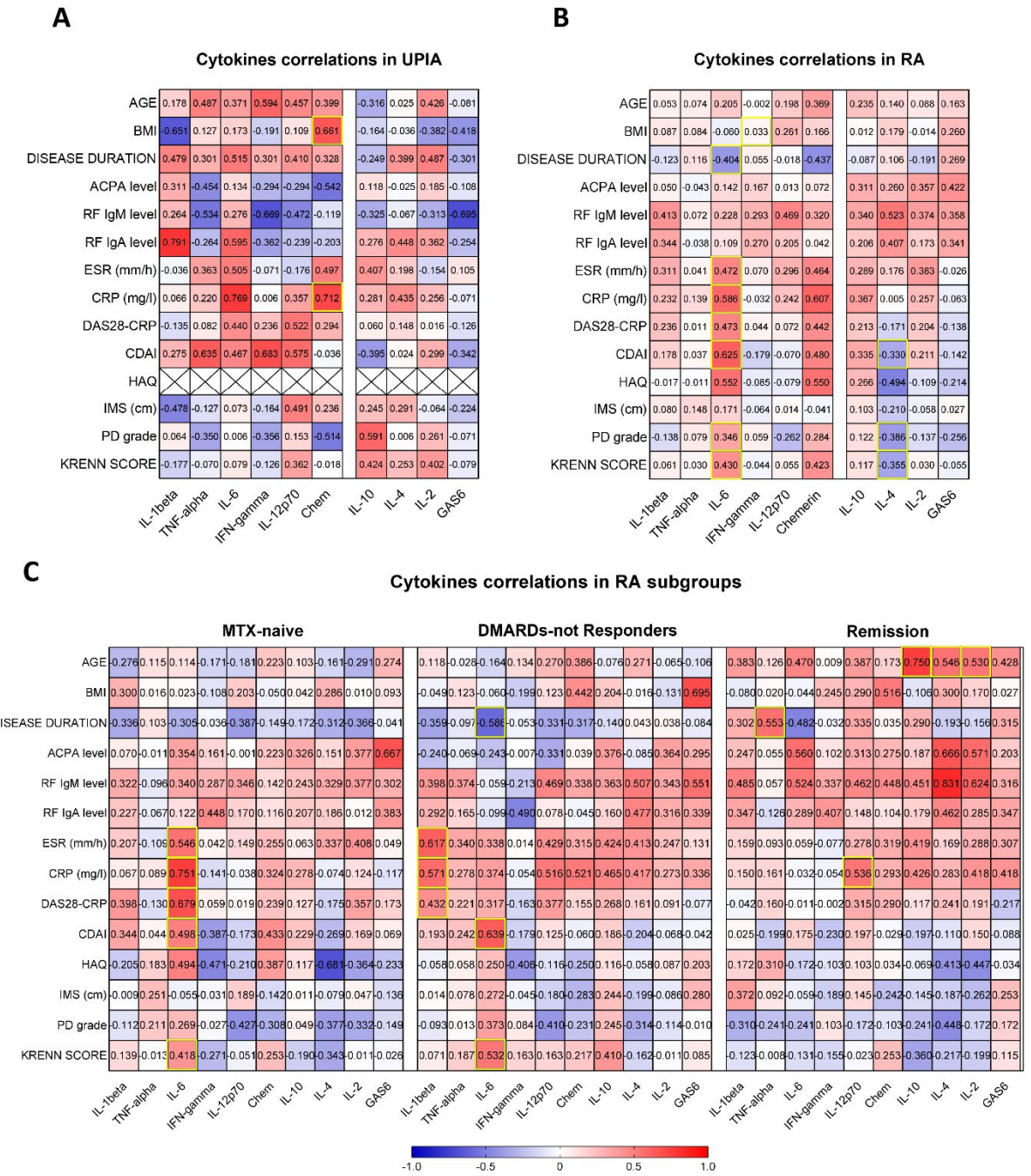


Figure 12. Correlation heatmaps of serum cytokines and chemokines plasma levels and demographics, clinical, immunological, ultrasound and histological features of UPIA (A) and RA groups (B-C).
UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index; RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre). Two-tailed Spearman's correlation are shown in every cell. Yellow square: p-value <0.05, considered statistically significant.

4.3.4 SPM LEVELS EVALUATION IN RA SYNOVIAL TISSUE

To assess the balance between pro-inflammatory and anti-inflammatory/pro-resolving molecules, a pilot study was performed to assess SPM and pro-inflammatory arachidonic acid-derived molecules in synovial tissue of one RA patient in sustained remission (REM; patient: REM1) and three RA patients in high disease activity (HDA; patients: HD1, HD2, HD3) (**Fig. 13A-13B**). These data showed that REM patient presented lower levels of pro-inflammatory molecules than HDA patients (101.3 vs 2153.00 (84.06-3333.00,) respectively; in pg/100 mg of synovial tissue) (**Fig. 13C**) with a SPM/pro-inflammatory molecules ratio of 0.19 vs 0.04 (0.04-0.05) (**Fig. 13D**).

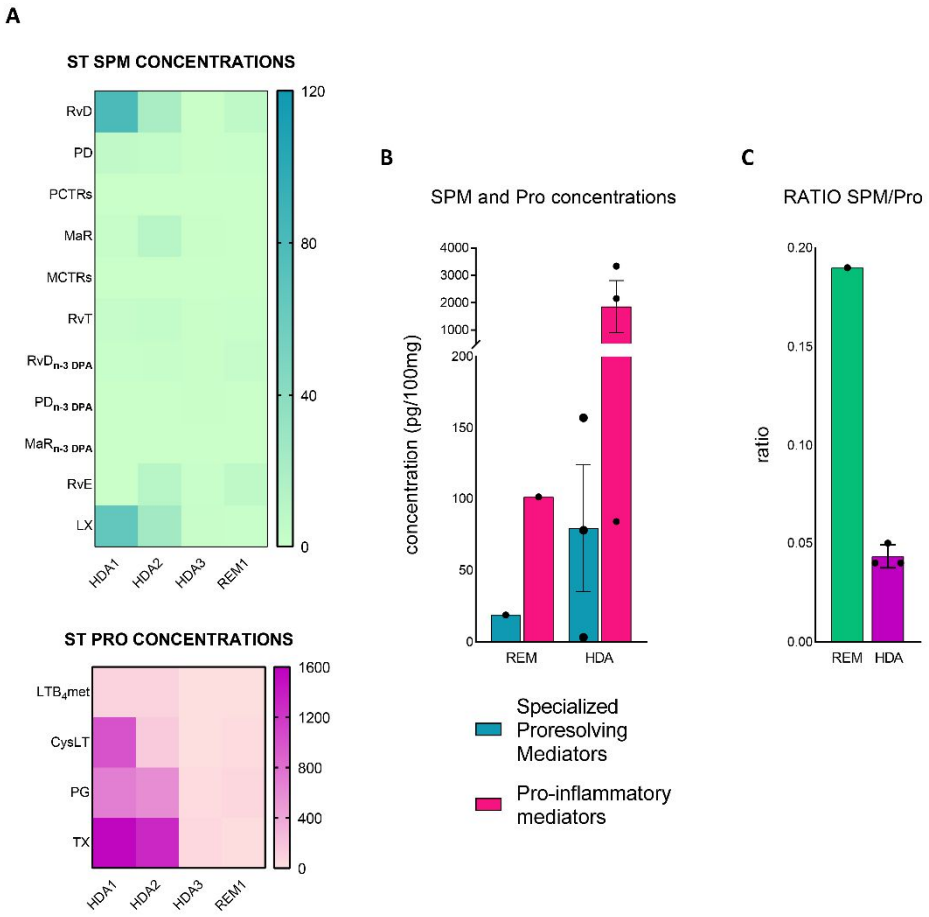


Figure 13. SPM and AA-derived pro-inflammatory mediators levels in RA synovial tissue.
A: concentration heatmap of synovial tissue SPM (green tone) and AA-derived pro-inflammatory mediators (pink tone) of studied patients. **B:** total concentrations of SPM (blue) and AA-derived pro-inflammatory mediators (red) of studied patients. **C:** ratio of total SPMs concentration and total AA-derived pro-inflammatory mediators in studied patients (green: remission patient; violet: HDA patients).
The values are expressed as mean ± SEM. Each dot represents a patient. ST: synovial tissue; SPM: specialized pro-resolving mediator; AA: arachidonic acid; Pro: AA-derived pro-inflammatory mediators; Rv: Resolvin series; PD: Protectin; MaR: Maresin; LX: Lipoxin series; LT: Leukotrien series; PG: Prostaglandins; TX: Tromboxan; HDA: high disease activity RA patients; REM: sustained remission RA patient.

4.3.5 ERV1, ALX/FPR2 AND BLT1 EXPRESSION IN RA SYNOVIAL TISSUE

Table 7 summarizes the demographic, immunological, clinical, ultrasound and histological findings of RA patients, who was divided between DAS28-CRP based disease activity as high disease activity (HDA, DAS28-CRP > 4.1) and sustained remission (REM, DAS28-CRP < 2.3) to assess the expression of ERV1, ALX/FPR2 and BLT1 in synovial tissue by RT-PCR (as fold changes), comparing to OA control group (**Fig. 14A-14B**). In synovial tissue, high expression of ERV1, ALX/FPR2 and BLT1 were found in HDA (4.40 (2.70-11.90); 4.90 (3.20-5.45); 5.90 (3.85-10.30) respectively) compared to REM (1.10 (0.60-5.70), p= 0.012; 1.50 (0.80-2.60), p= 0.0006; 1.60 (1.40-6.60), p= 0.016, respectively) and OA (1.20 (0.40-1.40, p= 0.005; 0.80 (0.50-2.70), p= 0.003; 1.10 (0.40-2.70), p= 0.002, respectively). As shown in **Fig. 14C-14E**, all the studied genes expressions were comparable between REM and OA condition.

Considering RA cohort, ERV1 and ALX/FPR2 expression inversely correlated with disease duration (r= -0.43, p= 0.037; r= -0.48, p= 0.016 respectively). Furthermore, the expression of the same genes directly correlated with DAS28-CRP, CDAI and PD grade (r= 0.60, p= 0.002, r= 0.75, p= 0.0001; r= 0.41, p= 0.049, r= 0.59, p= 0.003; r= 0.47, p= 0.033, r= 0.66, p= 0.001, respectively). Moreover, BLT1 expression directly correlated with DAS28-CRP (r= 0.58, p= 0.003) and ALX/FPR2 with KSS (r= 0.57, p= 0.003) (**Fig. 15**).

To assess if synovial tissue macroscopic phenotype could relate to SPM receptors gene expressions, the fold changes values of these genes were analysed stratifying RA patients by phenotype. The expression of ERV1, ALX/FPR2 and BLT1 was comparable in RA patients with compact vs villous synovitis (**Fig. 16A**).

Finally, to evaluate the potential predictive value of SPM receptors gene expression, the fold changes values of these genes were analysed stratifying REM-RA patients according to disease flares occurred within 12 months from the study enrolment (**Fig. 16B**). Although the expression of ERV1, ALX/FPR2 and BLT1 was comparable between patients flaring or not, ERV1 and BLT1 presented low fold change values in patients who experimented a flare disease than who did not (ERV1: 0.40 (0.30-1.10) vs 0.70 (0.40-4.40) respectively, p= 0.5714; BLT1: 0.60 (0.50-0.60) vs 0.90 (0.40-2.70) respectively, p= 0.4203) (**Fig. 16C**).

In conclusion, the expression of ERV1, ALX/FPR2 and BLT1 genes were influenced by disease activity and were higher in HDA than in REM status. The analysis failed to demonstrate a possible role of SPM receptors genes expression as predictive factor to have disease flare or to maintain remission status.

Table 7. Demographic, immunological, clinical, ultrasound and histological findings of enrolled subjects for RT-PCR analysis

RT-PCR ANALYSIS	OA (a, N: 7)	HDA (b, N: 9)	REM (c, N: 15)	p-value a vs b	p-value a vs c	p-value b vs c
Age	32.00 (30.63)	56.89 (11.38)	56.07 (12.72)		0.07	
Female (n, %)	4 (57%)	5 (56%)	11 (73%)		0.61	
BMI (Kg/mq)	27.80 (3.11)	23.19 (4.18)	25.09 (3.04)		0.43	
Smoking Status (n, %)	0 (0%)	3 (33%)	3 (20%)		0.24	
Disease Duration (mo)	78.00 (1.41)	30.78 (54.89)	120.47 (115.74)	0.070	**0.0001**	0.301
RF/ACPA positivity (n, %)	0 (0%)	5 (56%)	9 (60%)	**0.046**	**0.008**	0.831
ESR (mm/h)	11.25 (10.53)	65.11 (29.58)	12.64 (11.14)	**0.0001**	0.451	**0.0001**
CRP (mg/l)	0.80 (0.81)	70.46 (45.22)	1.38 (1.36)	**0.0001**	0.520	**0.0001**
TJC-28	2.50 (2.52)	8.78 (6.79)	0	**0.031**	0.082	**0.016**
SJC-28	3.50 (4.43)	8.33 (6.42)	0.20 (0.56)	**0.022**	0.063	**0.0001**
DAS28-CRP		6.50 (0.93)	1.66 (0.74)			**0.003**
CDAI		33.00 (14.86)	1.50 (2.14)			**0.0001**
SMH (cm)	0.97 (0.20)	1.12 (0.24)	0.83 (0.25)	0.655	0.726	0.511
Power-doppler Grade	0.67 (0.58)	2.50 (0.75)	0	**0.001**	0.454	**0.001**
Krenn Score	2.50 (1.00)	6.44 (1.94)	2.13 (1.55)	**0.014**	0.615	**0.011**
Steroids (n, %)	0 (0%)	1 (11%)	0 (0%)	0.362		0.187
csDMARDs (n, %)	0 (0%)	2 (22%)	11 (73%)	0.182	**0.001**	**0.01**
b/tsDMARDs (n, %)	0 (0%)	2 (22%)	11 (73%)	0.182	**0.001**	**0.01**

OA: osteoarthritis; HDA: high disease activity RA patients; REM: sustained remission RA patients; RA: rheumatoid arthritis; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic Disease Modifying Anti-Rheumatic Drugs; b/tsDMARDs: biological / target synthetic Disease Modifying Anti-Rheumatic Drugs. Mean ± SD or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

