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NEW THERAPIES IN THE FIELD OF RHEUMATOLOGY

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EDITORIAL
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Editorial: New Therapies in the Field of Rheumatology

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Editorial on the Research Topic

New Therapies in the Field of Rheumatology

Rheumatology is a relatively recent medical specialty: the term "rheumatology" was coined by two American physicians only in 1940 (Rodnan, 1977). Approximately five decades after, rheumatology experienced a great advancement: biological agents became commercially available, revolutionizing the therapeutic approach to patients with systemic autoimmune diseases. The first biological compounds introduced in the market neutralize tumor necrosis factor alpha (TNFα), a key molecule in the pathogenesis of inflammatory arthritides. Despite the incontrovertible evidence on the efficacy of TNF-inhibitors, there are still many aspects that warrant clarification in order to optimize the therapeutic management of patients. Some of these critical issues are discussed in the research topic entitled "New Therapies in the Field of Rheumatology". Laganà et al. investigate whether gender affects the response to TNF-inhibitors among subjects with spondyloarthropathies or inflammatory bowel diseases (IBD), evincing a higher discontinuation rate of adalimumab but not infliximab in women with IBD. Even body weight might impact anti-TNFα efficacy, as demonstrated by Giani et al. In a cohort of 110 children with juvenile idiopathic arthritis, the remission rate is lower among obese patients, both for conventional disease modifying anti-rheumatic drugs and TNF-inhibitors. The accumulating experience with biological agents has also led to a better elucidation of adverse events. In particular, paradoxical immune-mediated inflammatory reactions, which consist in the onset or the exacerbation, during anti-TNF treatment, of manifestations commonly responding to biologics, are increasingly recognized. As discussed by Garcovich et al., they frequently involve the skin, mainly with a psoriatic presentation. Paradoxical reactions should be adequately accounted by clinicians as, although rare, are regarded as an important cause of biological discontinuation. With the third millennium, the rheumatology community has welcomed several new biologicals, such as anakinra and belimumab. Anakinra is a recombinant form of human protein interleukin (IL)-1 receptor antagonist (IL-1Ra), and has been used in adult onset Still's disease (AOSD). Unfortunately, IL-1 blockade is not effective in all subjects, and in some cases loss of efficacy supervenes. It would be crucial to define the long-term efficacy of anakinra in AOSD, potentially identifying predictors of response. In a multi-centre study on 141 AOSD subjects, Vitale et al. (2019) report a retention rate at −60 and −120 months of 55.2 and 39.5%, respectively. In this study, only the number of swollen joints at baseline is predictive of secondary inefficacy of anakinra, an observation that might guide clinicians in the choice of treatment and follow-up of patients. Belimumab is the only biological agent approved for systemic lupus erythematosus (SLE). By targeting soluble B lymphocyte stimulator (BLyS, also known as

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BAFF), this monoclonal antibody modulates B cells, reducing the survival and the differentiation of B lymphocytes (Samotij and Reich, 2019). Regola et al. propose the usefulness of B cells immunophenotyping during belimumab therapy to monitor the response to treatment. In this study on 14 lupus patients receiving belimumab, a significant association between the decrease of B cells total number at 6 months and SLEDAI-2K improvement at 12 months emerges. More recently, the therapeutic armamentarium reserved to patients with psoriatic arthritis has been expanded significantly, thanks to the interests on the IL-23/IL-17 axis. IL-17 is a major pathogenic player in PsA, and IL-23 is upstream of IL-17. As reviewed by Sakkas et al., several pharmaceutical agents targeting the IL-23/IL-17 axis have been developed, with two compounds being already approved for PsA by regulatory agencies (secukinumab and ustekizumab).

The great progresses matured in the field of rheumatology thanks to the introduction of biological therapies do not imply neglecting the potential beneficial effects of conventional drugs. Achievement in clinical management of patients might be obtained by optimizing current treatments thanks to real-world evidence or by expanding the indications of drugs used in non rheumatological settings. This is the case of viscosupplementation in symptomatic hip osteoartrhritis. De Lucia et al. retrospectively assess the effectiveness and the tolerability of U.S.-guided intra-articular injection of hyaluronic acid. In a cohort of 122 patients, viscosupplementation reduces pain, intake of pain killers and joint stiffness, improving hip functionality. Benefits improve over 12 and 24 months, suggesting an additive effect of repeated injections. Sirolimus is a drug routinely used for the prevention of transplant rejection; by targeting the mammalian target of rapamycin (mTOR), sirolimus inhibits antigen-induced T cell proliferation and increases the number of circulating regulatory T cells. mTOR exerts a pivotal role in pathogenesis of lupus, a condition warranting novel therapeutic tools due to the high morbidity and mortality burden. Unsurprisingly, sirolimus has been tested as treatment for SLE. In a retrospective observational study on 27 patients with mildly active non-renal SLE, a Swedish group reports the efficacy of sirolimus on musculoskeletal manifestations, in particular arthritis and tendinitis (Eriksson et al.). Similarly, aminaphtone is a synthetic derivative of 4-aminobenzoic acid which has been widely used in case of microvascular impairment. Researchers from University of Genoa, Italy, evaluate the response to aminaphtone in a cohort of 46 patients with active Raynaud's phenomenon. This six-month study demonstrates that aminaphtone increases skin blood perfusion and improves clinical symptoms due to Raynaud's phenomenon, a condition still difficult to treat (Ruaro et al.). It can be also envisaged that, in the near future, a better diagnostic assessment of Raynaud's phenomenon might guide treatment choices (Ruaro et al.).

Rheumatological diseases yet acknowledge a poorly clarified aetiopathogenesis: a further elucidation of pathogenic mechanisms will hopefully guide the development of future therapeutic tools. The present Research Topic includes two studies investigating the pathogenic role and the potential pharmacological relevance in rheumatoid arthritis (RA) of matrix metalloproteinases (MMP), endopeptidases deputed to degradation of extra-cellular matrix and cleavage of non-matric peptides. Horvárth et al. show in a mouse model of chronic arthritis the increase activity of all MMP isoforms in the joints. They also explore the effects of a broad-spectrum inhibitor of MMP, doxycycline, administered at a subantimicrobial dose. Doxycycline does not influence clinical signs, mechanical hyperalgesia and joint function. However, a worsening of bone microarchitectural lesions emerges, but such effect is most probably independent of MMP inhibition. Conversely, a Chinese research group reports that astragalin, a flavonoid extracted from leaves of persimmon and green tea seeds, effectively prevents the evolution of synovial inflammation and joint destruction in a murine model of collagen-induced arthritis (CIA), and such protective effect is partly mediated by the inhibition of MMP. Astragalin exerts an even broader action: it down-regulates pro-inflammatory cytokines in chondrocytes and synovial cells from CIA mice and inhibits p38 and JNK in MH7A cells treated with TNFα (Jia et al.). Furthermore, this issue provides insights into the pathogenesis of SLE: Furini et al. ascertain the role of P2X7R in a cohort of 48 lupus patients. P2X7R is an extracellular receptor for adenosine triphosphate, which is expressed by all immune cells. Upon activation, P2X7R guides the assembly of NLRP3 inflammasome, which in turn leads to the release of IL-1β and IL-18 and, indirectly, of additional cytokines involved in SLE pathogenesis. P2X7R expression is reduced in PBMCs from SLE subjects, even though there is no significant correlation between P2X7R activity and disease activity or duration, and experiments exclude a P2X7R role in driving the inflammatory response. These observations exert obvious pharmacology implications, as several drugs target IL-1β. Lastly, Yacoub and Schulke propose a novel model to explain the regulation of T cells by apoptotic cells, previously thought to be "immunologically null". According to their theory, in a non-inflammatory context, apoptotic cells induce a immunosuppressive or tolerogenic response via CD80-CTLA4 coinhibitory signal to T cells, thus overriding costimulatory signals and ultimately counteracting T cell activation. Thanks to an extensive literature revision, these authors identify systemic autoimmune conditions in which this immunomodulatory model might be of therapeutic importance.

As a whole, the present Research Topic clearly pictures the complexity of the pharmacological approach in rheumatology. Despite the many progresses in the management of rheumatological diseases, clinicians are still far to offer optimal options to patients: the rates of treatment refractoriness are still high, side effects of drugs might be serious and costs are often burdensome. It is undeniable that much still remains to do, but the tremendous efforts of the research community give hope that many new advancements will be soon accomplished.

AUTHOR CONTRIBUTIONS

CBC, P-JJ and MG wrote the manuscript.

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BRIEF RESEARCH REPORT
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Sex Differences in Response to TNF-Inhibiting Drugs in Patients With Spondyloarthropathies or Inflammatory Bowel Diseases

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Spondyloarthritis (SpA) and inflammatory bowel diseases (IBD) are chronic inflammatory diseases characterized by an aberrant immune response and inflammation with a key role for TNF in their pathogenesis. Accordingly, TNF-inhibiting therapy (TNFi) has dramatically improved the management of these diseases. However, about 30% of patients discontinue TNFi for lack of response, loss of response, and side effects and/or adverse events. Thus, the possibility to identify in advance those patients who will have a good response to TNFi would be extremely beneficial. The aim of this study was to investigate differences between males and females with either SpA or IBD in response to TNFi molecules, i.e., infliximab (IFX) and adalimumab (ADA), considering the reasons for TNFi withdraw. Data of 594 patients, 349 with IBD (M/F: 194/155) and 245 with SpA (M/F: 123/122), previously unexposed to TNFi, were collected. In the IBD group, the rate of female patients discontinuing ADA was significantly higher than that of male patients ($p = 0.03$). No difference emerged according to the distribution of reason for discontinuation. Otherwise, a similar discontinuation rate between female and male patients receiving IFX therapy was observed. In the SpA group, the overall discontinuation rate was not different between males and females both for ADA and IFX. However, in patients treated with ADA, males interrupted therapy more frequently than females due to lack of response ($p = 0.03$). In conclusion, the assessment of sex differences in TNFi response could help physicians personalize the therapeutic approach in a sex-oriented perspective.

Keywords: spondyloarthritis, inflammatory bowel disease, sex differences, adalimumab, infliximab

Abbreviations: ADA, adalimumab; IBD, inflammatory bowel disease; IFX, infliximab; SpA, spondyloarthritis; TNFi, TNF-inhibiting therapy.

INTRODUCTION

Chronic inflammatory diseases are a group of pathologies with different clinical features, but with shared pathogenetic mechanisms leading to a not resolving inflammation. SpA refers to a group of inflammatory diseases that cause arthritis, consisting of predominantly axial or peripheral clinical manifestations (Proft and Poddubnyy, 2018). IBD is a group of chronic systemic diseases causing inflammation of the gastrointestinal tract. Ulcerative colitis affects only the large bowel (Ungaro et al., 2017) and Crohn's disease can involve the whole gastrointestinal tract (Torres et al., 2017). Several lines of evidence support a key pathogenic role for TNF in perpetuating chronic inflammation of SpA and IBD. In SpA, increased TNFα in synovial tissues and sacroiliac joints, associated with bony destruction, osteoproliferation, and synovitis, were found (Chou, 2013; Dubash et al., 2018). In IBD, elevated expression of TNF was detected in involved colonic tissue (Dionne et al., 1997; Matsuda et al., 2009). Moreover, high levels of TNF in serum and in the intestinal lamina propria were found to correlate with disease activity (Murch et al., 1991, 1993; Maeda et al., 1992). The pathogenic effect of TNF is related to its ability to disrupt the integrity of intestinal epithelium and affect the activity of regulatory T cells and regulatory macrophages (Ślebioda and Kmieć, 2014). In the treatment of SpA and IBD, corticosteroids and immunosuppressive agents are used to induce and maintain long-term remission. When these drugs fail to achieve sufficient disease control, biologic agents are used. Indeed, the introduction of TNFi has dramatically improved the management of SpA and IBD. However, about 30% of patients do not respond to TNFi at all or lose their initial response over time. In the time frame from initiation of therapy until response can be judged, non-responding patients suffer from uncontrolled disease. In addition, some patients have to discontinue TNFi because of severe side effects and/or adverse events (Kalden and Schulze-Koops, 2017; Lopetuso et al., 2017). Thus, the ability to identify in advance those patients who will have a good response to TNFi would be extremely beneficial.

Gender medicine deals with studying how diseases differ between men and women in terms of prevention, clinical signs, prognosis, and therapeutic approach (Baggio et al., 2013). In particular, there are sex-related differences in pharmacokinetics and pharmacodynamics, with evident consequence in the efficacy and side effects and/or adverse events of various drugs in the two sexes. This evidence should be considered before starting any therapy.

Of note, both SpA and IBD are characterized by the presence of sex differences in their onset, progression and response to therapy (Rusman et al., 2018a,b; Shah et al., 2018). Regarding TNFi, differences between males and females in response to these drugs have recently been suggested, with males responding better than females (Gonzalez-Lama et al., 2008a,b; Olivera et al., 2017; Rusman et al., 2018a,b). However, the relationship between sex and response to TNFi in SpA and IBD is far to be fully elucidated. Hence, aim of this study was to better investigate differences between males and females with either SpA or IBD in response to two TNFi molecules that dominate the biologic management of these diseases, i.e., IFX and ADA, taking into account the most common reasons for TNFi withdraw, including lack of response, loss of response at follow-up, and side effects and/or adverse events.

MATERIALS AND METHODS

This was a retrospective study enrolling IBD and SpA patients in 7 centers (4 Rheumatology Units and 3 Gastroenterology Units). In each center, diagnostic criteria recommended by International guidelines were followed (Gossec et al., 2016; Gomollon et al., 2017; Magro et al., 2017; van der Heijde et al., 2017). Only patients receiving TNFi therapy (ADA; IFX) were considered. Clinical records were reviewed and data were anonymously collected in a specific electronic database. For statistical analyses, the Mann-Whitney *U*-test, Chi-square test and the Fisher's exact test were used. The significance level was fixed at $p < 0.05$. This study was carried out in accordance with the recommendations of the Declaration of Helsinki and the ethics committee of Azienda Ospedaliera San Camillo Forlanini approved the study (2006/CE Lazio 1).

RESULTS

Data of 594 patients, 349 with IBD (M/F: 194/155) and 245 with SpA (M/F: 123/122) were collected.

Inflammatory Bowel Diseases

Clinical characteristics of IBD patients according to sex are shown in **Table 1**. There were 155 females (mean age 47 ± 16 years and disease duration 133 ± 110 months) and 194 males (mean age 46 ± 14 years and disease duration 144 ± 104 months) with IBD.

Overall, 80 (22.9%; 95% CI = 18.8–27.6) patients discontinued therapy for any reason. Among the female patients, 77 (50%) were treated with ADA and 78 (50%) with IFX. The rate (17/77, 22%) of female patients discontinuing ADA was not significantly different to that discontinuing IFX (25/78, 32%). Among the 194 male patients, 85 (44%) patients were treated with ADA and 109 (56%) with IFX. Of note, the rate (30/109, 27.5%) of male patients discontinuing IFX was significantly ($p = 0.0018$) higher than that discontinuing ADA (8/85, 9%). This observation suggested a better success of ADA than IFX treatment in male patients with IBD.

When comparing data according to sex (**Table 1**), the overall rate of female patients discontinuing ADA (17/77, 22%) was significantly ($p = 0.03$) higher than that of male patients (8/85, 9%). However, no difference emerged according to the distribution of reason for discontinuation, i.e., lack of response, loss of response at follow-up, and side effects and/or adverse events. No significant differences between female and male patients were detected for IFX discontinuation.

Analyzing treatment duration before drug discontinuation (**Figure 1**), we observed that this parameter was significantly shorter in female than male patients for IFX (median 6 months, range 1–50 months vs. 17 months, range 1–146 months,

TABLE 1 | Clinical characteristics of patients with IBD and SpA according to sex.

Patients	IBD		*P*	SpA		*P*
Sex	***F***	***M***		***F***	***M***	
Number, n	155	194		122	123	
Age, years (mean ± SD)	47 ± 16	46 ± 14	0.59	53 ± 14	55 ± 13	0.39
Disease duration, months (mean ± SD)	133 ± 110	144 ± 104	0.16	52 ± 71	64 ± 85	0.88
Adalimumab, n (%)	77/155 (50%)	85/194 (44%)	0.28	93/122 (76.3%)	76/123 (61.8%)	**0.02**
Treatment discontinuation, n (%)	17/77 (22%)	8/85 (9%)	**0.03**	26/93 (28%)	16/76 (21%)	0.37
Lack of efficacy, n (%)	6/17 (35.3%)	2/8 (25%)	1.0	7/26 (27%)	10/16 (62.5%)	**0.03**
Loss of efficacy, n (%)	7/17 (41.2%)	3/8 (37.5%)	1.0	14/26 (53.8%)	6/16 (37.5%)	0.35
Side/adverse effects, n (%)	4/17 (23.5%)	3/8 (37.5%)	0.6	5/26 (19.2%)	0/16 (0%)	0.14
Infliximab, n (%)	78/155 (50%)	109/194 (56%)	0.28	29/122 (23.7%)	47/123 (38.2%)	**0.02**
Infliximab discontinuation, n (%)	25/78 (32%)	30/109 (27.5%)	0.52	18/29 (62%)	22/47 (47%)	0.24
Lack of efficacy, n (%)	8/25 (32%)	3/30 (10%)	0.09	6/18 (33.3%)	8/22 (36.4%)	1.0
Loss of efficacy, n (%)	9/25 (36%)	16/30 (53.3%)	0.28	4/18 (22.2%)	4/22 (18.2%)	1.0
Side/adverse effects, n (%)	8/25(32%)	11/30 (36.7%)	0.78	8/18 (44.5%)	10/22 (45.4%)	1.0

Bolded values refer to significant values (P < 0.05).

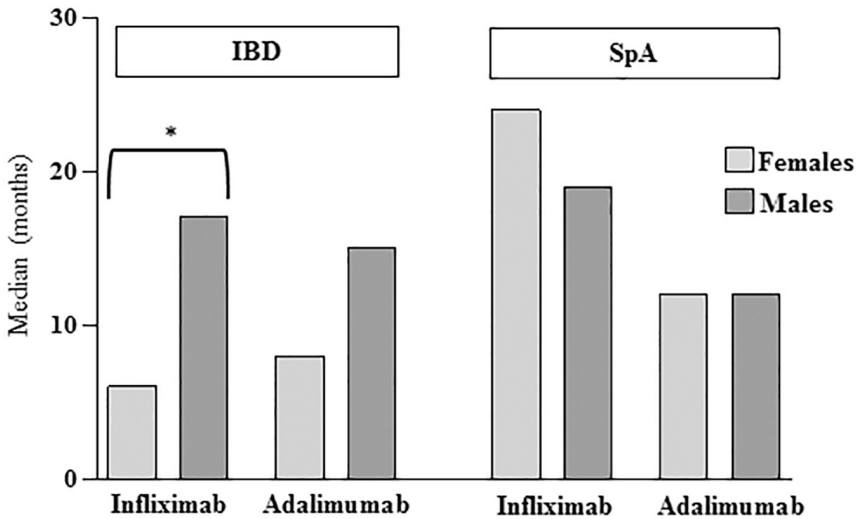

FIGURE 1 | Median of therapy duration before interruption in either Inflammatory Bowel Disease (IBD) or Spondyloarthritis (SpA) patients divided according to sex and type of drug. *p = 0.003 by Mann-Whitney *U*-test.

p = 0.003) but not for ADA (median 8 months, range 2–48 months vs. 15 months, range 2–35 months).

Spondyloarthritis

Clinical characteristics of SpA patients according to sex are shown in **Table 1**.

We enrolled 122 females (mean age 53 ± 14 years and disease duration 52 ± 71 months) and 123 males (mean age 55 ± 13 years and disease duration 64 ± 85 months) with SpA.