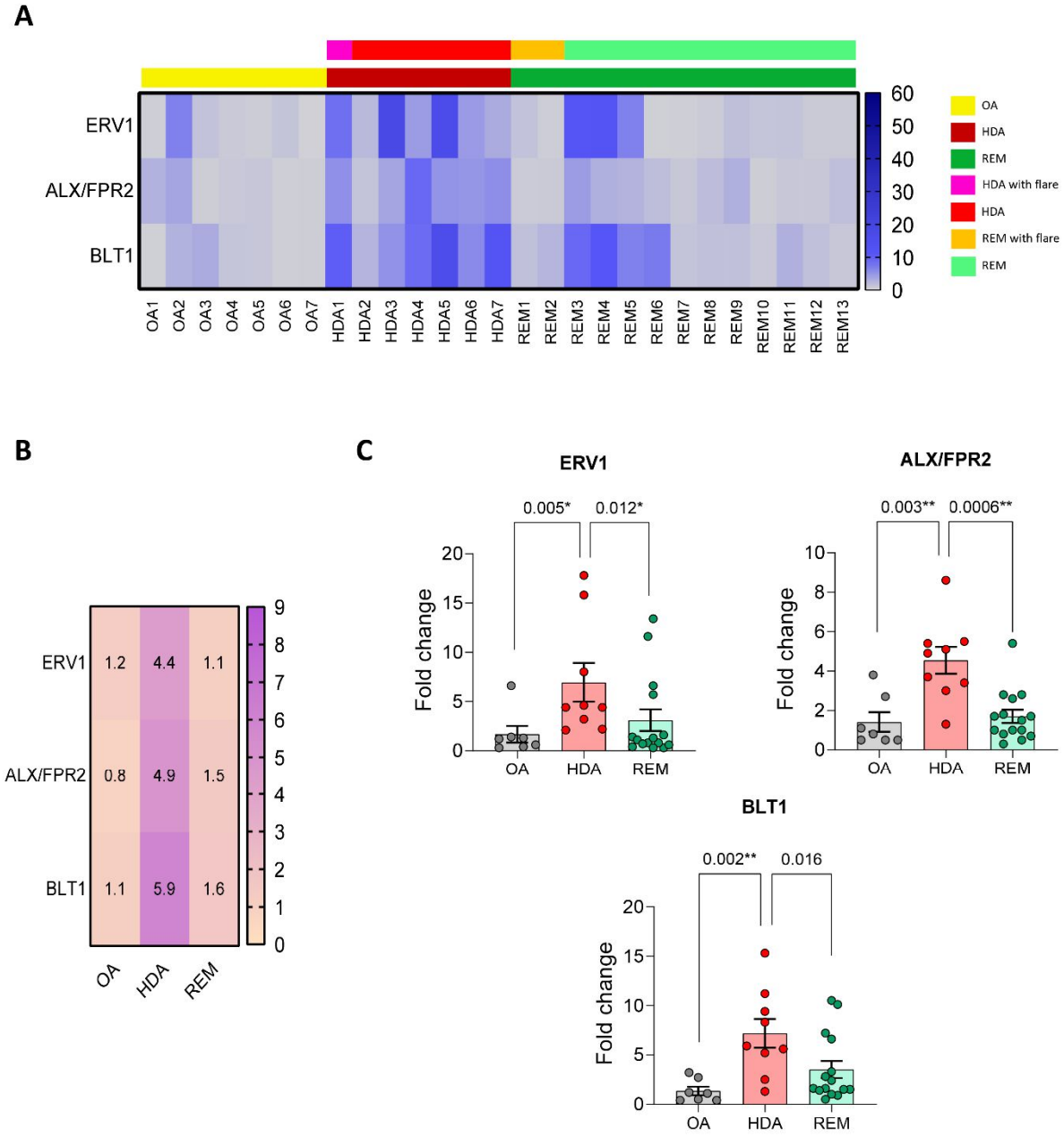


Figure 14. SPM receptors expression in RA synovial tissue determined by qPCR on the time of biopsy performance.

A: heatmap of ERV1, ALX/FPR2 and BLT1 receptor expression. Each row represents a gene within each cluster. Each bar represents a single patient. **B:** heatmap of ERV1, ALX/FPR2 and BLT1 receptor expression. Each row represents a gene. Each bar represents a group. **C:** ERV1, ALX/FPR2 and BLT1 receptor expression between each group. The expression of the studied SPM receptors was comparable between sustained remission RA (1.10 (0.60-5.70); 1.50 (0.80-2.60); 1.60 (1.40-6.60) respectively) and OA (1.20 (0.40-1.40); 0.80 (0.50-2.70); 1.10 (0.40-2.70) respectively) but lower than HDA condition (4.40 (2.70-11.90), p= 0.012; 4.90 (3.20-5.45), p= 0.0006; 5.90 (3.85-10.30), p= 0.016 respectively).

Gene expression is expressed as fold change value. The values are expressed as mean ± SEM. P-value <0.05 was considered statistically significant. OA: osteoarthritis; HDA: high disease activity RA group; REM: sustained remission RA group.

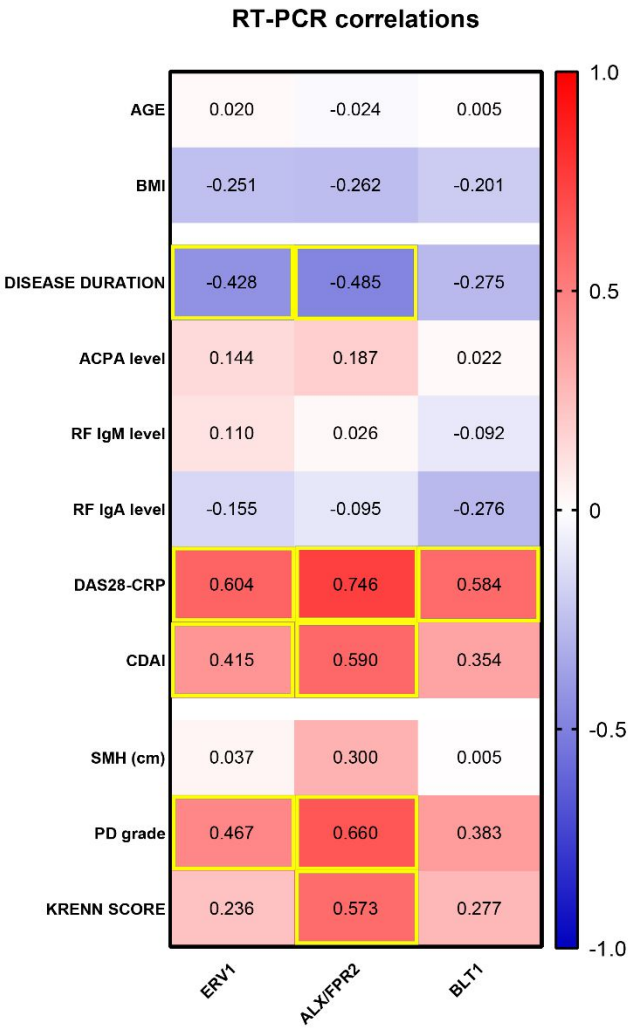


Figure 15. SPM receptors expression correlation with demographic, clinical, immunological, ultrasound and histological findings in RA groups.
BMI: Body Mass Index; RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre). Two-tailed Spearman's correlation are shown in every cell. Yellow square: p-value <0.05, considered statistically significant.

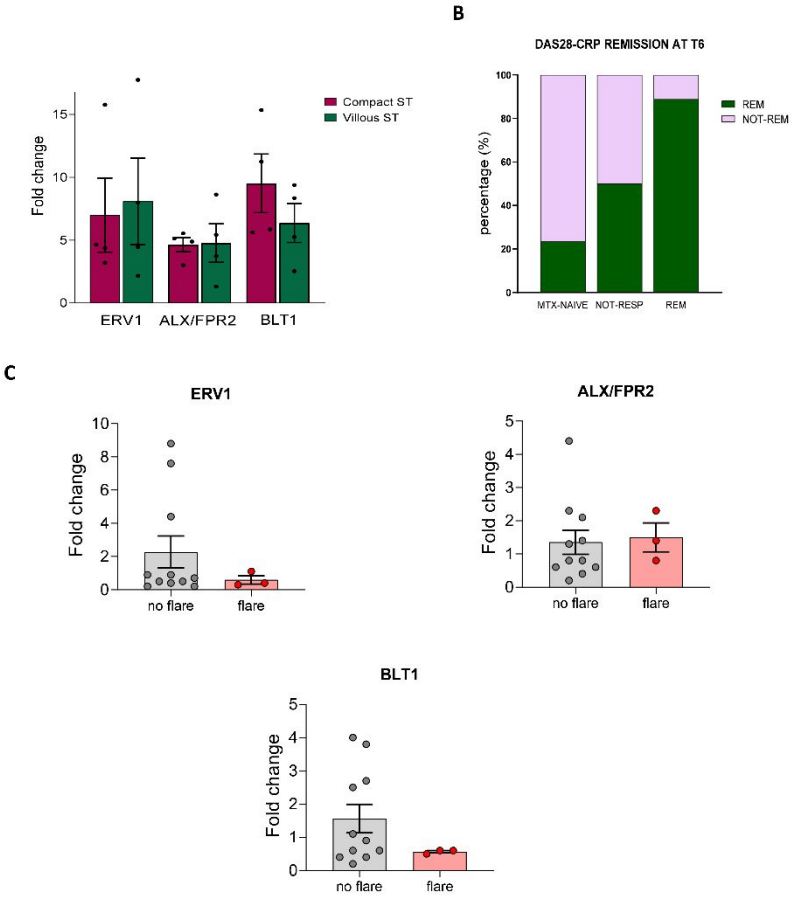


Figure 16. SPM receptors expression in patients stratified by macroscopic synovial tissue aspect and by disease flare within 12 months follow-up.
A: the expression of ERV1, ALX/FPR2 and BLT1 (as fold change) was comparable in RA patients with compact vs villous synovitis. **B:** cumulative histograms in which are shown the percentages of patients who experimented (pink) or not (green) a disease flare within 12 months from the study enrolment, across RA subgroups. **C:** the expression of ERV1, ALX/FPR2 and BLT1 was comparable between the RA patients who experimented a disease flare vs who did not and remained in sustained remission. Each dot is a patient. The values are expressed as mean ± SEM.

4.3.6 PERIPHERAL BLOOD AND SYNOVIAL TISSUE CELLULAR COMPOSITION ACROSS DISEASE PHASES

Table 8, Table 9, Table 10 and **Table 11** summarize the demographic, immunological, clinical, ultrasound and histological findings of enrolled subjects to assess how peripheral blood and synovial tissue cellular composition changed across disease phase. For this purpose, cell percentages of peripheral blood and synovial tissue were evaluated. In particular, considering peripheral blood cellular composition, REM presented lower neutrophils percentage (30.59 (24.27-47.07)) compared to MTX-naïve (52.24 (48.54-63.83), p= 0.0001) and DMARDs-non responder (53.58 (38.59-59.66), p= 0.011) RA but comparable to HC (40.28 (34.08-46.44)), OA (43.29 (32.89-53.14)) and UPIA (45.94 (33.51-50.39). Similarly, CD3 cells percentage was lower in REM (73.95 (68.36-80.31)) compared to RA DMARDs-non responder (79.40 (73.93-85.76), p= 0.020) but comparable to HC (77.87 (77.01-84.23)), OA (73.03 (69.24-80.47) and UPIA (72.25 (67.68-83.91). Moreover, CD14 cell percentage were comparable between REM (8.96 (7.44-12.28)) and MTX-naïve (9.25 (6.89-11.29)) or DMARDs-not responder (10.66 (8.26-12.31) patients, but lower when compared to HC (6.58 (3.71-7.38), p= 0.004) and UPIA (6.70 (4.68-9.33), p= 0.031). Finally, CD19 cell percentage in REM was higher (12.59 (7.08-16.62)) than DMARDs-not responder (7.40 (6.45-12.73), p= 0.043) but comparable to HC (12.79 (8.98-14.92)), OA (13.21 (10.69-14.55)) and UPIA (13.85 (10.26-17.49)). No variation was found between different condition in NK cell percentage (**Fig. 17**).
Considering the whole RA cohort, PB neutrophils percentage inversely correlated to disease duration (r= -0.385, p= 0.003) but directly correlated to HAQ (r= 0.547, p= 0.002), DAS28-CRP (r= 0.497, p= 0.0001), CDAI (r= 0.445, p= 0.001), PD grade (r= 0.476, p= 0.0001), KSS (r= 0.294, p= 0.023) and IL-6 (r= 0.302, p= 0.020) and Chemerin (r= 0.284, p= 0.038) levels. Furthermore, PB CD3 cells percentage directly correlated with IL-12p70 (r= 0.277, p= 0.034) and Chemerin (r= 0.295, p= 0.030). Finally, PB CD19 cell percentage inversely correlated to age (r= -0.351, p= 0.005) (**Fig. 18A**). Considering UPIA cohort, few correlations was found with PB CD14 cell percentage and disease duration (r= -0.636, p= 0.048) and neutrophils percentage with IL-12p70 (r= 0.645, p= 0.032) (**Fig. 18B**). In RA sustained remission, PB CD19 cell percentage inversely correlated to age (r= -0.620, p= 0.014) (**Fig. 18C**).