Overall, 82 (33.5%; 95% CI = 27.9–39.6) patients discontinued therapy for any reason. Among female patients, 93 patients (76.3%) were treated with ADA and 29 (23.7%) with IFX. The rate of female patients discontinuing IFX was significantly higher than that discontinuing ADA (18/29, 62% vs. 26/93, 28%, p = 0.002). Among male patients, 76 (61.8%) were treated with ADA and 47 (38.2%) with IFX. The rate of male patients discontinuing IFX was significantly higher than that discontinuing ADA (22/47, 47% vs. 16/76, 21%, p = 0.005). This observation suggested a better success of ADA than IFX treatment in both female and male patients with SpA.

Considering sex differences (**Table 1**), regarding the choice of TNFi a higher percentage of females than males started ADA and a higher percentage of males than females started IFX (p = 0.02 in both cases). The overall discontinuation rate was not different between males and females, however in the ADA therapy group male patients interrupted therapy more frequently than females due to lack of response (10/16, 62.5% vs. 7/26, 27%, p = 0.03). As shown in **Figure 1**, the median of therapy duration before interruption was similar between female and male patients, for both IFX (24 months, range 1–66 months vs. 19 months, range

4–150 months) and ADA (12 months, range 3–90 months vs. 12 months, range 3–72 months).

DISCUSSION

To investigate whether differences exist between males and females as concerned the response to IFX and ADA, we considered patients with different chronic inflammatory diseases, such as IBD and SpA, disaggregating data for sex and type of drug. Overall, in the IBD patients, we observed that female sex appeared to be a negative predictive factor for ADA response, confirming previous reported studies (Zelinkova et al., 2012; Olivera et al., 2017; Tanaka et al., 2018). In some studies (Zelinkova et al., 2012; Tanaka et al., 2018) ADA discontinuation was observed to be associated to a higher risk in female than in male patients for skin reactions, infections, and arthralgia. In contrast, we did not find any difference between males and females regarding adverse effects underlying ADA discontinuation. Considering that women have a stronger innate and acquired immune response than men, thus being more resistant to infections but more susceptible to autoimmune and allergic reactions (Klein and Flanagan, 2016), the controversial data regarding adverse events needs to be further investigated in a larger patient population. Regarding IFX treatment, no significant differences between female and male patients were detected for drug discontinuation. However, accordingly to Olivera et al. (Olivera et al., 2017), we observed that, among patients discontinuing IFX, females stayed on IFX for a significantly shorter period compared to males. Interestingly, carrying out a head to head comparison of the two TNFi investigated, ADA seems to achieve better results than IFX in males, whereas no differences were observed within the female population.

In the SpA patients, ADA represents the mainstay of management independently from sex. Interestingly, more female patients were treated with ADA whereas more male patients were treated with IFX. No differences for ADA or IFX discontinuation were detected between males and females. Regarding the reason for ADA discontinuation, lack of drug efficacy is more frequent in males than in females. Our data are in contrast with those reported by literature showing that treatment efficacy of TNFi in SpA was lower in women compared to men and that switching TNFi treatment was more frequent in female than in male patients (Rusman et al., 2018a,b). In this regard, one important limitation of our retrospective study is the lack of information regarding a range of variables able to impact TNFi response, such as smoking, body mass index, and glucocorticoid use. Hence, a larger prospective study, taking into account all these variables, is strongly needed to better define the role of sex in TNFi response.

CONCLUSION

In conclusion, our data found that patients with chronic inflammatory diseases may have different outcomes linked to the type of drug and the disease, as well as to the sex. In this context, the assessment of sex differences in TNFi response could help physicians personalize the therapeutic approach in a sex-oriented perspective in different inflammatory diseases.

AUTHOR CONTRIBUTIONS

BL, AZ, MS, MC, AM, APD, RL, PS, and LR contributed to patient enrolment, data collection, and interpretation. VB provided intellectual input throughout the study. MP and EO provided important contribution to the conception of the work as well manuscript writing. All the authors read and approved the final manuscript.

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Clinical Experience of Sirolimus Regarding Efficacy and Safety in Systemic Lupus Erythematosus

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New treatment options constitute unmet needs for patients diagnosed with systemic lupus erythematosus (SLE). Inhibition of the mammalian target of rapamycin (mTOR) pathway by sirolimus, a drug approved and in clinical use to prevent transplant rejection, has shown promising effects in lupus animal models as well as in patients with both antiphospholipid syndrome and SLE. Sirolimus inhibits antigen-induced T cell proliferation and increases the number of circulating regulatory T cells. Recently, sirolimus was tested in an open label phase 1/2 trial, including 43 patients with active SLE, resistant or intolerant to conventional medications. The results were encouraging showing a progressive improvement, including mucocutaneous and musculoskeletal manifestations. At our university unit, we have more than 16 years' experience of sirolimus as treatment for non-renal manifestations of SLE. Herein, we retrospectively evaluated data on tolerance, dosage, affected organ systems, disease activity measures, corticosteroid reduction, concomitant immunosuppressive therapies, and patient-reported outcome measures (PROMs) such as pain intensity, fatigue, well-being and quality-of-life (QoL) in 27 Caucasian patients with mildly active SLE. Musculoskeletal manifestation was the main reason for sirolimus treatment followed by skin involvement and leukocytopenia. Mean time on sirolimus was 47.1 (range 2–140) months. Decreasing global disease activity was observed, as measured by the clinical SLE disease activity index-2000, with a mean reduction of 2.5 points (range −10 to 0) and a corresponding mean reduction of the physician's global assessment (0–4) of 0.64 (range −2 to 0). The mean daily dose of corticosteroids (prednisolone) was reduced by 3.3 mg (−12.5 to 0). Non-significant trends toward improvements of QoL and pain intensity were found. Serious side-effects were not seen during sirolimus treatment, but early withdrawal due to nausea (n = 4) and non-serious infections (n = 2) appeared. This observational study, including longtime real-life use of sirolimus in SLE, is the largest to date and it essentially confirms the results of the recent phase 1/2 trial. Our data indicate that sirolimus is efficient in patients with musculoskeletal SLE manifestations, particularly arthritis and tendinitis. Further randomized controlled trials evaluating the potential benefits of sirolimus in SLE are warranted, but should aim to enroll patients with shorter disease duration, less accrued damage, and more diverse ethnicities.

Keywords: sirolimus, arthritis, tendinitis, musculoskeletal pain, quality-of-life, systemic lupus erythematosus

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INTRODUCTION

Novel therapies aiding patients with systemic lupus erythematosus (SLE) constitute an unmet need since the available drugs often are limited to efficacy in certain disease phenotypes, and may have significant side-effects (Lateef and Petri, 2012). In fact, only few of the medications used in clinical practice today are approved for SLE, and several new candidate drugs have recently failed to meet their primary end-points in randomized controlled trials (Doria et al., 2017; Geh and Gordon, 2018). Instead, current therapeutic strategies for SLE mainly rely on clinical experience of older therapies used in other rheumatic conditions, or originate from the area of transplantation.

The pathogenesis of SLE is multifactorial. Genetic susceptibility and environmental factors play important roles and are accompanied by the involvement of T and B cells, dendritic cells, macrophages and neutrophils (Bengtsson and Rönnblom, 2017). The profound T cell dysfunction found in SLE has partly been attributed to activation of the mammalian target of rapamycin (mTOR), representing an intracellular serine/threonine receptor which regulates cell growth, proliferation and survival. mTOR is formed by a protein complex which includes mTORC1 and mTORC2 (Perl, 2016). mTORC1 drives the expansion of T helper (Th) type 1 cells, Th17 T cells, and CD4-CD8- (double-negative) T cells. mTORC2, as well as mTORC1, inhibit the development of CD4+CD25+FoxP3+ T regulatory cells. In addition, the differentiation of macrophages and dendritic cells is influenced by mTOR (Thomson et al., 2009; Perl, 2016).

Mammalian target of rapamycin is thus implicated in the pathogenesis of SLE in several ways. Patients with SLE have a reduced number of regulatory T cells (T_{regs}) with impaired suppressive activity (Banica et al., 2017; Kato and Perl, 2018). Follicular helper T (Tfh) cells are critical for germinal center formation and B cell activation. Tfh cells are expanded in SLE, and mTOR1 may be of importance for Tfh differentiation, although results are conflicting (Oaks et al., 2016). Furthermore, the B cell stimulating factor BAFF promotes B cell activation via mTOR activation (Ke et al., 2014), and inhibition of mTOR in plasmacytoid dendritic cells limits production of type I interferons, which has obvious implications for SLE (Cao et al., 2008; Bengtsson and Rönnblom, 2017).

Rapamycin, under the generic designation sirolimus, is a drug approved and in clinical use to prevent transplant rejection. Rapamycin has been shown to prevent the development of nephritis in lupus-prone mice (Warner et al., 1994; Lui et al., 2008). Just recently, sirolimus was tested in an open label phase 1/2 trial, including 43 patients with active SLE, resistant or intolerant to conventional medications. The results were encouraging showing a progressive improvement in several disease phenotypes, including mucocutaneous and musculoskeletal manifestations (Lai et al., 2018). In addition, involvement of the mTOR pathway in vascular lesions associated with the antiphospholipid syndrome (APS) has also been suggested and may be of high relevance also in SLE (Canaud et al., 2014). In a recently published case series of 16 patients with active or quiescent lupus nephritis where 7 had a previous history of malignancy, Yap and co-authors described an auspicious response to sirolimus treatment (Yap et al., 2018). However, to our knowledge, larger compilations on longterm real-life experience of sirolimus in SLE have so far not been reported.

At our university unit, we have more than 16 years' experience of sirolimus as treatment for non-renal manifestations of SLE. Herein, we systematically evaluated our retrospective sirolimus data in relation to tolerance, dosage, affected organ systems, disease activity measures, corticosteroid reduction, concomitant immunosuppressive therapies, and patient-reported outcome measures (PROMs). Reduction of global SLE disease activity as defined by the physician's global assessment (PGA) and the clinical SLE Disease Activity Index 2000 (cSLEDAI-2K) score (Scott, 1993; Uribe et al., 2004) constituted the primary outcome of the present study.