Table 8. Demographic, immunological, clinical, ultrasound and histological findings of enrolled subjects for FACS analysis of PB derived cells

PB FACS ANALYSIS	HC (N: 10)	OA (N: 5)	UPIA (N: 11)	RA (N: 62)	p-value
Age	50.90 (10.77)	51.20 (13.37)	57.36 (9.52)	58.02 (13.42)	0.464
Female (n, %)	8 (80%)	4 (80%)	8 (73%)	46 (74%)	0.968
BMI (Kg/mq)	26.43 (5.27)	27.13 (2.48)	28.3 (5.19)	24.84 (4.71)	0.301
Smoking Status (n, %)	4 (40%)	0 (0%)	5 (45%)	15 (24%)	0.185
Disease Duration (mo)		78.00 (1.41)	48.20 (46.98)	82.91 (105.84)	**0.022**
RF/ACPA positivity (n, %)		0 (0%)	3 (30%)	34 (58%)	**0.017**
ESR (mm/h)		11.25 (10.53)	33.64 (21.56)	38.07 (28.42)	**0.0001**
CRP (mg/l)		0.8 (0.81)	6.58 (14.99)	24.80 (35.78)	**0.006**
TJC-28		2.00 (2.45)	1.60 (1.78)	5.36 (4.90)	**0.0001**
SJC-28		2.80 (4.15)	2.10 (0.994)	4.90 (4.63)	**0.0001**
DAS28-CRP			3.25 (1.21)	4.46 (2.06)	**0.013**
CDAI			13.41 (4.10)	20.57 (15.37)	**0.004**
SMH (cm)		0.97 (0.20)	0.93 (0.19)	0.98 (0.26)	0.891
Power-doppler Grade		0.67 (0.58)	1.00 (1.00)	1.21 (0.99)	**0.023**
Krenn Score		2.50 (1.00)	2.91 (2.12)	3.87 (2.55)	**0.011**
Steroids (n, %)		0 (0%)	0 (0%)	13 (21%)	0.134
csDMARDs (n, %)		0 (0%)	0 (0%)	30 (48%)	**0.002**
b/tsDMARDs (n, %)		0 (0%)	0 (0%)	16 (26%)	0.074

PB: peripheral blood compartment; HC: healthy controls; OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic Disease Modifying Anti-Rheumatic Drugs; b/tsDMARDs: biological/target synthetic Disease Modifying Anti-Rheumatic Drugs. Mean ± SD or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

Table 9. Demographic, immunological, clinical, ultrasound and histological findings of enrolled RA patients divided by clinical phases for FACS analysis of PB derived cells

PB FACS ANALISYS	MTX-NAÏVE (a) (N: 26)	DMARDs-NOT RESPONDER (b) (N: 21)	REMISSION (c) (N: 15)	p-value a vs b	p-value a vs c	p-value b vs c
Age	59.00 (14.01)	58.62 (13.99)	55.47 (12.01)		0.734	
Female (n, %)	20 (77%)	16 (76%)	10 (67%)		0.367	
BMI (Kg/mq)	24.26 (4.23)	26.07 (5.34)	24.01 (4.41)		0.439	
Smoking Status (n, %)	7 (27%)	4 (19%)	4 (27%)		0.211	
Disease Duration (mo)	27.04 (47.40)	113.35 (121.82)	135.21 (115.42)	**0.0001**	**0.0001**	0.351
RF/ACPA positivity (n, %)	11 (42%)	13 (68%)	10 (71%)	0.181	0.133	0.769
ESR (mm/h)	47.35 (30.45)	42.86 (25.13)	13.64 (10.96)	0.731	**0.0001**	**0.0001**
CRP (mg/l)	34.74 (38.99)	28.25 (37.51)	1.14 (1.18)	0.454	**0.0001**	**0.0001**
TJC-28	7.00 (3.91)	6.96 (5.31)	0.07 (0.26)	0.643	**0.0001**	**0.0001**
SJC-28	6.56 (3.49)	6.33 (5.19)	0.13 (0.35)	0.262	**0.0001**	**0.0001**
DAS28-CRP	5.29 (1.44)	5.45 (1.39)	1.62 (0.73)	0.591	**0.0001**	**0.0001**
CDAI	27.71 (9.34)	27.09 (14.27)	1.00 (1.78)	0.466	**0.0001**	**0.0001**
SMH (cm)	1.04 (0.26)	0.99 (0.23)	0.84 (0.25)	0.654	**0.041**	**0.047**
Power-doppler Grade	1.95 (0.84)	1.11 (0.58)	0	**0.013**	**0.0001**	**0.0001**
Krenn Score	5.12 (2.36)	3.85 (2.43)	1.87 (1.64)	0.061	**0.0001**	**0.001**
Steroids (n, %)	4 (15%)	9 (43%)	0 (0%)	**0.037**	0.110	**0.003**
csDMARDs (n, %)	1 (4%)	18 (86%)	11 (73%)	**<0.0001**	**<0.0001**	0.355
b/tsDMARDs (n, %)	0 (0%)	6 (28%)	10 (67%)	**0.003**	**<0.0001**	**0.023**

PB: peripheral blood compartment; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic DMARDs; b/tsDMARDs: biological/target synthetic DMARDs. Mean ± SD or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

Table 10. Demographic, immunological, clinical, ultrasound and histological findings of enrolled subjects for FACS analysis of ST derived cells

ST FACS ANALISYS	OA (N: 4)	UPIA (N: 8)	RA (N: 36)	p-value
Age	41.75 (29.31)	53.87 (7.16)	58.36 (11.46)	0.074
Female (n, %)	4 (80%)	5 (62%)	27 (75%)	0.423
BMI (Kg/mq)	25.60 (3.68)	27.6 (5.43)	24.75 (4.19)	0.308
Smoking Status (n, %)	0 (0%)	3 (37%)	12 (33%)	0.221
Disease Duration (mo)	78.00 (1.41)	35.14 (40.67)	72.25 (69.26)	**0.027**
RF/ACPA positivity (n, %)	0 (0%)	2 (29%)	21 (62%)	**0.016**
ESR (mm/h)	12.67 (12.42)	32.50 (25.86)	38.23 (33.14)	**0.0001**
CRP (mg/l)	0.40 (0.17)	8.11 (17.63)	27.62 (41.14)	**0.003**
TJC-28	2.67 (3.05)	2.43 (1.68)	5.03 (5.76)	**0.0001**
SJC-28	4.00 (5.29)	2.43 (0.98)	4.69 (5.41)	**0.003**
DAS28-CRP		3.05 (1.42)	4.11 (2.31)	**0.0001**
CDAI		13.25 (4.19)	19.53 (18.11)	**0.0001**
SMH (cm)	0.87 (0.03)	0.94 (0.20)	0.99 (0.29)	0.939
Power-doppler Grade	0.50 (0.71)	1.00 (1.26)	1.22 (1.09)	**0.0001**
Krenn Score	3.00 (0)	3.00 (2.20)	4.25 (2.72)	**0.0001**
Steroids (n, %)		0 (0%)	8 (22%)	0.114
csDMARDs (n, %)		0 (0%)	18 (50%)	**0.009**
b/tsDMARDs (n, %)		0 (0%)	16 (44%)	**0.018**

ST: synovial tissue compartment; OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic Disease Modifying Anti-Rheumatic Drugs; b/tsDMARDs: biological / target synthetic Disease Modifying Anti-Rheumatic Drugs. Mean ± SD or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

Table 11. Demographic, immunological, clinical, ultrasound and histological findings of enrolled RA patients divided by clinical phases for FACS analysis of ST derived cells

ST FACS ANALISYS	MTX-NAÏVE (a) (N: 12)	DMARDs-NOT RESPONDER (b) (N: 11)	REMISSION (c) (N: 13)	p-value a vs b	p-value a vs c	p-value b vs c
Age	63.09 (8.08)	57.09 (11.94)	55.08 (13.00)		0.551	
Female (n, %)	8 (67%)	8 (73%)	11 (85%)		0.572	
BMI (Kg/mq)	23.58 (2.64)	25.95 (5.73)	24.63 (3.63)		0.371	
Smoking Status (n, %)	6 (50%)	3 (27%)	3 (23%)		0.317	
Disease Duration (mo)	24.25 (39.69)	85.55 (87.87)	105.31 (49.61)	**0.0001**	**0.0001**	0.071
RF/ACPA positivity (n, %)	4 (33%)	8 (73%)	9 (69%)	0.058	0.073	0.851
ESR (mm/h)	58.00 (37.86)	44.91 (26.36)	12.33 (11.32)	0.331	**0.0001**	**0.0001**
CRP (mg/l)	52.09 (48.13)	29.68 (40.58)	1.25 (1.25)	0.291	**0.0001**	**0.0001**
TJC-28	8.00 (3.69)	7.73 (7.04)	0	0.641	**0.0001**	**0.0001**
SJC-28	7.33 (4.07)	7.36 (6.28)	0	0.266	**0.0001**	**0.0001**
DAS28-CRP	5.90 (0.74)	5.35 (1.59)	1.41 (0.69)	0.491	**0.0001**	**0.0001**
CDAI	31.04 (9.23)	30.61 (17.58)	0.67 (0.98)	0.467	**0.0001**	**0.0001**
SMH (cm)	1.02 (0.29)	1.11 (0.27)	0.84 (0.27)	0.891	**0.017**	**0.021**
Power-doppler Grade	2.00 (0.81)	1.54 (0.93)	0.10 (0.32)	**0.038**	**0.0001**	**0.0001**
Krenn Score	5.72 (2.19)	5.27 (2.83)	2.15 (1.62)	0.142	**0.0001**	**0.001**
Steroids (n, %)	2 (17%)	6 (54%)	0 (0%)	0.057	0.125	**0.002**
csDMARDs (n, %)	0 (0%)	8 (73%)	10 (77%)	**<0.0001**	**<0.0001**	0.813
b/tsDMARDs (n, %)	0 (0%)	7 (64%)	9 (69%)	**0.002**	**<0.0001**	0.772

ST: synovial tissue compartment; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic DMARDs; b/tsDMARDs: biological/target synthetic DMARDs. Mean ± SD or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

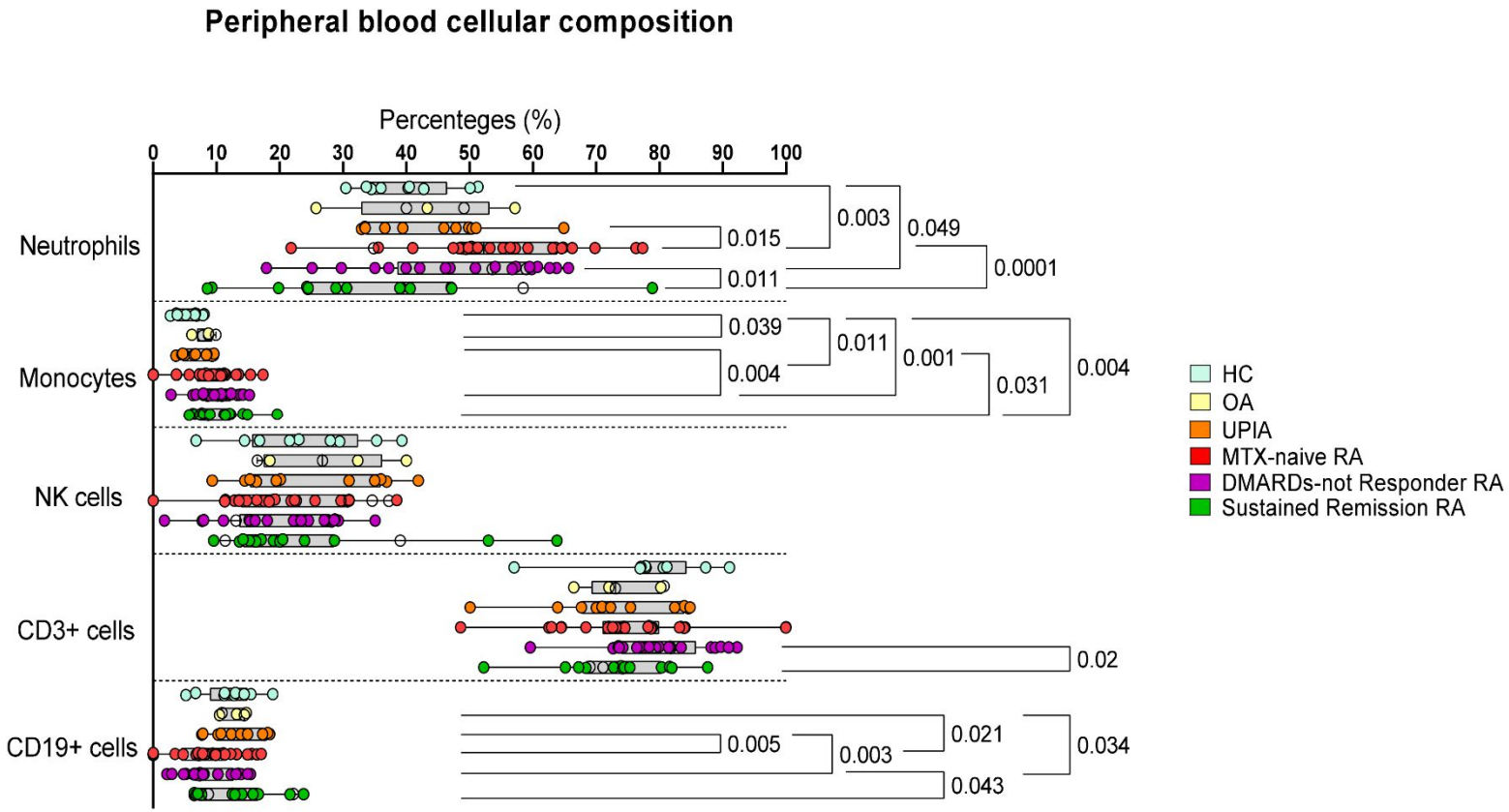


Figure 17. Peripheral blood cellular composition across disease phases

RA patients in sustained remission presented lower neutrophils percentage (30.59 (24.27-47.07)) compared to MTX-naïve (52.24 (48.54-63.83), p= 0.0001) and DMARDs-not responder (53.58 (38.59-59.66), p= 0.011). Similarly, CD3 cells percentage was lower in RA sustained remission (73.95 (68.36-80.31)) compared to RA DMARDs-not responder (79.40 (73.93-85.76), p= 0.020). Moreover, CD14 cell percentage where lower between RA sustained remission and HC (6.58 (3.71-7.38), p= 0.004) and UPIA (6.70 (4.68-9.33), p= 0.031). Furthermore, CD19 cell percentage in RA sustained remission was higher (12.59 (7.08-16.62)) than DMARDs-not responder (7.40 (6.45-12.73), p= 0.043). No variation was found between different condition in NK cell percentage. Finally, sustained remission RA presented comparable percentages of PB derived cells with UPIA, OA and HC.

The values are expressed as median and IQR (grey bar). Minimal and maximal values are also shown. Each dot represents a patient. P-value <0.05 was considered statistically significant. HC: healthy controls; OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs.