MATERIALS AND METHODS

Patients

The University Hospital in Linköping constitutes a tertiary referral center serving two other regional public hospitals in the county council of Östergötland, Sweden. At the Rheumatology outpatient clinic, we have long experience of monitoring patients with SLE by a prospective, structured follow-up program "KLURING" (Swedish acronym for *Clinical LUpus Register In Northeastern Gothia*), including registration of disease phenotypes, ongoing medication, and comorbidities (Frodlund et al., 2013).

As part of the KLURING cohort, a total of 27 patients with SLE, classified according to the 1982 American College of Rheumatology (ACR) criteria (Tan et al., 1982; Ighe et al., 2015), received sirolimus in daily doses of 1–3 mg between June 2002 and August 2018, and were followed from initiation of treatment until withdrawal, death or end of study period. All patients had previously been intolerant, or were judged as inadequate responders, to at least two disease-modifying anti-rheumatic drugs (DMARDs). Patient characteristics at the initiation of sirolimus treatment are further detailed in **Table 1**.

Assessments

Systemic lupus erythematosus disease activity was assessed by the use of PGA (graded 0–4) (Scott, 1993) and the cSLEDAI-2K score (which excludes items for low complement and positive anti-dsDNA antibodies) (Uribe et al., 2004). Acquired organ damage, required to have been persistent for at least 6 months, was recorded by the Systemic Lupus International Collaborating Clinics (SLICC)/ACR damage index (SDI) encompassing damage in 12 defined organ systems (Gladman et al., 1996). Continuous data on PROMs were collected. The PROMs included data on quality-of-life (QoL) captured by the EQ-5D score (Leidl, 2009), functional ability estimated by the health assessment questionnaire (HAQ) (Lomi et al., 1995), as well as pain intensity, fatigue and well-being, all measured using the visual analog scale (VAS; graded 0–100 mm) (Hallert et al., 2003).

Laboratory Measurements

Safety was continuously monitored by blood cell counts, liver enzymes (including alanine aminotransferase and aspartate aminotransferase), plasma creatinine, and blood lipids (including total cholesterol and triglycerides). Inflammatory and serological disease activity were followed by the erythrocyte sedimentation rate (ESR), and plasma analyses of C-reactive protein (CRP), creatine phosphokinase (CK), complement protein 3 (C3), and 4 (C4).

TABLE 1 | Characteristics of the included patients at the start of sirolimus treatment.

Patient characteristics	Mean (range) or %
	All (n = 27)
Background variables	
Females	100
Age (years)	44.3 (20–65)
Duration of SLE (years)	9.8 (2–34)
Weight (kg)	65.6 (47–93)
Length (cm)	165.7 (147–176)
Caucasian ethnicity	100
cSLEDAI (score)	4.5 (1–12)
PGA (score)	1.3 (0–2)
SDI (score)	1.0 (0–6)
Number of fulfilled ACR criteria	5.5 (4–8)
Concomitant medication	
Prednisolone, daily dose (mg)	7.5 (0–20)
Hydroxychloroquine	59.2
Methotrexate	7.4
Mycophenolate mofetil	11.1
Warfarin	14.8
Acetylsalicylic acid	29.6
Statins	0
Clinical phenotypes (ACR-82 definitions)	
(1) Malar rash	48.1
(2) Discoid rash	18.5
(3) Photosensitivity	63.0
(4) Oral ulcers	22.2
(5) Arthritis	100
(6) Serositis	48.1
(a) Pleuritis	48.1
(b) Pericarditis	3.7
(7) Renal disorder	25.9
(8) Neurologic disorder	3.7
(a) Seizures	3.7
(b) Psychosis	0
(9) Hematologic disorder	66.7
(a) Hemolytic anemia	3.7
(b) Leukocytopenia	37.0
(c) Lymphocytopenia	44.4
(d) Thrombocytopenia	14.8
(10) Immunologic disorder	51.9
(a) Anti-dsDNA antibody	44.4
(b) Anti-Smith antibody	7.4
(11) IF-ANA	100

ACR, American College of Rheumatology; IF-ANA, immunofluorescence microscopy antinuclear antibodies; cSLEDAI-2K, clinical Systemic lupus erythematosus disease activity index 2000; SDI, SLICC/ACR damage index.

Statistics

The GraphPad software (version 4.0; GraphPad Software Inc., San Diego, CA, United States) and the Python Language Reference (version 3.7, available at http://www.python.org, Python Software Foundation, Wilmington, DE, United States) were used for preparing figures and for statistical evaluation. Since the number of observations was different between many visits, repeated paired t-tests were used to examine differences in laboratory variables overtime and Wilcoxon's test for paired samples was used to evaluate disease activity scores. Correlation analysis was performed using Pearson's correlation coefficient. Two-tailed $p < 0.05$ was considered significant.

RESULTS

Patients Treated With Sirolimus

As demonstrated in **Table 2**, 27 unique female SLE patients at our unit were prescribed sirolimus between June 2002 and August 2018 (study period). The mean daily dose was 1.5 mg (range 1–3). Before start of sirolimus, the mean number of failed DMARDs was 3.6 (range 2–6). The mean time on sirolimus was 47.1 (range 2–140) months. Six of 27 (22%) withdraw the drug due to nausea (n = 4) and non-serious infections (n = 2) before the 3-month evaluation visit (early cessation indicated by asterisks in **Table 2**), which was the reason why these six cases were excluded from efficacy analyses. At the last follow-up in August 2018, seven patients were still on treatment with sirolimus, and one individual (who had reached remission after 70 months on sirolimus) was not considered in need of the drug anymore; this corresponds to a drug survival of 38% regarding cases that passed the 3-month evaluation visit.

Organ Manifestations

As shown in **Table 2**, musculoskeletal involvement was the target for sirolimus treatment (96%), followed by cutaneous lupus (37%), and leukocytopenia (7%). Regarding specific musculoskeletal manifestations, arthritis (54%) was the dominating reason for sirolimus, but tendinitis (15%) and arthralgia (31%) were also common. Seven of 27 (26%) patients had a history of renal involvement, but none had signs of active lupus nephritis at the initiation of sirolimus. 5 (19%) had concomitant APS. Sirolimus was frequently combined with corticosteroids, hydroxychloroquine (HQ) and/or other DMARDs as indicated in **Table 2**.

Efficacy

Inflammatory and serological disease activity was followed over time by measurement of ESR, CRP, C3, C4, and CK. As illustrated in **Figure 1**, levels of C4 increased slightly over time, whereas CRP and ESR were rather stable. CK did not change significantly (not shown). As shown in **Figure 2A**, a decreased

TABLE 2 | Individual descriptions of the 27 female pations.

Patient number	Age at start (years)	Target organs	Sirolimus exposure (months)	Daily dose (mg) of sirolimus	Combining DMARDs	Cause of cessation	Number of DMARDs ahead of sirolimus	SDI at start	SDI at last follow-up on sirolimus
1	58	Musculoskeletal, leukocytopenia	117	2	HQ	Treatment ongoing	2	1	1
2	65	Musculoskeletal, discoid lupus	10	3	None	Rash, swelling of legs	3	6	6
3	43	Musculoskeletal	18	1	HQ, MMF	Decreasing effect	6	3	3
4	53	Musculoskeletal	96	1	None	Treatment ongoing	4	0	1
5	62	Musculoskeletal	35	2	HQ, MTX	Itching, headache	5	1	3
6*†	51	Musculoskeletal, leukocytopenia	3	1	None	Infection, lack of efficacy	6	2	2
7	59	Musculoskeletal, malar rash	13	1	HQ	Increased liver enzymes	3	0	1
8	37	Musculoskeletal, alopecia, pleuritis	4	2	None	Nausea	5	1	1
9	27	Musculoskeletal	129	1	HQ	Treatment ongoing	4	0	2
10†	56	Musculoskeletal	31	2	None	Malignancy	3	1	1
11	35	Musculoskeletal	7	1	None	Itching, fatigue	2	1	1
12	61	Musculoskeletal	63	2	HQ	Treatment ongoing	2	1	2
13	44	Musculoskeletal	140	3	HQ, MMF	Treatment ongoing	4	1	1
14	52	Musculoskeletal	111	2	None	Treatment ongoing	3	1	2
15†	50	Musculoskeletal, malar rash	127	2	None	Infections	5	2	7
16*	32	Musculoskeletal	2	1	HQ	Nausea	3	0	0
17	38	Musculoskeletal, photosensitivity	4	1	HQ	Lack of efficacy	4	0	0
18*	48	Musculoskeletal	3	1	HQ	Nausea	2	0	0
19	20	Musculoskeletal	70	1	HQ	Reached remission	4	0	0
20	50	Musculoskeletal	104	1	HQ	Angioedema	4	0	1
21	48	Musculoskeletal	7	1	HQ, MMF	Nausea	3	1	1
22	21	Lupus profundus	27	1	HQ	Diarrhea	6	1	1
23*	38	Musculoskeletal, malar rash	3	1	None	Nausea	2	0	0
24	34	Musculoskeletal, photosensitivity	135	1	HQ	Treatment ongoing	2	0	0
25*†	44	Musculoskeletal	3	1	None	Infections	2	0	0
26*†	29	Musculoskeletal, alopecia	2	2	MTX	Nausea	5	3	3
27	40	Musculoskeletal, oral/genital ulcers, acute cutaneous lupus	10	2	HQ	Lack of efficacy	5	0	0

**Early cessation (≤3 months). †Deceased at the end of study period. HQ, hydroxychloroquine; MMF, mycophenolate mofetil; MTX, methotrexate.*

global disease activity was observed over time using cSLEDAI-2K (p = 0.0002) with a mean reduction of 2.5 (range −10 to 0) comparing the time-point of initiation with the last observation. A corresponding reduction of 0.64 (−2 to 0) regarding PGA (**Figure 2B**) was also found (p = 0.0005). Sirolimus appeared to be especially effective against arthritis and tendinitis, whereas

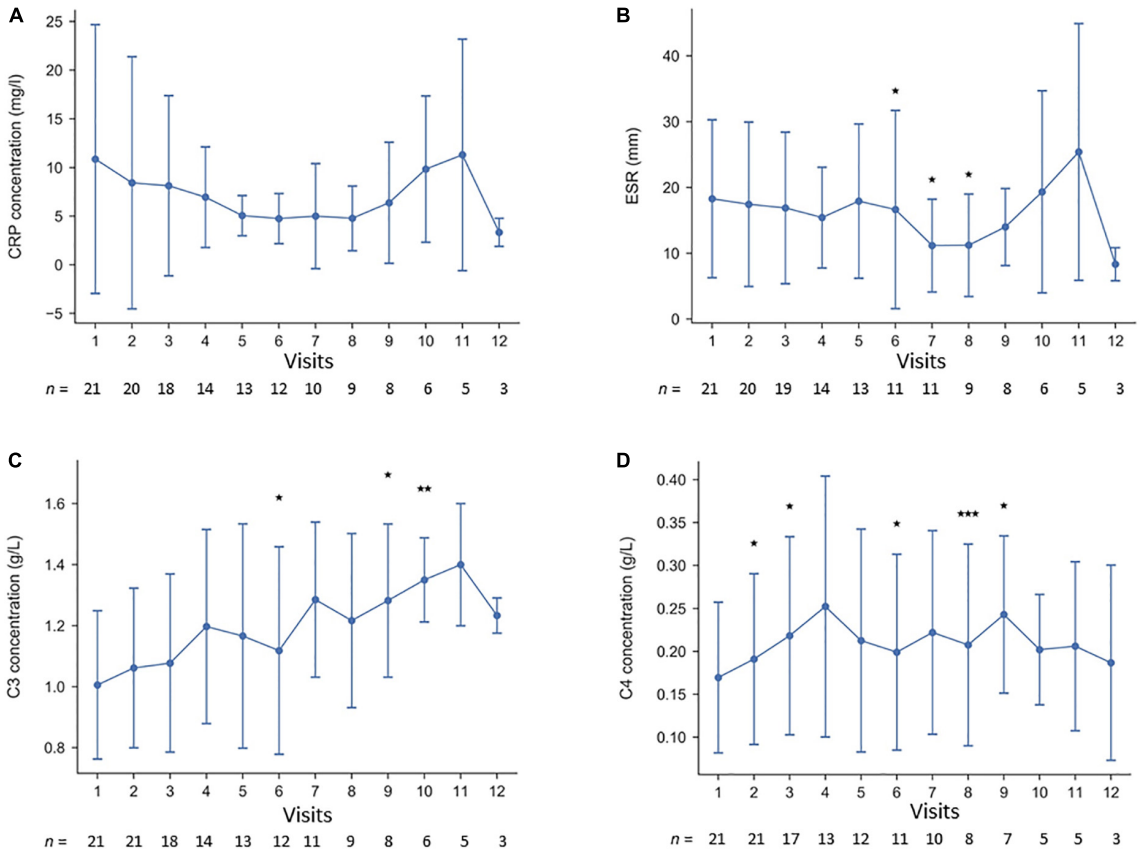

FIGURE 1 | (A–D) Longitudinal laboratory efficacy data at the first 12 visits of the 21 cases that passed the 3-month evaluation visit; **(A)** C-reactive protein, **(B)** erythrocyte sedimentation rate, **(C)** complement protein 3, and **(D)** complement protein 4. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$.

patients with arthralgia did not respond (**Figure 2C**). The mean daily dose of corticosteroids (prednisolone) at start was 7.5 mg (**Table 1**), but it was reduced by 3.3 mg (range −12.5 to 0) comparing the time-point of sirolimus initiation with the last observation ($p < 0.001$). The correlation between exposure to sirolimus and reduction of prednisolone dose was highly significant ($r = -0.7$, $p < 0.0004$) (**Figure 2D**). No significant improvements of PROMs (EQ-5D, HAQ, VAS pain intensity/fatigue/well-being) were observed. SDI scores at the initiation, and at the time-point of last follow-up on sirolimus, are demonstrated in **Table 2**. As shown in **Table 1**, the mean SDI at initiation of sirolimus was 1.0 (0–6), and at last follow-up 1.5 (0–7). The mean annual accrual of SDI on sirolimus was 0.1 (range 0–0.9).

Safety

At end of the study period, 22 of 27 cases were alive. The five deceased patients (mean age 53.4 years, range 33–63) had been on sirolimus for a mean time of 33.2 months (range 2–133). The cause of death was malignancy in three cases (adenocarcinoma of the lung, ovarian cancer, acute myeloid leukemia) of which two patients had early cessations of sirolimus (before the 3-month evaluation visit). Sepsis was the cause of death in the other two cases, whereof one patient had an early withdrawal. All five patients had discontinued sirolimus at the time-point of death, and none of the deaths were considered related to the drug. No renal flares, or onset of new lupus nephritis, were observed in any of the 27 patients.

No myocardial infarctions were registered, but two minor strokes were observed. Patient number 9, with SLE since 1983, was started on sirolimus because of arthritis in October 2007. Due to a new onset of seizure, a brain magnetic resonance imaging (MRI) was performed and showed new ischemic lesions. Antiphospholipid antibodies were detected and she was, in addition to SLE, diagnosed with APS which led to continuous treatment with warfarin. This event was indeed considered as an SLE exacerbation. However, the patient is still on sirolimus and has not had further strokes since then. Patient number 15, with multiple sclerosis since the 90s and SLE combined with APS since 2000, was started on sirolimus due to arthritis in June 2002. In 2009, she developed a minor warfarin-dependent cerebrovascular bleeding but the treatment with sirolimus was not discontinued until 2013.

Drug safety was continuously monitored by blood tests. As demonstrated in **Figure 3**, no alarming signals regarding blood cell counts or renal function were noted. None of the patients developed hypercholesterolemia or triglyceridemia, leading to treatment with statins. After 13 months, patient number 7 ended

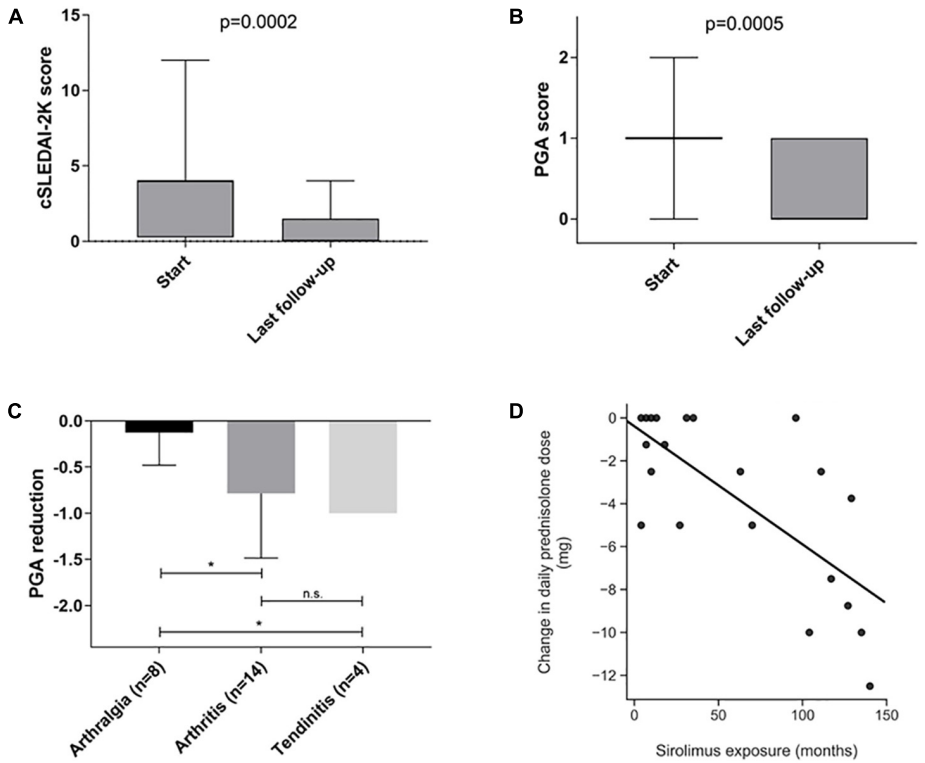

FIGURE 2 | (A–D) Differences in global disease activity between start/initiation of sirolimus therapy with regard to **(A)** clinical SLEDAI-2K, and **(B)** physician's global assessment (PGA). **(C)** Illustrates the reduction of PGA scores with regard to type of musculoskeletal manifestation. **(D)** Demonstrates the correlation between reduction of daily corticosteroid dose and the exposure of sirolimus in months. $^*p < 0.05$.

treatment with sirolimus due to elevated liver enzymes. However, both alanine aminotransferase and aspartate aminotransferase normalized shortly after cessation.

DISCUSSION

Current knowledge on the performance of sirolimus in autoimmune diseases is increasing, but observational data are mainly missing in rheumatology. Thus, we aimed to retrospectively compile the 16-years of clinical experience we have at our university unit on sirolimus in SLE. As a drug with potency of blocking T cell activation, sirolimus has clear-cut implications for the SLE pathogenesis. In support of sirolimus as a suitable treatment option in SLE, blockade of the mTOR pathway has shown promising effects in lupus animal models (Warner et al., 1994; Bonegio et al., 2005; Lui et al., 2008) as well as in patients (Fernandez et al., 2006; Yap et al., 2012, 2018; Lai et al., 2013; Bride et al., 2016; Herold et al., 2018). The recent phase 1/2 trial showed effects primarily in the mucocutaneous and musculoskeletal organ systems (Lai et al., 2018). Reduced number of new episodes of rash was also reported but quite few patients developed cutaneous lupus over the study period why distinct conclusions were not possible. However, very interestingly, Lai et al. (2018) also observed that low levels of T_{regs} were reversed and high levels of interleukin (IL) 4 and IL-17 from other T cells than T_{regs} were reduced during the sirolimus treatment. Previously, Bride with coauthors reported beneficial effects of sirolimus on severe autoimmune cytopenias (Bride et al., 2016) and satisfactory response in individual patients with refractory discoid lupus erythematosus have been observed (Herold et al., 2018).