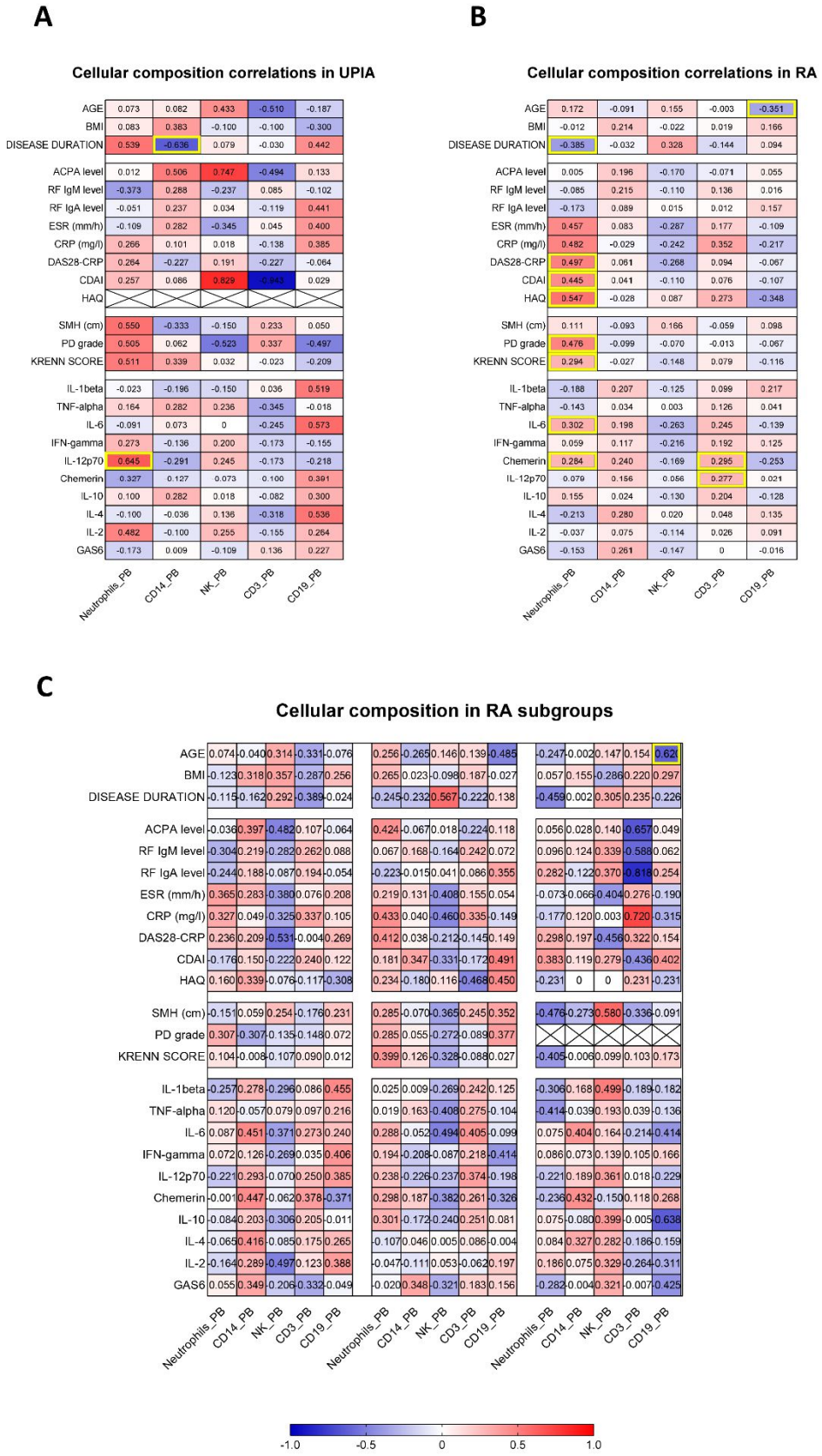


Figure 18. Correlation heatmaps of peripheral blood cellular composition vs demographics, clinical, immunological, ultrasound and histological features of UPIA (A) and RA groups (B-C).
UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index; RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre). Two-tailed Spearman's correlation are shown in every cell. Yellow square: p-value <0.05, considered statistically significant.

Considering synovial tissue cellular composition, REM presented lower ST macrophages percentage (5.35 (3.06-8.30)) compared to MTX-naïve (15.41 (10.14-30.80), p= 0.004) and DMARDs-non responder (10.58 (8.07-18.08), p= 0.029) RA but comparable to UPIA (6.83 (4.13-12.46)). No variation was found between different condition in ST CD3 and ST CD19 cells percentage (**Fig. 19**). Considering the whole RA cohort, ST macrophages percentage inversely correlated to disease duration (r= -0.556, p= 0.001) but directly correlated to DAS28-CRP (r= 0.609, p= 0.0001), CDAI (r= 0.522, p= 0.003), PD grade (r= 0.417, p= 0.020), KSS (r= 0.343, p= 0.043), IL-6 (r= 0.340, p= 0.043) and Chemerin (r= 0.416, p= 0.018) levels. Furthermore, ST CD3 cells percentage directly correlated with DAS28-CRP (r= 0.447, p= 0.012) and CDAI (r= 0.381, p= 0.050). (**Fig. 20A**). Considering UPIA cohort, few correlations was found with ST macrophages and age (r= -0.975, p= 0.005) and IL-10 (r= 0.900, p= 0.037) (**Fig. 20B**). In REM, ST CD3 cell percentage directly correlated to IL-10 levels (r= 0.699, p= 0.011) (**Fig. 20C**).

In conclusion, both PB and ST cellular fractions were influenced by inflammatory status assessed by clinical indexes. In particular, in PB the percentages of innate and adaptive immune cells returned at the levels of HC in sustained remission RA, except for CD14+ monocytes which persisted in high percentage. Furthermore, in ST the percentages of macrophages progressively decreased across RA phases from MTX-naïve to REM. In contrast, the percentages of CD3+ and CD19+ cells remained comparable between the different conditions.

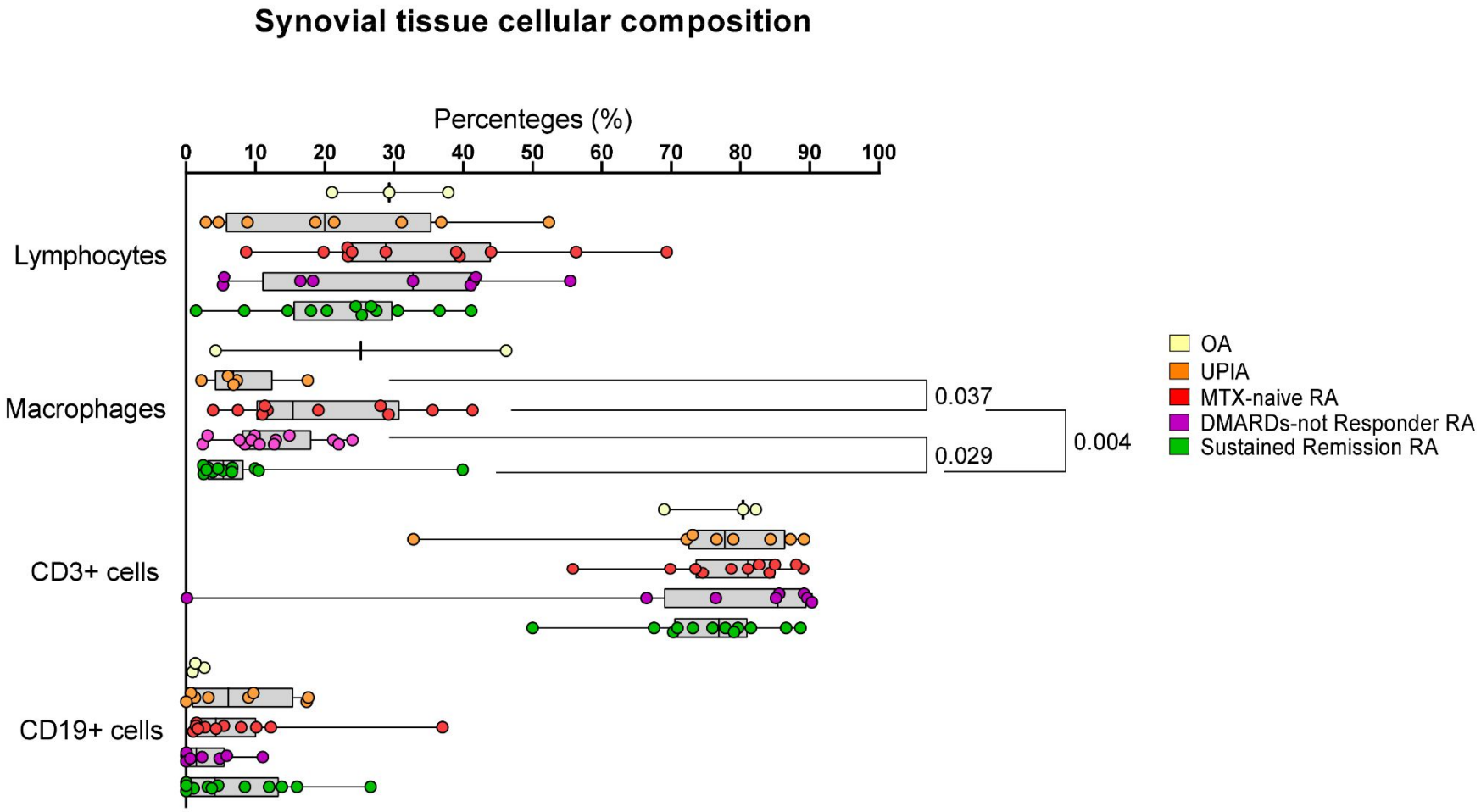


Figure 19. Synovial tissue cellular composition across disease phases
RA patients in sustained remission presented lower ST macrophages percentage (5.35 (3.06-8.30)) compared to MTX-naïve (15.41 (10.14-30.80), p= 0.004) and DMARDs-not responder (10.58 (8.07-18.08), p= 0.029) RA but comparable to UPIA. No variation was found between different condition in ST CD3+ and ST CD19+ cells percentage. The values are expressed as median and IQR (grey bar). Minimal and maximal values are also shown. Each dot represents a patient. P-value <0.05 was considered statistically significant. OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs.

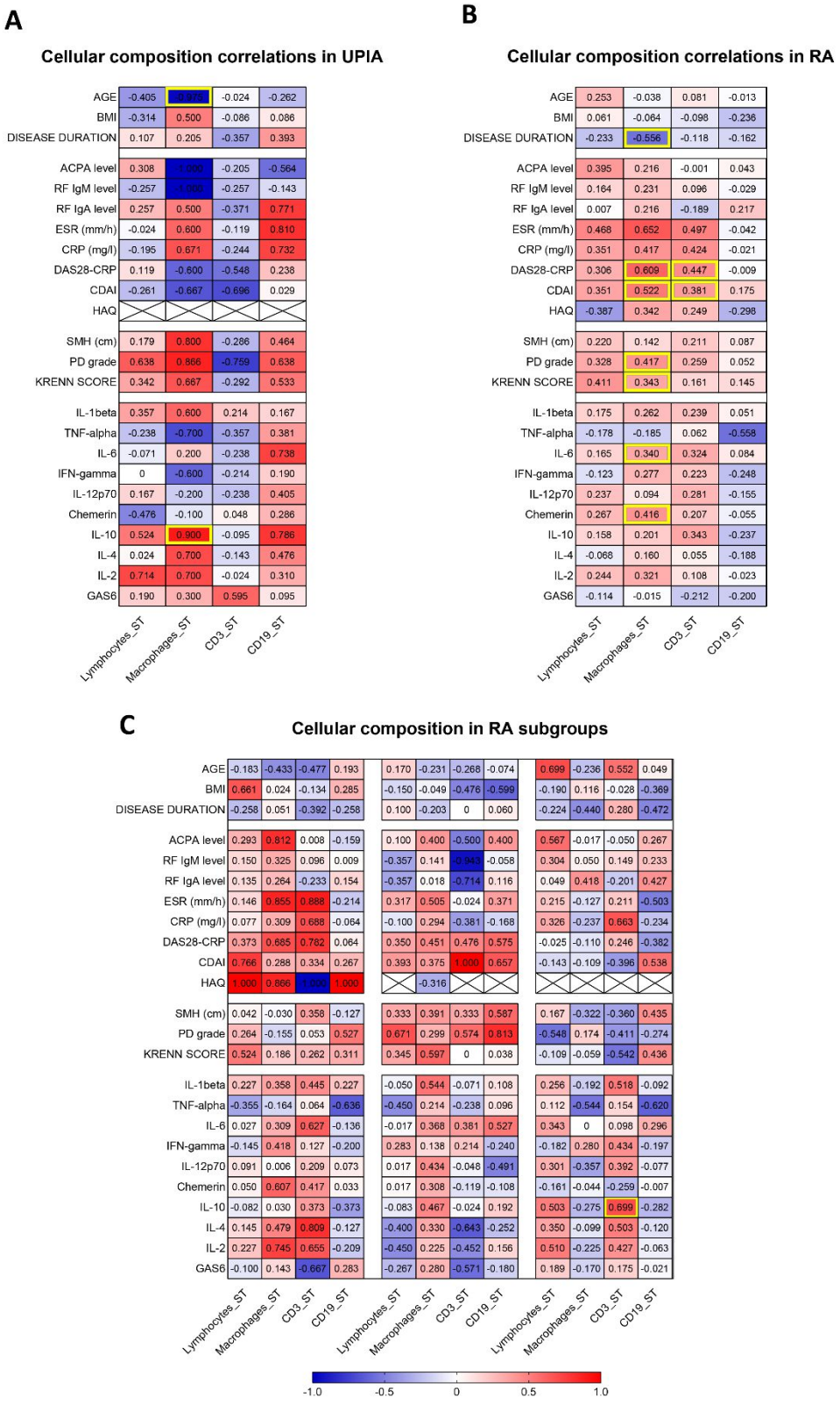


Fig.20. Correlation heatmaps of synovial tissue cellular composition vs demographics, clinical, immunological, ultrasound and histological features of UPIA and RA groups.

UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index; RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre). Two-tailed Spearman's correlation are shown in every cell. Red square: p-value <0.05, considered statistically significant.

4.3.7 ERV1, ALX/FPR AND BLT1 PERCENTAGES OF POSITIVE CELLS AND MFI VALUES ON PERIPHERAL BLOOD AND SYNOVIAL TISSUE CELLULAR FRACTIONS IN RHEUMATOID ARTHRITIS ACROSS DISEASE PHASES

To evaluate the different expression of SPM receptors in PB and ST derived cells in RA across disease phases, ERV1, ALX/FPR2 and BLT1 percentages of positive cells and MFI values were collected comparing with HC (only for PB), OA and UPIA diseases (for both PB and ST). The cell fractions taken considered were neutrophils, NK cells, CD14+, CD3+ and CD19+ cells for PB and macrophages, CD3+ and CD19+ cells for ST.

Considering ERV1 receptor (**Fig. 21**), in REM neutrophils low percentage of MFI values (0.67 (0.58-0.87)) and percentages of positive cells (24.77 (17.77-43.21)) was found comparing to HC (4.62 (2.21-5.52), p= 0.0001; 58.36 (50.93-65.86), p= 0.0001 respectively) but comparable to other conditions. Furthermore, only in REM higher MFI value in NK cells (1.45 (0.89-1.68)) was found comparing to HC (0.10 (0.10-0.14), p= 0.0001) and DMARDs-not responder RA (0.78 (0.48-1.36), p= 0.026). In contrast, in REM lower percentage of ERV1+ positive cells (70.25 (48.45-74.14)) was found with respect to HC (79.32 (63.06-89.63), p= 0.040). Similarly, in CD3+ cells all condition presented higher MFI values and lower percentages of positive cells comparing to HC (0.10 (0.10-0.10); 40.97 (30.85-51.95), respectively), including REM (0.45 (0.38-0.54), p= 0.0001; 15.83 (10.10-23.02), respectively). Finally, in CD19+ cells all condition presented higher MFI values comparing to HC (0.10 (0.10-0.11)), including REM (0.96 (0.72-1.18), p= 0.003). Any differences in MFI values and in percentages of positive cells were found in PB monocytes and in ST derived cells.