The Swedish healthcare system is tax funded and offers universal access, limiting the risks of patient selection bias, and drugs may be prescribed off label. As far as we know this observational study of sirolimus in SLE, including long time follow-up, is the largest to date. However, this is not a clinical trial and the included 27 patients with mildly active non-renal SLE were intolerant, or previously had an inadequate response, to at least two DMARDs. Some cases had tested multiple DMARDs without success and eventually failed on sirolimus as well. In addition, the study population was limited to cases without active renal or CNS involvement which is reflected by the rather low cSLEDAI-2K scores at start. Albeit, it is encouraging that none of our patients developed new (or incident) renal flares over the study period and the accrual of further damage was modest. Lack of longitudinal data on anti-dsDNA antibody levels and 28-joint disease activity scores constitute limitations of the study.

Comparing retrospective data with results from a clinical trial is challenging, but the outcome with reduced global disease activity (cSLEDAI-2K and PGA) essentially confirm the promising results of the phase 1/2 trial (Lai et al., 2018) and

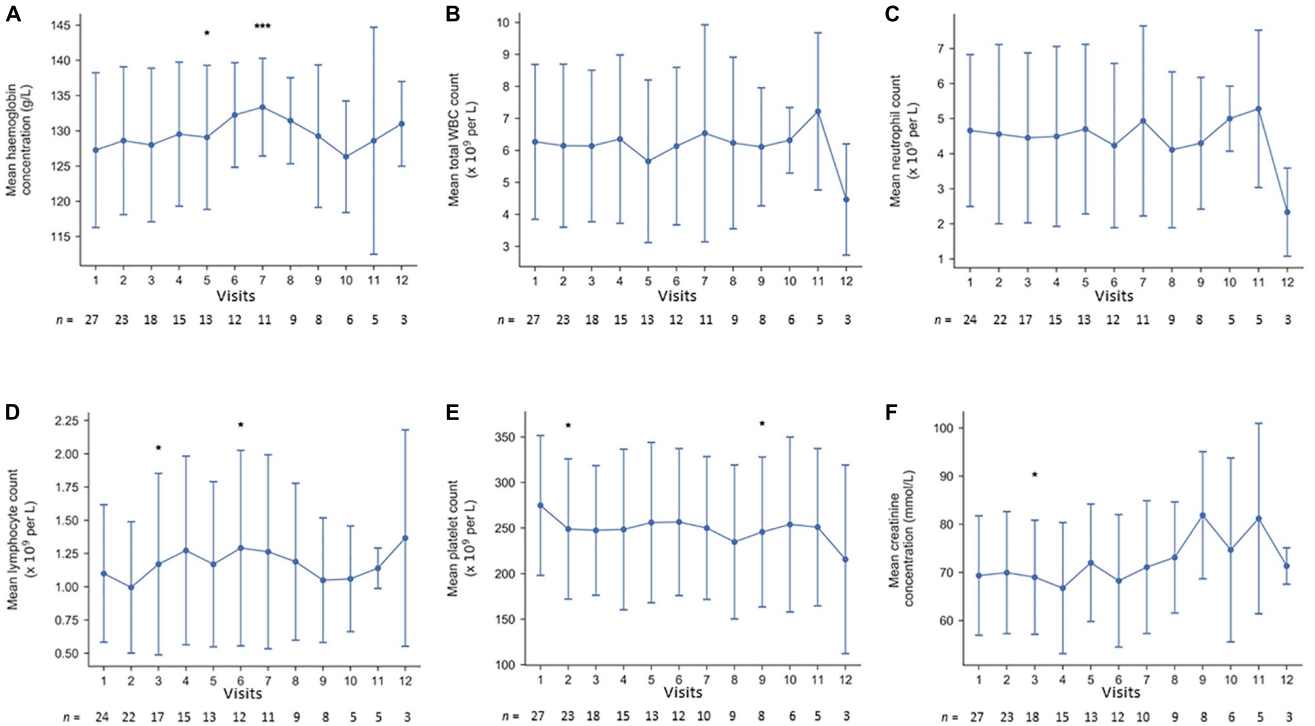

FIGURE 3 | (A–F) Longitudinal laboratory safety data at the first 12 visits of the 27 cases; **(A)** hemoglobin, **(B)** white blood cell count, **(C)** neutrophil count, **(D)** lymphocyte count, **(E)** platelet count, and **(F)** plasma creatinine. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

indicate that sirolimus is efficient in patients with musculoskeletal manifestations (i.e., arthritis and tendinitis). Whereas 2 mg sirolimus daily was used in the trial, we used slightly lower doses (mean 1.5 mg, with range 1–3 mg). Both dose regimens are lower than the doses usually prescribed in renal transplantation, a fact that has experimental support from lupus-prone mice (Warner et al., 1995).

The sirolimus trial did not include any PROMs (Lai et al., 2018). Although our patients with longtime follow-up showed trends toward improved QoL and less reported pain, the data were not statistically significant. The composition of the study population may be one of several reasons for this. Fifteen of 27 (56%) patients were already affected by irreversible organ damage (SDI>0), which has a proven impact on both QoL and activity limitations in SLE (Björk et al., 2015). Failure of up to 6 DMARDs before the initiation of sirolimus probably also led to a bias in term of selection of refractory cases. Thus, for future studies, there may be better options to record improvements on QoL and other PROMs if cases with more recent-onset SLE were eligible.

Premature atherosclerosis in SLE may be related to type I interferons, whereas traditional risk factors seem to be of less importance (Kahlenberg and Kaplan, 2013; Leonard et al., 2018). Thus, pharmacological intervention preventing vascular events in SLE would obviously be of interest. Sirolimus inhibits smooth muscle hypertrophy in vessel walls (Gallo et al., 1999; Elloso et al., 2003), which may outweigh the transient hyperlipidemia sometimes reported in transplanted patients treated with higher doses of sirolimus (Asleh et al., 2018). mTOR signaling is also the major pathway in inhibition of endothelial autophagy which is implicated in atherogenesis (Xiong et al., 2014). Furthermore, as concomitant APS occurs in approximately one third of SLE patients and the mTOR pathway is involved in the vascular lesions related to APS, sirolimus may be of high importance regarding future studies of vascular disease in SLE (Canaud et al., 2014). Our retrospective case series do not permit any conclusions concerning vascular disease, but on the other hand neither hypercholesterolemia nor triglyceridemia were observed among our patients taking low doses of sirolimus. Another action of sirolimus with important implications for lupus nephritis and its longtime prognosis is the anti-fibrotic effects, possibly mediated via E-cadherein in experimental renal fibrosis (Liu, 2006).

A non-negligible proportion of the patients (>20%) experienced non-serious side-effects or general discomfort and stopped sirolimus soon after its introduction. However, major side-effects were not seen and routine laboratory follow-up was normal in almost all cases. The rate of malignancies (11%) may appear high, but none of them occurred during sirolimus therapy and in two of the cases exposure to the drug was very short. A causative effect of sirolimus is unlikely but cannot be entirely excluded. The question is also hampered by the fact that the longterm risk of several types of cancers in SLE is increased (Bernatsky et al., 2013). Data from organ transplant recipients show that longterm immunosuppressive regimens which include mTOR inhibitors are associated with an overall

reduced cancer risk when compared to patients not treated with mTOR inhibitors (Yanik et al., 2015). In transplantation, however, use of sirolimus has been associated with pneumonitis, microangiopathy, thrombocytopenia, hypercholesterolemia, liver toxicity, lymphangioleiomyomatosis, and increased proteinuria in nephrotic patients (Marti and Frey, 2005; Takada et al., 2016). None of the above appeared in our series.

CONCLUSION

In summary, low doses of sirolimus were efficient in reducing global disease activity, especially regarding musculoskeletal manifestations, for patients with established mildly active SLE. Corticosteroids could be withdrawn or significantly reduced in many patients. Serious side-effects were not seen, although some patients stopped medication early due to non-serious discomfort. Only Caucasian patients were enrolled herein why it is warranted that further randomized controlled trials evaluating the potential benefits of sirolimus in SLE encompass larger and more mixed groups of cases.

ETHICS STATEMENT

In Sweden, drugs are allowed to be used off label. Nevertheless, informed consent was obtained from all subjects. The research protocol was approved by the Regional Ethics Review Board in Linköping, Sweden (Decision No. M75-08/2008).

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content and approved the final version to be published. CS had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. PE and CS conceived and designed the study. PE, PW, and CS acquired the data and analyzed and interpreted the data.

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Astragalin Suppresses Inflammatory Responses and Bone Destruction in Mice With Collagen-Induced Arthritis and in Human Fibroblast-Like Synoviocytes

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Astragalin, as a bioactive flavonoid with anti-inflammatory, antioxidant, and protective properties, provides a potential agent for rheumatoid arthritis (RA). In this study, its therapeutic efficacy and the underlying mechanisms were explored using DBA/1J mice with collagen-induced arthritis (CIA). It was demonstrated that astragalin could significantly attenuate inflammation of CIA mice. The effects were associated with decreased severity of arthritis (based on the arthritis index), joint swelling and reduced bone erosion and destruction. Furthermore, astragalin treatment suppressed the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8), and inhibited the expression of matrix metalloproteinases (MMP-1, MMP-3, and MMP-13) in chondrocytes and synovial cells of CIA mice. Fibroblast-like synoviocytes derived from RA patients (MH7A cells) were applied to verify these effects. *In vitro*, astragalin inhibited the expression of matrix metalloproteinases (MMP-1, MMP-3, and MMP-13) dose-dependently in TNF-α-induced MH7A cells, with no apparent cytotoxicity. Furthermore, astragalin suppressed the phosphorylation of p38, JNK, and the activation of c-Jun/AP-1 in TNF-α-induced MH7A cells. In conclusion, it has proven that astragalin could attenuate synovial inflammation and joint destruction in RA at least partially by restraining the phosphorylation of MAPKs and the activating of c-Jun/AP-1. Therefore, astragalin can be a potential therapeutic agent for RA.

Keywords: astragalin, rheumatoid arthritis, matrix metalloproteinase, CIA, fibroblast-like synoviocytes

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune joint disease, which is characterized by inflammation of synovial tissue and the destruction of bone and cartilage in multiple peripheral joints (Smolen et al., 2016). RA is estimated to affect approximately 1% of the adult population worldwide and is a major source of disability (Myasoedova et al., 2010). Despite traditional disease-modifying anti-rheumatic drugs and biological agents have proven to improved clinical outcomes

in patients with RA. However, these beneficial effects are far from satisfaction because of their obvious adverse effects with a high frequency and high cost of treatment (Guo et al., 2018). Therefore, further efforts are required to develop new therapeutic agents with fewer side effects for treatment of RA. Of the potential cellular participants in RA, fibroblast-like synoviocytes (FLSs) play a critical role by regulating the secretion of inflammatory mediators and expression of MMPs, which cause changes in chondrocyte metabolism and matrix degradation (Bartok and Firestein, 2010; Korb-Pap et al., 2016). Hence, exploring new anti-rheumatic arthritis drugs to alleviate the destructive behavior of RA-FLSs may be a reasonable and effective method for the treatment of RA.

Astragalin, also known as kaempferol-3-O-glucoside, is a newly found flavonoid from leaves of persimmon and green tea seeds and has been used for treating many diseases for a long time (Riaz and Rasul, 2018). Several biological functions of astragalin have been studied, including anti-inflammatory, anti-oxidant, and anti-cancer. The previous study has proven that astragalin can downregulate lipopolysaccharide (LPS)-induced inflammatory responses by suppressing the NF-κB signaling pathway (Kim and Kim, 2011; Nhiem et al., 2011). Astragalin can also increase the survival from lethal endotoxemia and reduce acute lung injury in a murine asthma model (Li et al., 2013; Liu et al., 2015). In addition, astragalin can inhibit ovalbumin (OVA)-induced allergic inflammation and eosinophilia in lung tissues (Cho et al., 2014; Kim et al., 2017). However, the therapeutic benefits of astragalin in RA remain unknown. Thus, we speculated that astragalin may be a potential anti-rheumatic arthritis drug.

In this study, we investigated the role of astragalin in the regulation of synovial inflammation and joint destruction in a collagen-induced arthritis (CIA) mouse model *in vivo* and its underlying mechanisms in MH7A RA-derived FLSs *in vitro*.

MATERIALS AND METHODS

Drugs and Reagents

Astragalin (PubChem CID:5282102; purity >98.0%; **Figure 1B**) was purchased from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China); methotrexate (MTX) from Shanghai Oriental Medicine Science and Technology Industry Co., Ltd. (Shanghai, China, cat. # 100138); bovine type II collagen (cat. # 20021), Freund's complete adjuvant (CFA, cat. # 7001), Freund's incomplete adjuvant (IFA, cat. # 7002) from Chondrex (Redmond, WA, United States); MMP-1 (cat. # ab215083), MMP-3 (cat. # ab189572), MMP-13 (cat. # ab100605) antibodies and ELISA kit from Abcam (Cambridge, United Kingdom); TNF-α (cat. # EMC102a), IL-1β (cat. # EMC001b), IL-6 (cat. # EMC004), IL-8 (cat. # EMC104) ELISA kit from Neobioscience (Shanghai, China); recombinant human tumor necrosis factor (TNF-α) from PeproTech (Rocky Hill, CT, United States, cat. # 300-1A); anti-c-Jun (cat. # 9165), anti-phospho-c-Jun (cat. # 3270), anti-c-Fos (cat. # 2250), anti-phospho-c-Fos (cat. # 5348), anti-p38 (cat. # 8690), anti-phospho-p38 (cat. # 4511), anti-extracellular signal regulated kinase (ERK, cat. # 4695), anti-phospho-ERK (cat. # 4370), anti-c-Jun N-terminal kinase (JNK, cat. # 4252), anti-phospho-JNK (cat. # 4255) from Cell Signaling Technology (Beverly, NJ, United States); goat anti-rabbit IgG H&L (Alexa Fluor® 488) (Abcam, Cambridge, United Kingdom, cat. # ab150077); Actinomycin D (cat. # A1410), 4′,6-diamidino-2-phenylindole (cat. #D9564) from Sigma (Sigma-Aldrich, United States); cell culture medium, fetal bovine serum and trypsin from Gibco (Gland Island, NE, United States).

Animals

Specific pathogen-free, DBA/1J male mice (7∼8-week-old) were provided by the Vital River company (Beijing, China). Ten of these mice were assigned to the negative control group and thirty to the experimental group. This study was approved by the Medical Ethics Committee of Shanghai University of Traditional Chinese Medicine. The methods applied in this study were carried out in accordance with the approved guidelines and regulations.

Induction of Collagen-Induced Arthritis

Collagen-induced arthritis model was established according to a previous protocol (Brand et al., 2007). Briefly, bovine collagen type II was dissolved in 10 mM acetic acid to 2 mg/ml. This solution was then emulsified in equal volumes of complete Freund's adjuvant (CFA, 4 mg/ml M. tuberculosis). CIA mice were immunized intradermally by 100 μl of emulsion at the base of the tail on day 0. To ensure a high incidence of RA induction in the CIA model, 100 μl of bovine type II collagen emulsified in incomplete Freund's adjuvant was used as a booster on day 21 after the first immunization. Typically, the first signs of arthritis appeared in this model at 21–28 days after the first immunization.

In vivo Drug Administration

DBA/1J mice were randomly divided into four groups (10 mice/group). Group 1 was used the non-immunized mice (Control), whereas mice in group 2–4 were used the CIA mice. Group 2: mice treated with PBS, 0.2 ml/day/intraperitoneally (CIA-Veh); Group 3: mice treated with MTX, 0.1 mg/kg/3 day/intraperitoneally (CIA-MTX); Group 4: mice treated with astragalin, 5 mg/kg/day/intragastrically (CIA-Ast). All the mice from these groups received additional treatments between day 22 and day 50. The time diagram of the process of CIA induction and treatment is shown in **Figure 1A**.

Arthritis Assessment

Collagen-induced arthritis was considered to have successfully developed when swelling was observed in at least one digit or paw. The global assessment, arthritis index, swollen joints count, and hind paw thickness were scored and recorded every 5 days in a blinded manner as reported before. The severity of arthritis in each of the four paws was scored with a 0–4 scale by visual evaluation of each paw as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the tarsals or ankle joint; 2, erythema and mild

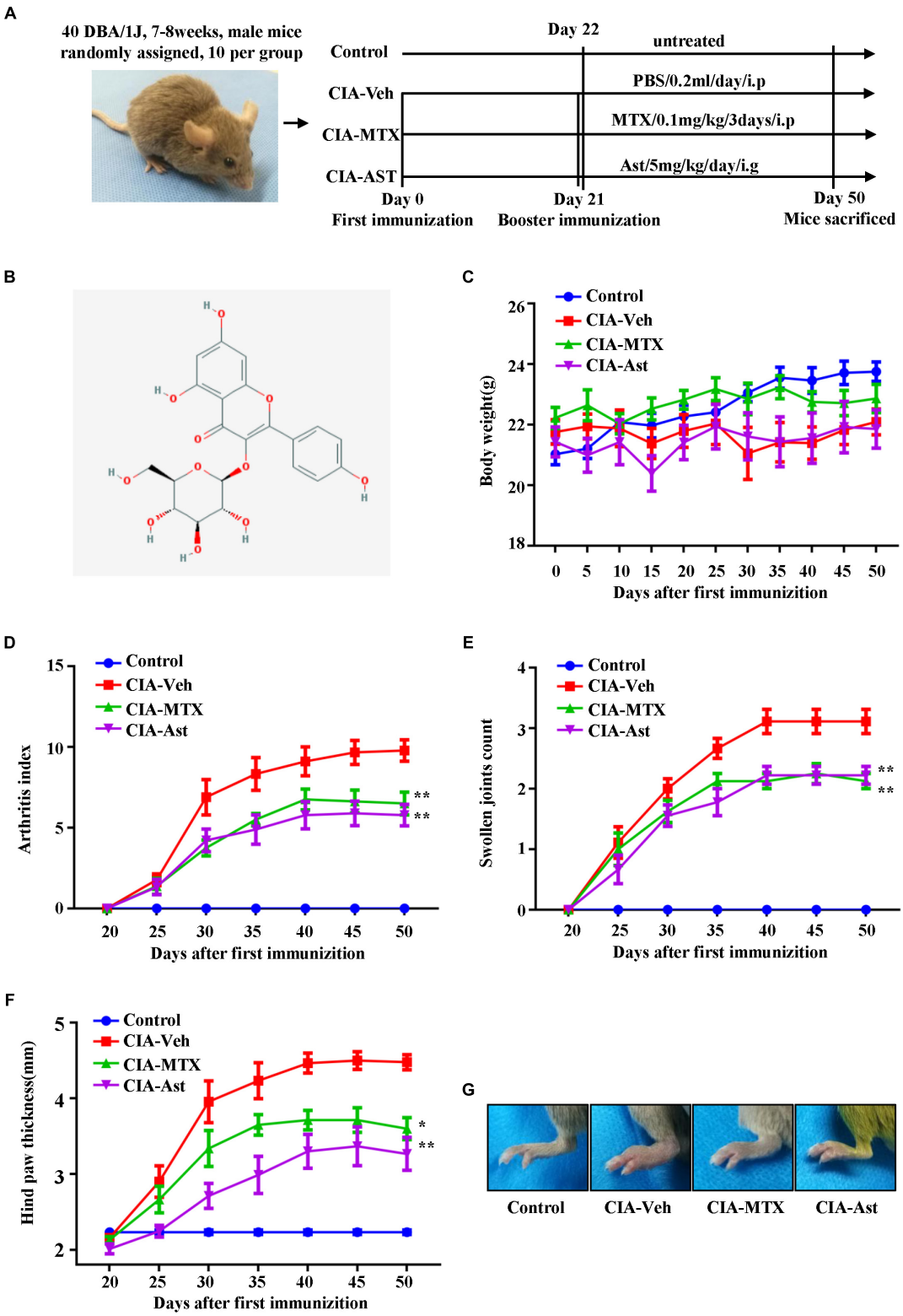

FIGURE 1 | Astragalin attenuated the symptoms of CIA mice. The time diagram of the process of CIA induction and treatment **(A)**. The chemical structure of astragalin **(B)**. Mice with established CIA were injected either with PBS or 0.1 mg/kg/3 day MTX intraperitoneally or with oral dose of astragalin 5 mg/kg/day after day 21 booster immunization. The body weight **(C)**, arthritis index **(D)**, swollen joints count **(E)**, and hind paw thickness **(F)** were scored and recorded every 5 days in a blinded manner. The representative pictures of the hind paws of CIA mice **(G)**. Results are shown as mean ± SEM of ten mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ versus CIA-Veh group.