Considering ALX/FPR2 receptor (**Fig. 22**), in NK cells despite comparable MFI values between different conditions, in REM low percentages of positive cells (1.55 (0.31-3.14)) were found comparing to HC (4.19 (3.34-21.71) but similar to other conditions. Similarly, in CD14+ monocytes REM presented lower MFI value (0.10 (0.10-3.15) and percentages of positive cells (1.17 (0.47-3.91) comparing to HC (2.18 (1.64-2.61), p= 0.027; 82.29 (74.85-93.64), p= 0.016) but not dissimilar to other conditions. Moreover, in CD19+ cells despite lower MFI value in REM (0.23 (0.20-0.27)) comparing to HC (0.28 (0.25-0.36); p= 0.031), no difference was found between percentages of positive cells of these groups (0.87 (0.57-3.52), 1.97 (1.42-4.36), respectively). In contrast, in CD3+ cells despite lower percentage of positive cells in REM (0.05 (0.04-0.10)) comparing to HC (0.44 (0.12-0.85); p= 0.005), no difference was found between MFI values of these groups (0.25 (0.24-0.27), 0.27 (0.25-0.28), respectively). No differences in percentages of positive cells and MFI values were found in PB neutrophils and in ST derived cells.

Finally, considering BLT1 receptor (**Fig. 23**), all conditions including REM presented in neutrophils

higher MFI values (6.76 (5.41-7.56)) and percentage of positive cells (99.94 (99.83-99.98)) when compared to HC (1.04 (0.94-1.18), p= 0.0001; 97.35 (94.37-98.86), p= 0.0001 respectively). Furthermore, in NK cells despite comparable MFI values between REM and HC, percentages of positive cells were higher in UPIA (10.25 (6.93-14.97)) and it reduced from MTX-naïve (8.35 (4.30-15.80), p= 0.001) and DMARDs-not responder RA (5.16 (3.96-8.37), p= 0.023) to REM (5.16 (3.96-8.37)), which was comparable with HC (6.81 (5.87-25.66)) and OA (6.02 (2.66-6.92)) but lower than MTX-naïve RA (p= 0.011). Moreover, both in PB CD3+ and CD19+ the highest values were found in HC (0.81 (0.70-0.96); 1.11 (0.81-1.28), respectively) comparing to other condition, including REM (0.10 (0.10-0.10) p= 0.0001 for both comparison), in contrast with comparable values of percentages of positive cells across different conditions. Interestingly, in ST CD3+ despite no changes in percentages of positive cells between all the studied conditions, MFI value of REM was very lower (0 (0-0)) when compared to OA (3.88 (0.44-6.78), p= 0.017), UPIA (3.91 (1.13-9.01), p= 0.002) and MTX-naïve RA (1.75 (0-3.00), p= 0.010) but comparable to DMARDs-not responder (0 (0-4.03)). Any differences in percentages of positive cells and MFI values were found in PB monocytes and in ST macrophages and CD19+ cells.

Fig. 24 and **Fig. 25** show the correlation found between the SPMs receptors and demographic, clinical, immunological, ultrasound and histological features in UPIA and RA cohorts. In particular, considering the whole RA cohort, in PB setting ERV1$^+$CD19$^+$ percentage of positive cells and MFI value directly correlated with IL-10 (r= 0.394, p= 0.005; r= 0.394, p= 0.004 respectively) and GAS6 (r= 0.335, p= 0.023; r= 0.329, p= 0.022 respectively). Moreover, ALX/FPR2$^+$CD14$^+$ and ALX/FPR2$^+$ in NK cells percentage of positive cells percentage of positive cells directly correlated to GAS6 (r= 0.490, p= 0.0001; r= 0.323, p= 0.021 respectively). Finally, BLT1$^+$CD14$^+$ percentage of positive cells and MFI value directly correlated with CDAI (r= 0.312, p= 0.037; r= 0.387, p= 0.007 respectively) as BLT1$^+$CD14$^+$ percentage of positive cells directly correlated to IL-6 (r= 0.317, p= 0.018). In ST setting, ERV1$^+$ in macrophages inversely correlated to disease duration (r= -0.376, p= 0.037). Furthermore, ALX/FPR2$^+$CD19$^+$ MFI value inversely correlated to ACPA, RF IgM and IgA levels (r= -0.413, p= 0.040; r= -0.378, p= 0.048; r= -0.385, p= 0.043 respectively). Finally, BLT1$^+$CD3$^+$ MFI value directly correlated to DAS28-CRP, CDAI, HAQ, PD grade and KSS (r= 0.582, p= 0.0001; r= 0.389, p= 0.049; r= 0.537, p= 0.026; r= 0.388, p= 0.041; r= 0.392, p= 0.029 respectively). All those correlations were not present in UPIA disease.

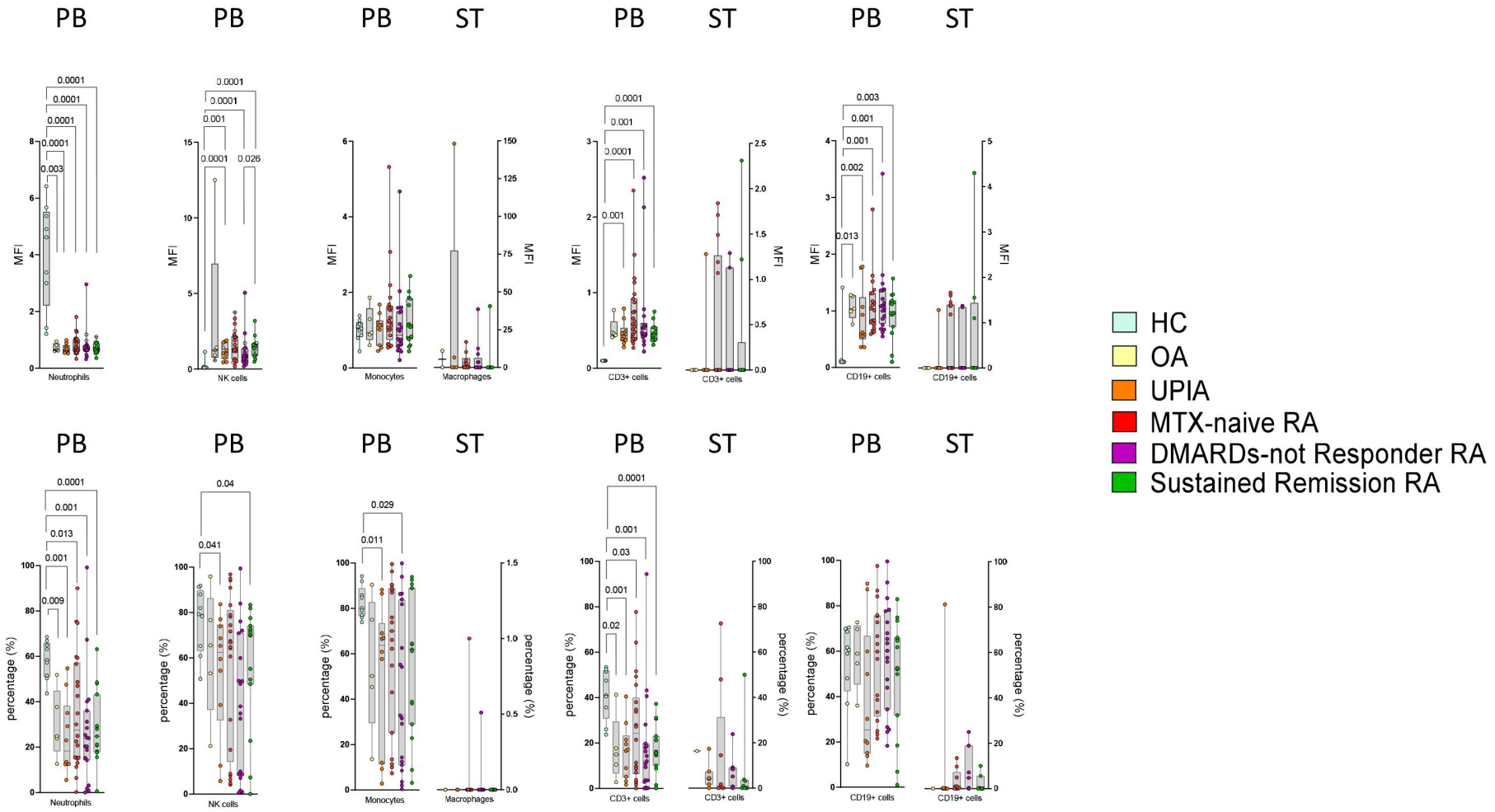


Figure 21. ERV1 expression in PB and ST compartment expressed as percentage of positive cells and MFI value

In sustained remission RA neutrophils low percentage of MFI values (0.67 (0.58-0.87)) and percentages of positive cells (24.77 (17.77-43.21)) was found comparing to HC (4.62 (2.21-5.52), p= 0.0001; 58.36 (50.93-65.86), p= 0.0001 respectively) but comparable to other conditions. Furthermore, only in REM higher MFI value in NK cells (1.45 (0.89-1.68)) was found comparing to HC (0.10 (0.10-0.14), p= 0.0001) and DMARDs-not responder RA (0.78 (0.48-1.36), p= 0.026). In contrast, in REM lower percentage of ERV1+ positive cells (70.25 (48.45-74.14)) was found with respect to HC (79.32 (63.06-89.63), p= 0.040). Similarly, in CD3+ cells all condition presented higher MFI values and lower percentages of positive cells comparing to HC (0.10 (0.10-0.10); 40.97 (30.85-51.95), respectively), including REM (0.45 (0.38-0.54), p= 0.0001; 15.83 (10.10-23.02), respectively). Finally, in CD19+ cells all condition presented higher MFI values comparing to HC (0.10 (0.10-0.11)), including REM (0.96 (0.72-1.18), p= 0.003). Median, IQR (grey bar), minimal and maximal values are shown. Each dot represents a patient. P-value <0.05 was considered statistically significant. HC: healthy control; OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs.

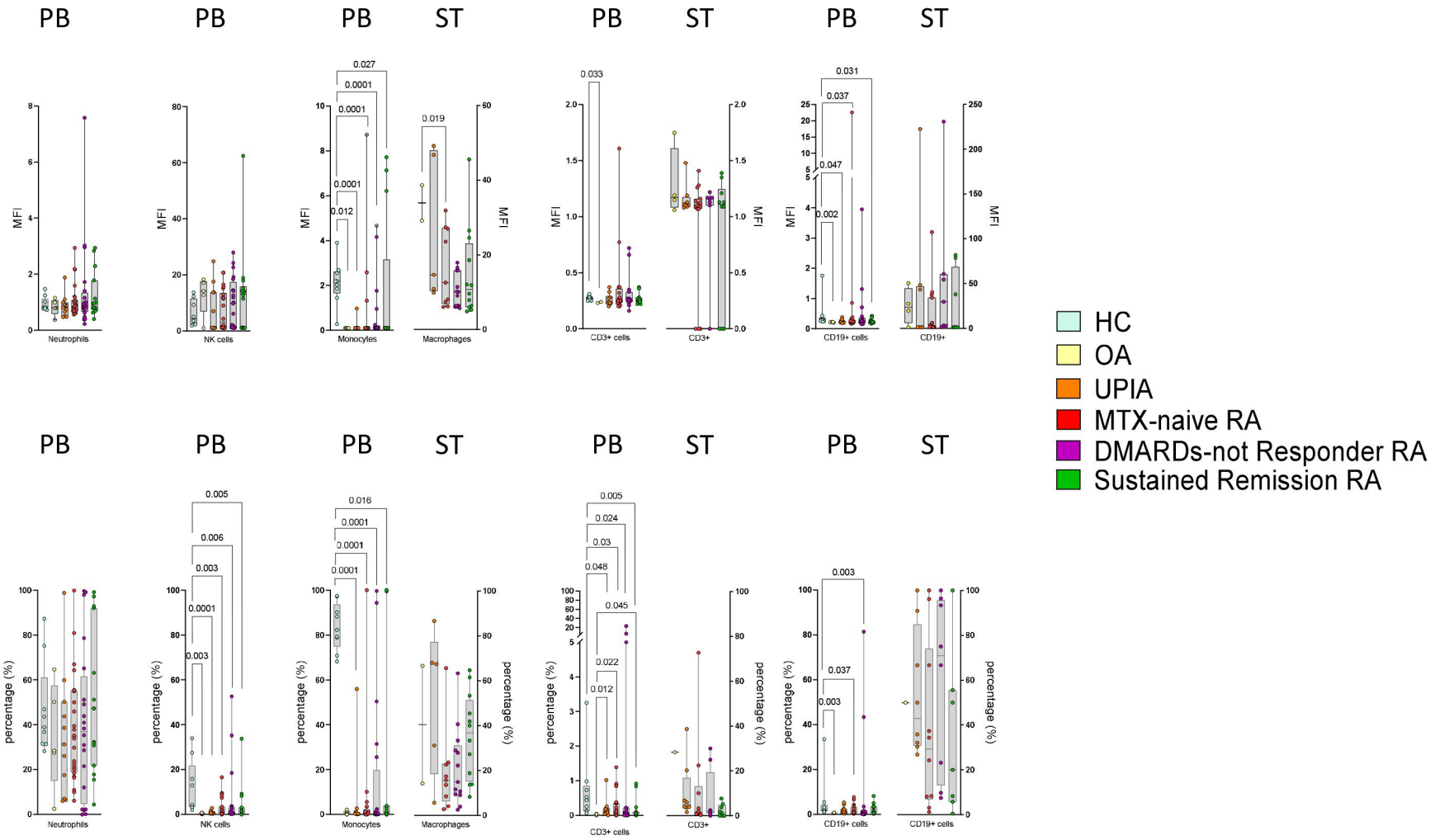


Figure 22. ALX/FPR2 expression in PB and ST compartment expressed as percentage of positive cells and MFI value
In sustained remission RA NK cells low percentages of positive cells (1.55 (0.31-3.14)) were found comparing to HC (4.19 (3.34-21.71). Similarly, in CD14+ monocytes sustained remission presented lower MFI value (0.10 (0.10-3.15) and percentages of positive cells (1.17 (0.47-3.91) comparing to HC (2.18 (1.64-2.61), p= 0.027; 82.29 (74.85-93.64), p= 0.016). Moreover, in CD19+ cells despite lower MFI value in REM (0.23 (0.20-0.27)) comparing to HC (0.28 (0.25-0.36); p= 0.031). In CD3+ cells lower percentage of positive cells in REM (0.05 (0.04-0.10)) comparing to HC (0.44 (0.12-0.85); p= 0.005) was found. Median, IQR (grey bar), minimal and maximal values are shown. Each dot represents a patient. P-value <0.05 was considered statistically significant. HC: healthy control; OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs.

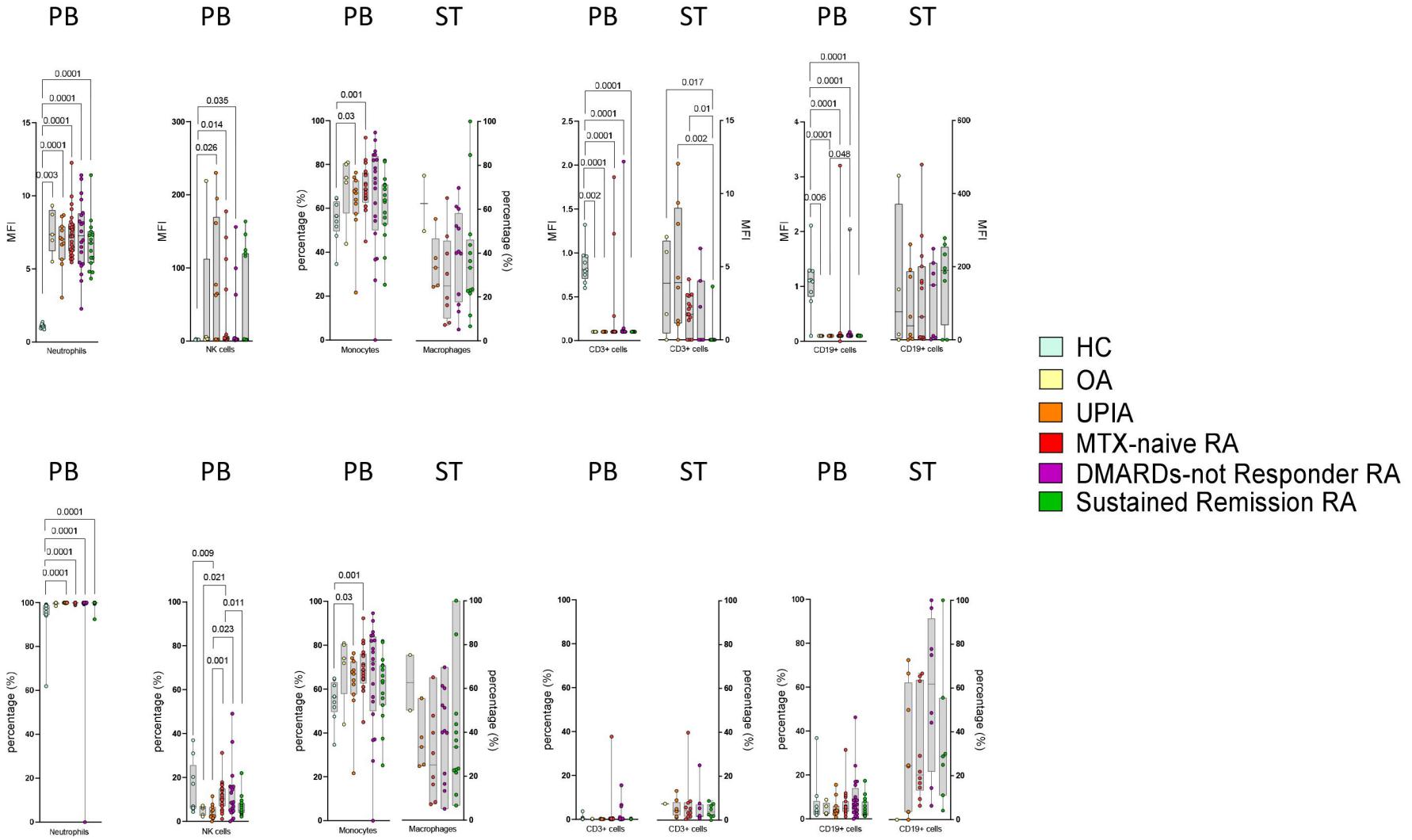


Figure 23. BLT1 expression in PB and ST compartment expressed as percentage of positive cells and MFI value

In sustained remission RA neutrophils presented higher MFI values (6.76 (5.41-7.56)) and percentage of positive cells (99.94 (99.83-99.98)) when compared to HC (1.04 (0.94-1.18), p= 0.0001; 97.35 (94.37-98.86), p= 0.0001 respectively). Furthermore, NK cells percentages of BLT1+ cells were higher in UPIA (10.25 (6.93-14.97)) and it reduced from MTX-naïve (8.35 (4.30-15.80), p= 0.001) and DMARDs-not responder RA (5.16 (3.96-8.37), p= 0.023) to REM (5.16 (3.96-8.37)) but lower than MTX-naïve RA (p= 0.011). Moreover, both in PB CD3+ and CD19+ the highest values were found in HC (0.81 (0.70-0.96); 1.11 (0.81-1.28), respectively) comparing to sustained remission RA (0.10 (0.10-0.10) p= 0.0001 for both comparison). Interestingly, in ST CD3+ despite no changes in percentages of positive cells between all the studied conditions, MFI value of REM was very lower (0 (0-0)) when compared to OA (3.88 (0.44-6.78), p= 0.017), UPIA (3.91 (1.13-9.01), p= 0.002) and MTX-naïve RA (1.75 (0-3.00), p= 0.010). Median, IQR (grey bar), minimal and maximal values are shown. Each dot represents a patient. P-value <0.05 was considered statistically significant. HC: healthy control; OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs.

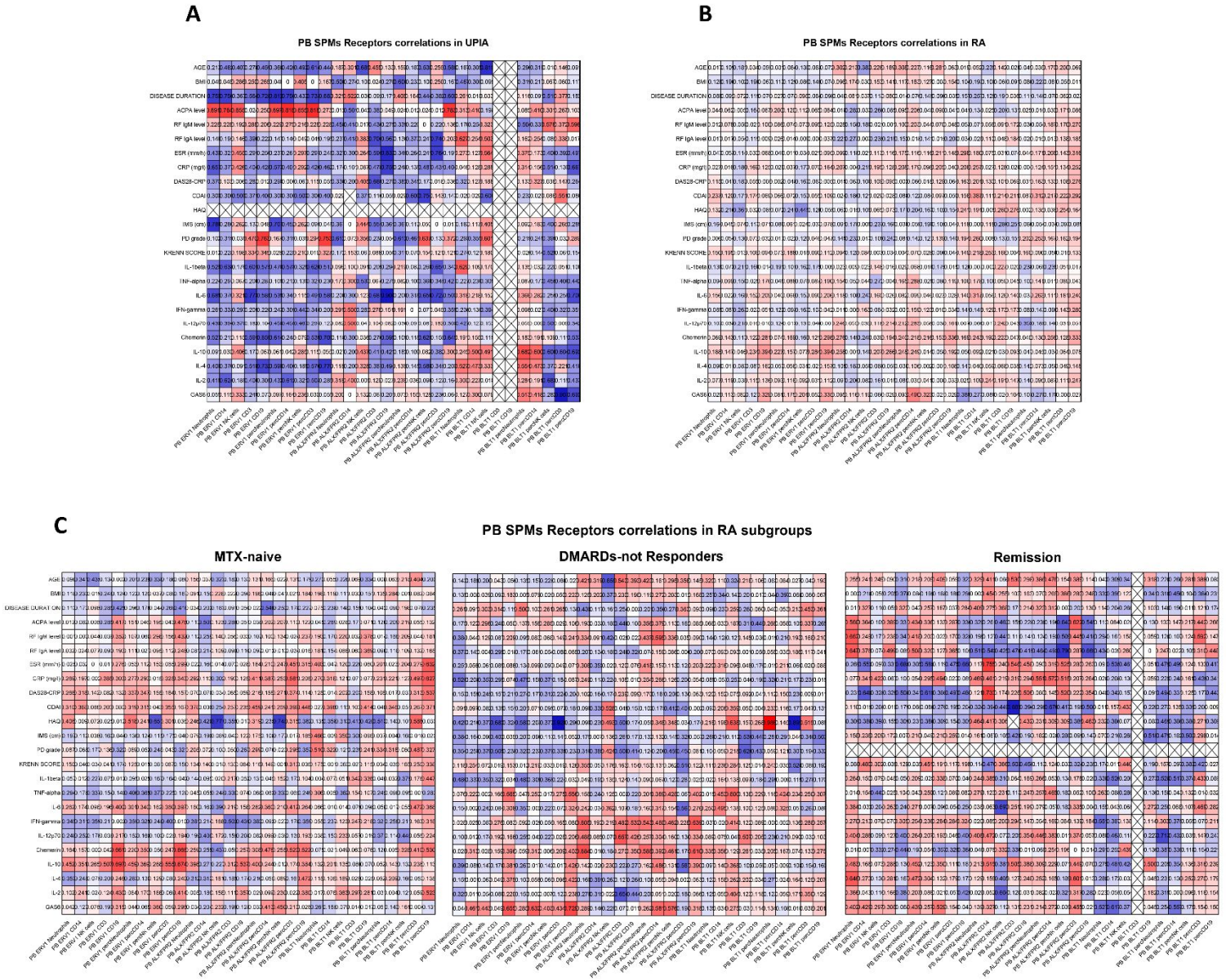


Fig.24. Correlation heatmaps of PB SPMs receptors vs demographics, clinical, immunological, ultrasound and histological features of UPIA (A) and RA groups (B-C).
PB: peripheral blood compartment; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index; RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre). Two-tailed Spearman's correlation are shown in every cell. Yellow square: p-value <0.05, considered statistically significant.

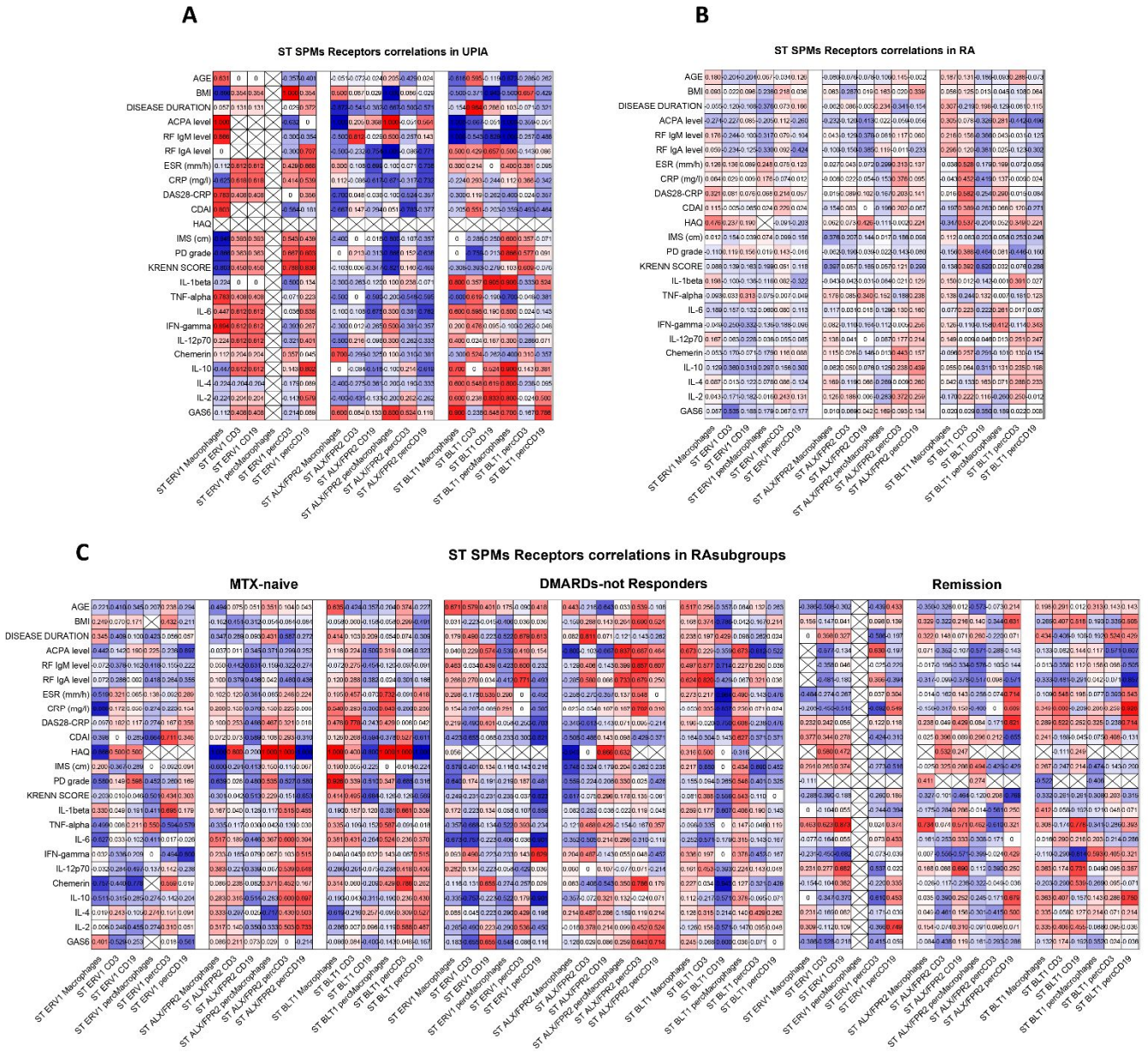


Fig.25. Correlation heatmaps of ST SPMs receptors vs demographics, clinical, immunological, ultrasound and histological features of UPIA (A) and RA groups (B-C). ST: synovial tissue compartment; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; BMI: Body Mass Index; RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre). Two-tailed Spearman's correlation are shown in every cell. Yellow square: p-value <0.05, considered statistically significant.

4.3.8 TREATMENT IMPACT ON SPM RECEPTORS EXPRESSION IN SUSTAINED REMISSION RA

To assess the influence of cs- and b/ts-DMARDs treatment on SPM receptors expression in REM, this group was stratified based on treatment used at the time of synovial biopsy as mono (csDMARDs or b/tsDMARDs) or combo (bDMARDs and csDMARDs) therapy groups. Among 18 RA patients in sustained remission, 6 were treated with csDMARDs, 4 with b/tsDMARDs and 8 with combo therapy. **Table 11** shows the demographic, clinical, immunological, ultrasound and histological characteristics of RA remission subgroups.

Table 11. Demographic, immunological, clinical, ultrasound and histological findings of sustained remission RA patients in mono or combo therapy

	csDMARDs THERAPY (a) (N: 6)	b/tsDMARDs THERAPY (b) (N: 4)	COMBO THERAPY (c) (N: 8)	p-value a vs b	p-value a vs c	p-value b vs c
Age	56.50 (48.25-63.00)	51.00 (40.75-53.75)	57.50 (43.75-72.75)	0.198	0.519	0.126
Female (n, %)	4 (67%)	3 (75%)	6 (75%)	0.710	0.710	0.710
BMI (Kg/mq)	26.5 (21.5-29.4)	23.5 (19.9-27.8)	24.0 (23.0-24.6)	0.336	0.272	0.671
Smoking Status (n, %)	3 (50%)	1 (25%)	1 (12%)	0.137	0.137	0.137
Disease Duration (mo)	80.00 (45.00-120.00)	143.00 (63.25-423.00)	108.00 (58.50-156.75)	0.327	0.380	0.396
RF/ACPA positivity (n, %)	4 (67%)	2 (50%)	6 (75%)	0.999	0.999	0.999
ESR (mm/h)	19.00 (12.50-24.50)	5.00 (2.00-17.00)	8.00 (3.00-15.00)	0.162	0.085	0.635
CRP (mg/l)	0.50 (0.50-2.12)	0.50 (0.50-1.01)	0.50 (0.50-2.50)	0.600	0.745	0.381
TJC-28	0 (0-0.25)	0 (0)	0 (0)	0.414	0.248	0.999
SJC-28	0 (0-1.00)	0 (0)	0 (0)	0.221	0.471	0.480
DAS28-CRP	2.03 (1.18-2.56)	1.39 (0.92-1.92)	1.54 (1.11-2.26)	0.392	0.245	0.865
CDAI	0 (0-3.50)	1 (0-2.75)	1 (0-2.00)	0.788	0.662	0.921
SMH (cm)	0.74 (0.53-0.77)	0.91 (0.85-0.96)	0.83 (0.70-1.05)	**0.020**	0.128	0.197
PD Grade	0 (0-0.25)	0 (0)	0 (0)	0.480	0.317	0.999
Krenn Score	1.00 (0.75-1.25)	2.50 (1.25-5.25)	1.50 (1.00-2.75)	0.052	0.114	0.328

RA: rheumatoid arthritis; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic Disease Modifying Anti-Rheumatic Drugs; b/tsDMARDs: biological / target synthetic Disease Modifying Anti-Rheumatic Drugs; PD: Power Doppler. Median and IQR or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

Despite the difference in terms of SMH (csDMARDs therapy: 0.74 (0.53-0.77) vs b/tsDMARDs therapy: 0.91 (0.85-0.96), p= 0.020) (**Fig. 26A**), no other difference was found in terms of serum levels of cytokines and chemokines, ERV1, ALX/FPR2 and BLT1 gene (**Fig. 26B**) and cellular expression.

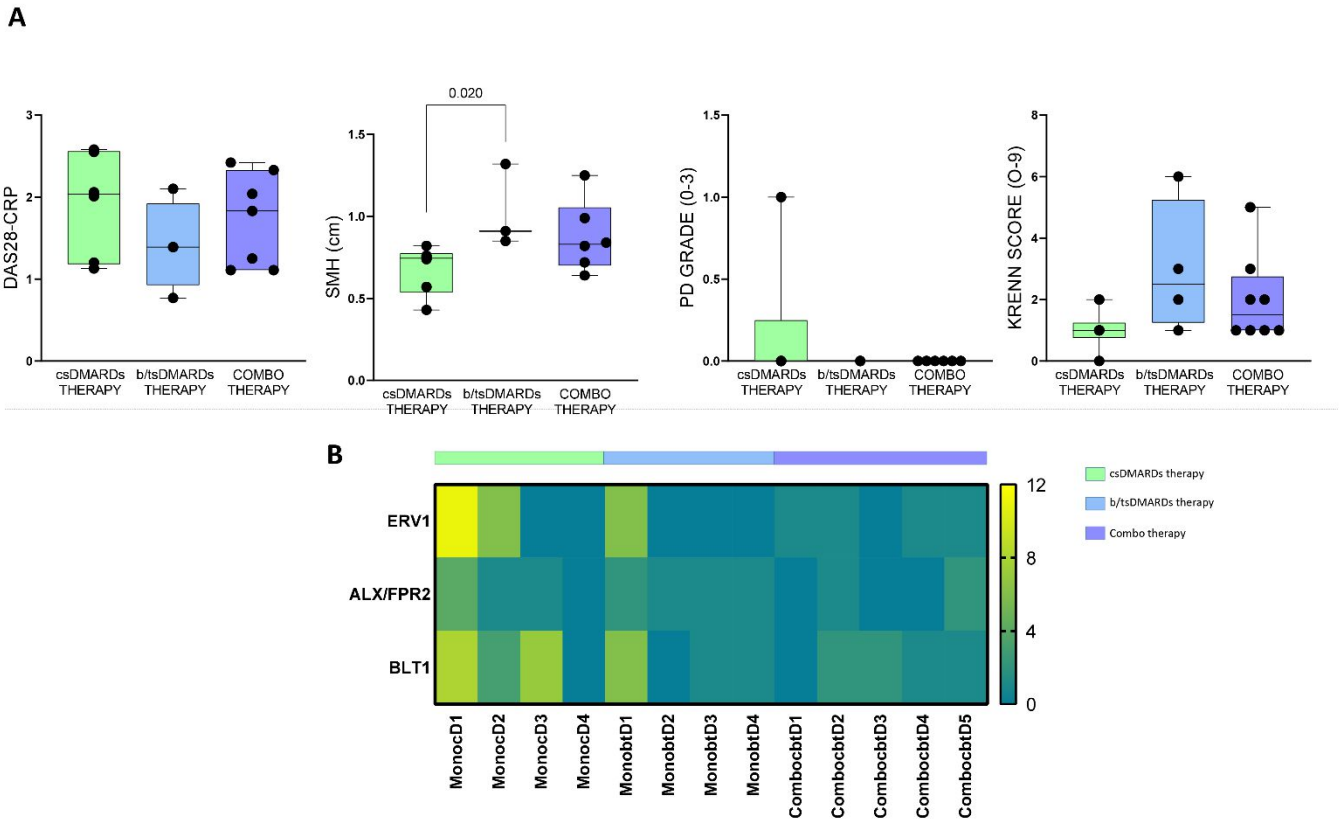


Figure 26. Clinical, ultrasound, histological and SPM expression findings of RA patients stratified by treatment.
A: clinical (as DAS28-CRP), ultrasound (as SMH and PD Grade) and histological (as Krenn Score) finding between RA patient in mono therapy (green: csDMARDs, blue: b/tsDMARDs) and combo therapy (violet). SMH: csDMARDs user (0.74 (0.53-0.77) vs b/tsDMARDs user (0.91 (0.85-0.96), p= 0.020). Every dot represents a patient. Median, IQR (bar) and minimal and maxima values are shown. **B**: heatmap of genetic expression of ERV1, ALX/FPR2 and BLT1. Each row represents a gene. Each bar represents a single patient. RA: rheumatoid arthritis; DAS28-CRP: disease activity score CRP-based of 28 joints; SMH: synovial membrane hyperplasia (in cm: centimetre); PD: Power Doppler csDMARDs: conventional synthetic Disease Modifying Anti-Rheumatic Drugs; b/tsDMARDs: biological / target synthetic Disease Modifying Anti-Rheumatic Drugs; MonocD: RA patient in csDMARDs therapy; MonobtD: RA patient in b/tsDMARDs therapy; CombocbtD: RA patient in combo therapy (b/tsDMARDs and csDMARDs);

4.3.9 DEMOGRAPHIC, CLINICAL, IMMUNOLOGICAL, ULTRASOUND AND HISTOLOGICAL CHARACTERISTICS OF RA PATIENTS IN BOOLEAN REMISSION

To assess the impact of a deeper remission on resolution mechanisms, RA patients were stratifying based on DAS28-CRP defined sustained remission. Among 17 patients with RA in DAS28-defined sustained remission, 9 also met Boolean remission criteria at the time of synovial biopsy. **Table 12** shows the demographic, clinical, immunological, ultrasound and histological characteristics of RA remission subgroups.

Table 12. Demographic, immunological, clinical, ultrasound and histological findings of sustained remission RA patients

	BOOLEAN REM (N: 9)	NOT BOLEAN REM (N: 8)	p-value
Age	53.00 (36.50-54.50)	60.00 (54.25-71.25)	**0.011**
Female (n, %)	6 (67%)	7 (87%)	0.312
BMI (Kg/mq)	24.2 (23.0-28.5)	23.9 (20.1-28.2)	0.630
Smoking Status (n, %)	4 (44%)	1 (12%)	0.149
Disease Duration (mo)	106.00 (65.00-143.50)	118.00 (49.00-168.00)	0.874
RF/ACPA positivity (n, %)	6 (67%)	5 (62%)	0.858
ESR (mm/h)	8.00 (4.00-19.00)	13.50 (2.75-26.00)	0.595
CRP (mg/l)	0.50 (0.50-0.50)	1.60 (0.67-3.47)	**0.002**
TJC-28	0 (0)	0 (0)	0.289
SJC-28	0 (0)	0 (0-0.75)	0.426
DAS28-CRP	1.39 (1.15-2.08)	2.02 (1.19-2.40)	0.531
CDAI	0 (0-1.50)	1 (0-3.00)	0.495
SMH (cm)	0.3 (0.71-0.88)	0.74 (0.66-0.89)	0.401
Power-doppler Grade	0 (0-0.25)	0 (0)	0.248
Krenn Score	1.00 (1.00-1.50)	2.00 (1.00-2.75)	0.248
Steroids (n, %)	0 (0%)	0 (0%)	
csDMARDs (n, %)	7 (78%)	5 (62%)	0.490
b/tsDMARDs (n, %)	6 (67%)	5 (62%)	0.858

REM: sustained remission status; RA: rheumatoid arthritis; BMI: Body Mass Index (Kg/mq: kilogram per metre squared); RF: Rheumatoid Factor; ACPA: Anti-Citrullinated Protein Antibody; ESR: erythrocyte sedimentation rate (in mm/h: millimetre in the first hour); CRP: C-reactive protein (in mg/l: milligram per litre); TJC-28: tender joint counts on 28 joints; SJC-28: swollen joint counts on 28 joints; DAS28-CRP: disease activity score CRP-based of 28 joints; CDAI: clinical disease activity index; SMH: synovial membrane hyperplasia (in cm: centimetre); csDMARDs: conventional synthetic Disease Modifying Anti-Rheumatic Drugs; b/tsDMARDs: biological / target synthetic Disease Modifying Anti-Rheumatic Drugs. Median and IQR or frequency and percentages are shown as appropriated. P-value <0.05 was considered statistically significant.

Despite the two groups differed in terms of DAS28-CRP, lower in Boolean remission (1.08 ± 0.13) compared to non-Boolean remission (1.66 ± 0.44; p< 0.001), when considered US and histological findings, the two groups were comparable (**Fig. 27A**). Furthermore, comparing the serum levels of cytokines and chemokines of the two groups, no difference was found. Similarly, synovial tissue ERV1, ALX/FPR2 and BLT1 gene expression was comparable between Boolean and non-Boolean remission RA patients, also if it is possible to identify two clusters with different pattern of SPM receptors expression (**Fig. 27B**). Finally, evaluating SPM receptors by FACS analysis, ST CD3+ showed a difference in the percentage of ERV1+ cells. In particular, in Boolean remission ST CD3+ERV1+ cells percentage was higher (3.77 (3.08-50)) than in not-Boolean remission (0 (0-0.7275); p= 0.05) (**Fig. 27C**).

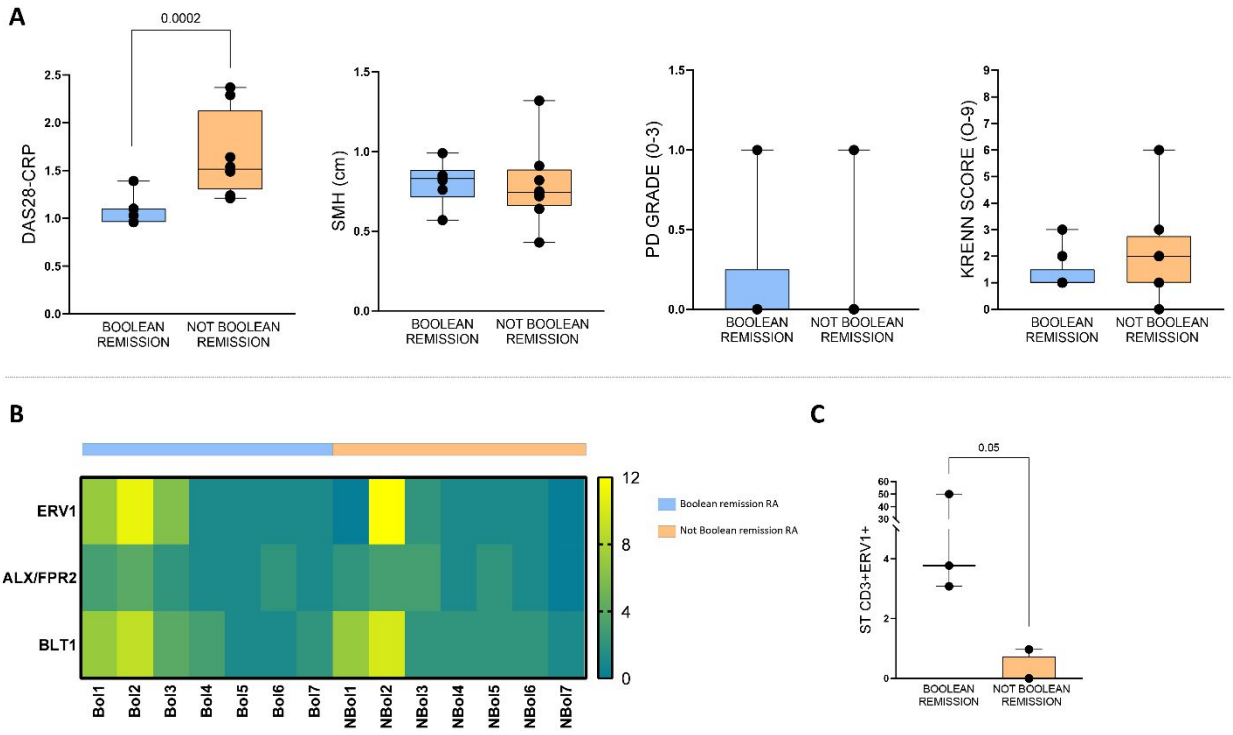


Figure 27. Clinical, ultrasound, histological and SPM expression findings of RA patients in Boolean remission vs not-Boolean remission.
A: clinical (as DAS28-CRP), ultrasound (as SMH and PD Grade) and histological (as Krenn Score) finding between Boolean remission (blue) vs not-Boolean remission (orange) RA patients. DAS28-CRP: Boolean remission (1.08 ± 0.13) compared to not-Boolean remission (1.66 ± 0.44; p< 0.001). Every dot represents a patient. Median, IQR (bar) and minimal and maxima values are shown. **B**: heatmap of genetic expression of ERV1, ALX/FPR2 and BLT1. Each row represents a gene. Each bar represents a single patient. Bol: Boolean remission RA patient; NBol: not-Boolean remission RA patient. **C:** percentages of ST CD3+ERV1+ cells comparing Boolean remission (3.77 (3.08-50)) to not-Boolean remission (0 (0-0.7275); p= 0.05). Every dot represents a patient. Median, IQR (bar) and minimal and maxima values are shown. RA: rheumatoid arthritis; DAS28-CRP: disease activity score CRP-based of 28 joints; SMH: synovial membrane hyperplasia (in cm: centimetre); PD: Power Doppler csDMARDs: conventional synthetic Disease Modifying Anti-Rheumatic Drugs; b/tsDMARDs: biological / target synthetic Disease Modifying Anti-Rheumatic Drugs.

4.4. DISCUSSION

This translational study aimed to identify novel soluble or tissue biomarkers in RA useful for patient stratification in clinical practice, improving their therapeutic management. In this contest, the expression of SPMs and their own receptors (ERV1, ALX/FPR and BLT1) were investigated in synovial membrane and in peripheral blood derived leukocytes of RA patients with different disease phases (MTX-naïve, not-responder to DMARDs and sustained remission respectively) and were correlated with the inflammatory status. The major focus of the study was to investigate SPMs expression in the contest of sustained clinical remission, for which biomarkers are still lacking.

In the experimental plan that was designed, firstly RA remission group was dissected (**Fig. 28A**). In particular, the assessment of clinical and imaging parameters mirrored the disease status with significantly lower clinical activity in terms of DAS28-CRP (due to the inclusion criteria) and US findings (SMH and PD grade respectively). Moreover, the assessment of the inflammatory status defined by serum cytokines and chemokines levels, revealed that IL-6, Chemerin, IL-10 and IL-4 directly correlated with the patients' clinical status in terms of DAS28-CRP (or CDAI), PD grade and KSS respectively. However, none of these soluble markers arose as unique parameter associated with sustained remission in RA. In particular, despite clinical and US remission fulfilment, 12% RA patients showed a high grade residual synovitis, as previously described (76). Similarly, despite the fulfilment of sustained clinical and US remission (76), RA patients might have heterogeneous cytokines and chemokines serum signatures as other conditions leading the management of these patients difficult and costly.

To overcome these limits, other studies already demonstrated the role of omega-3 and omega-6 EFA within the pathophysiology at RA onset or in the contest of high inflammatory conditions (3, 120-124) without information about the human synovial compartment in the remission stage. Therefore, in this study we run a pilot attempt of SPMs quantification in ST using LC-MS/MS technology, showing that synovial tissue of remission RA has an increased ratio between SPM and AA-derived pro-inflammatory molecules compared to synovial tissue of RA patients with high disease activity (**Fig. 28B**). Moreover, to investigate the cellular targets of SPMs in the peripheral blood and synovial tissue compartments we tested the expression of ERV1, ALX/FPR2 and BLT1 receptors showing that the expression of ERV1, ALX/FPR2 and BLT1 were contingent on disease activity, being lower in sustained remission stage, comparable to OA controls and higher in high disease activity (**Fig. 28C**). Furthermore, we tested the ability of SPMs receptors genes expression on synovial compartment as

putative novel biomarker of disease flare prediction in RA patients in remission without finding any relation about the clinical outcome.

Subsequently, FACS analysis assessing the expression of SPMs on PB- and ST-derived immune cells, revealed that, in PB compartment, ERV1, ALX/FPR2 and BLT1 expression was lower in terms of percentage of positive cells and MFI comparing to HC but comparable to different disease phases for innate immune cells with the exclusion of monocytes. Conversely, in adaptive immune cells ERV1 expression was lower as percentages of positive cells but higher as MFI values. On the other hand, in adaptive immune cells ALX/FPR2 and BLT1 expressions was lower both in terms of percentages of positive cells and MFI respectively. Considering ST compartment, despite no changes in percentage of CD3+BLT1+ cells between all the studied conditions, CD3+BLT1+ MFI value of RA sustained remission was almost depleted comparing to OA, UPIA and MTX-naïve RA (**Fig. 28D**). Considering whole RA cohort, CD3+BLT1+ MFI value directly correlated to DAS28-CRP, CDAI, PD grade and KSS. Furthermore, it directly correlated to Patients Reported Outcome (PRO) as HAQ, VAS-Pain and GH. All those correlations were not present in UPIA disease and in sustained remission RA groups.

Within RA subgroups, the use of steroids, csDMARDs and b/tsDMARDs was a relevant difference. Focalising to sustained remission RA, to assess the influence of pharmacological treatments on SPM receptors expression, this group was stratified based on the treatment used at the time of synovial biopsy performance without any significant difference. Furthermore, to assess the impact of a deeper clinical-defined remission on SPMs expression on both compartments, RA patients in Boolean-defined remission had ST-derived CD3+ cells enriched of ERV1 cells compared to RA patients with DAS28-CRP-defined remission.

BLT1 is a G-protein coupled receptor to which LTB4 (pro-inflammatory omega-6 derived mediator) and RvE1 binds (90, 153). On monocytes Pettersson (154) showed that BLT1 and its mRNA were down-modulated by the proinflammatory cytokines stimulation (like IFN-gamma and TNF-alpha) but up-regulated by anti-inflammatory molecules (like IL-10 and dexamethasone). Furthermore, the down-regulating action of proinflammatory stimuli (as IFN-gamma and LPS) on BLT1 receptor mRNA expression is described also in macrophages in vitro (28). Conversely, on PB CD3+ lymphocytes of asthmatic patients, Chung (155) showed that BLT1+ were more expressed in CD8+ than CD4+ cells and, in particular, in resistant-to-treatment disease rather than responsive-to-treatment and controls.

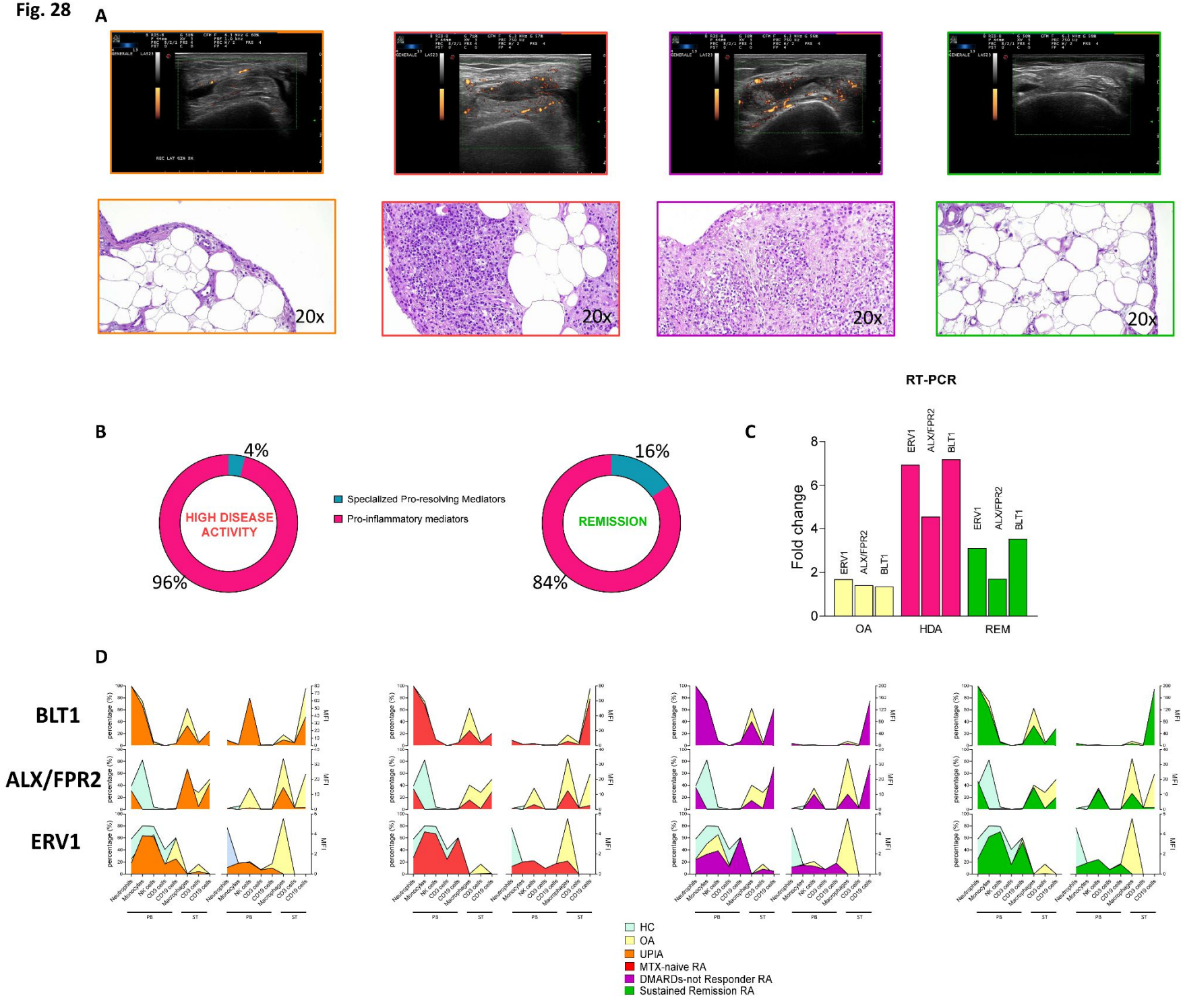
ERV1 is also a G-protein coupled receptor to which Chemerin (pro-inflammatory chemokines) and RvE1 binds on macrophages, DCs, neutrophils and NK cells (94, 156-158). The interaction between RvE1 and its receptor promotes the decrease of pro-inflammatory TNF-alpha and IL-12 synthesis,

reduces neutrophil transmigration and activation, stimulates efferocytosis and promotes anti-inflammatory phenotype conversion of macrophage (89, 94, 98, 157, 159-161). Furthermore, on PB CD4+ lymphocytes of psoriatic patients, Wang (162) described a higher ERV1 expression in disease condition than in HC, implying in Th9/Treg response.

Based on these data, the expression of BLT1 and ERV1 in ST-derived CD3+ cells might be considered a biomarker of disease activity in RA. In particular, the former could help to discern and confirm a remission from not-remission status, the latter a Boolean from a DAS28-CRP remission in outpatient setting in which clinical, US and histological findings are not concordant. We might speculate that SPMs pathways might contribute in the regulation of RA inflammatory activity at tissue level, where ERV1 and BLT1 receptors might be affected by pro- and anti-inflammatory/pro-resolving mediators and raising in case of high disease activity. Based on these issues, we could hypothesize that the change in the pro-inflammatory and anti-inflammatory/pro-resolving soluble ligands balance, within the synovial compartment might impact SPMs expression and activity on different target immune cells. On the other hands, in blood the highest expression of SPM receptors was described in healthy donors and it was strongly decreased in RA, even in remission status. This lack of restoration of SPM receptors in remission to the levels of those in healthy is interesting and epigenetic mechanisms could be implied in that regulation.

This study presents some limitations. In particular, the experimental cross-sectional design did not allow to use longitudinal approach to assess the expression of SMPs overtime. Moreover, this study was limited to the assessment of CD45+ cells in PB and ST compartments, excluding FLS and different clustering of resident macrophages. Therefore, further studies, expanding sample size and including the lipidomic analysis and *in vitro* functional studies, are needed to validate these data in order to explain the intrigue mechanisms behind inflammation and its resolution in RA.

Fig. 28


A
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HIGH DISEASE ACTIVITY
Specialized Pro-resolving Mediators
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Fold change
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BLT1
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BLT1
ALX/FPR2
ERV1
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MFI
Neutrophils
Monocytes
NK cells
CD19 cells
Macrophages
CD3 cells
PB
ST
HC
OA
UPIA
MTX-naive RA
DMARDs-not Responder RA
Sustained Remission RA

Figure 28. Summary representation of results.
A: example photos of ultrasound assessment with PD Grade and H&E staining of ST obtained by minimal invasive US-guided biopsy of each patients' category border colour identifies each category: orange for UPIA, red for MTX-naïve, violet for DMARDs-not responder and green for remission RA patients). **B:** percentages of the total concentrations of SPM (blue) and AA-derived pro-inflammatory mediators (red) of studied patients. **C:** ERV1, ALX/FPR2 and BLT1 receptor expression between REM (green) and HDA (red) with OA (yellow) control. The expression of the studied SPM receptors was comparable between sustained remission RA (1.10 (0.60-5.70); 1.50 (0.80-2.60); 1.60 (1.40-6.60) respectively) and OA (1.20 (0.40-1.40); 0.80 (0.50-2.70); 1.10 (0.40-2.70) respectively) but lower than HDA condition (4.40 (2.70-11.90), p= 0.012; 4.90 (3.20-5.45), p= 0.0006; 5.90 (3.85-10.30), p= 0.016 respectively). **D:** SPM receptors expression in PB (neutrophils, monocytes, NK cells, CD3 and CD19 cells) and ST compartment (macrophages, CD3 and CD19) expressed as percentages of positive cells (left) and MFI values (right) for each patients' category, overlying HC (light blue) and OA (yellow) controls.
The values are expressed as median. OA: osteoarthritis; UPIA: undifferentiated peripheral inflammatory arthritis; RA: rheumatoid arthritis; MTX: methotrexate; DMARDs: Disease Modifying Anti-Rheumatic Drugs; REM: sustained remission RA; PD: Power Doppler; H&E: hematoxylin and eosin stain.

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