swelling extending from the ankle to the tarsals; 3, erythema and moderate swelling extending from ankle to metatarsal joints; 4, erythema and severe swelling encompass the ankle, foot, and digits, or ankylosis of the limb. The final score for each mouse was the sum of the four paws. Thickness of the ankle was measured with digital calipers placed across the ankle joint at the widest point.

Ultrasound Analysis

After 7 weeks of treatment, the knee and ankle joints of these mice were examined using the Vevo 2100 imaging system (Vevo LAB, FUJIFILM Visual-Sonics, Toronto, ON, Canada). Both B mode and color Doppler were scanned with the 550 scan head, 40–50 MHz probes, wall filter = 3 mm/s, scan speed = 2 mm/s, dynamic range = 65.0 dB, the pulses to radiofrequency cycle number = 2, the pulse repetition frequency = 6 kHz, after the 2-dimensional (2D) images were obtained in real time, the images were analyzed and measurements manually determined and calculated using the Vevo LAB software studio measurement package. The Vevo LAB software was then used to construct the scans into a 3-dimensional (3D) image, which allowed for accurate volume measurement and image sculpting creating a visual representation of the knee and ankle joints.

Histopathological Assessment

On day 50, mice were sacrificed, the left knee and ankle joint tissues were collected and fixed in 4% paraformaldehyde, then decalcified in 10% EDTA for 20 days. Thereafter, the tissues were embedded in paraffin and sectioned using routine methods, and stained with hematoxylin and eosin (H&E). The joint sections were measured using a scale of 0–3 for grading the synovial inflammation, pannus formation, and destruction of bone and cartilage.

Micro-Computed Tomography (Micro-CT) Imaging

On day 50, the mice were sacrificed, and their right legs were excised and fixed in 4% formalin for 1 day. A micro-CT (SCANCO μCT80) was used to estimate the structural status in the right knee and ankle joints. The X-ray tube voltage was 55 kV and, the current was 72 μA, with a voxel size of 10 μm. The segmented images were 3D-reconstructed using the SCANCO proprietary software. Joint bone radiological destruction was scored on a scale from 0 to 3: 0, no damage; 1, minor; 2, moderate; 3, severe.

Immuno-Histochemical Analysis

The above-described paraffin-embedded knee and ankle joint tissues were deparaffinized with ethanol and xylene. After being hydrated in PBS, tissues were blocked with 0.3% hydrogen peroxide (H_2O_2) for 10 min. Next, the tissues were incubated with primary antibodies against MMP-1, MMP-3, MMP-13 at 4°C overnight. After washing the tissues with PBS for three times, secondary antibodies were added and incubated for another 1 h; then the signal was amplified with HRP-conjugated streptavidin using a Vectastain Elite ABC kit (Vector, United States).

Cell Culture

MH7A, a human RA-FLSs cell line (Miyazawa et al., 1998), purchased from the Riken cell bank (Tsukuba, Japan), was gifted by the Chinese Academy of Sciences, Shenzhen. The cells were maintained in 1640 medium (Hyclone, United States) with 100 IU/ml of penicillin/streptomycin (Sigma-Aldrich, United States) and 10% heat-inactivated fetal bovine serum (Gibco, United States) in an incubator with 5% CO2 at 37°C.

Cell Viability Assay

Cell viability was determined using the CCK-8 assay as described previously (Jia et al., 2015). Briefly, MH7A cells were seeded at $5*10^3$ cells/well in 96-well culture plates, incubated overnight, and then exposed at various concentrations of astragalin for 24, 48, and 72 h. Then, CCK-8 (Dojindo, Japan) was added to each well of the plate and incubated at 37°C for 1.5 h. The resulting optical density was detected at 450 nm by a microplate reader.

Quantitative RT-PCR Analysis

MH7A cells were pretreated with astragalin (0, 50, 100, 200 μM) for 2 h and then incubated for another 24 h in the presence or absence of TNF-α (10 ng/mL). Total RNA was extracted using Trizol reagent according to the manufacturer's instructions and each sample was reverse transcribed using the cDNA synthesis kit according to the manufacturer's protocol. Real-time PCR analysis was performed using SYBR Green PCR Premix Ex Taq II reagents on a CFX96 real-time system (Thermo Fisher Scientific, United States). Relative gene expression was calculated by the $^{\Delta\Delta}$Ct method. The primer sequences (forward and reverse) were as follows: for MMP-1, 5′-CTCAATTTCACTTCTGTTTTCTG-3′ and 5′-CATCTCTGTCGGCAAATTCGT-3′; for MMP-3, 5′-GGCTTCAGTACCTTCCCAGG-3′ and 5′-GCAGCAACC AGGAATAGGTT-3′; for MMP-13, 5′-CAAGATGCGGGG TTCCTGAT-3′, 5′-AATGCCATCGTGAAGTCTGGT-3′.

Enzyme Linked Immunosorbent Assay

On day 50, the mice were sacrificed, serum samples were extracted from peripheral blood and stored at −80°C until analysis. MH7A cells were seeded into 6-well plates ($1*10^6$ cells/well) for 24 h, then pretreated with astragalin (0, 50, 100, 200 μM) for 2 h, then incubated for another 24 h in the presence or absence of TNF-α (10 ng/ml). Cell culture supernatants were collected and stored at −80°C until analysis. The concentrations of the cytokines in serum and culture supernatants were determined by ELISA using a commercial kit according to the manufacturer's instructions.

Western Blot Analysis

MH7A cells were pretreated for 2 h with various concentrations of astragalin (0, 50, 100, 200 μM), and then exposure to TNF-α (10 ng/ml) for 0.5 h. The protein was collected, 30 μg of protein from each sample was separated by 15% SDS-PAGE and transferred to polyvinylidene fluoride membrane.

After blocking with 5% bovine serum albumin in TBST at room temperature for 2 h, the membranes were incubated with the corresponding primary antibodies overnight at 4°C. After washing with TBST for three times, the membranes were incubated with the secondary antibodies. Proteins were scanned using the ECL detection system. The gray values of protein bands were analyzed using ImageJ software.

Immunofluorescence Analysis

MH7A cells were seeded on a round coverslips in 24-well plates for 24 h, and then pretreated with astragalin (200 μM) for 2 h, stimulated with TNF-α for 0.5 h, fixed with 4% paraformaldehyde for 15 min and then permeated with 0.2% Triton X for 20 min. After blocking with 5% bovine serum albumin at room temperature for 0.5 h, cells were incubated with the anti-phospho-c-Jun antibody overnight at 4°C and then incubated with the goat anti-rabbit IgG H&L secondary antibody for 1 h at room temperature. The nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAP). The coverslips were mounted onto glass slides and images were recorded by an Olympus BX-51 microscope.

Statistical Analysis

The data were reported as mean ± standard error of the mean (SEM). Significant differences were assessed using GraphPad Prism 7.0 software (GraphPad, La Jolla, CA, United States). One-way ANOVA followed by Dunnett's *t*-test were used to determine differences between groups. As to three groups treated at different times points, two-way ANOVA comparison was performed. $p < 0.05$ was considered statistically significant.

RESULTS

Astragalin Attenuated the Symptoms of CIA Mice

To investigate the therapeutic effects of astragalin on RA *in vivo*, we established CIA mouse model. Treating mice with astragalin initiated at 22 days after the first immunization. Assessment of the severity of the arthritis was observed every 5 days after the booster injection. Compared with the negative control group, the CIA model mice showed a slight body weight loss (**Figure 1C**), and with higher scores in arthritis index, the number of swollen joints, and hind paw thickness. Both astragalin and MTX treatment had positive effects on attenuating the arthritis index, swollen joints count, and hind paw thickness of CIA mice (**Figures 1D–G**). These results indicated that astragalin effectively inhibited the development of arthritis.

Astragalin Inhibited Joint Space Widening and Synovial Vascularity

Ultrasound (US) in combination with the Doppler technique has already been used to quantify synovial inflammation by measuring the increased joint space volume and blood flow in CIA mice (Clavel et al., 2008; Elhai et al., 2015). The volumetric changes of joint space and vascularity in knee and ankle were measured with the Vevo 2100 imaging system. After 7 weeks of treatment, the knee and ankle joints of these mice were evaluated. The results of 3D high-frequency ultrasonography and color Doppler indicated that astragalin significantly inhibited joint space widening and synovial vascularity in the knee and ankle joints (**Figures 2A–E**).

Astragalin Alleviated Synovial Inflammation and Joint Destruction

A histopathological evaluation of the knee and ankle joints was performed to examine the degrees of arthritic damage. Severe synovial hyperplasia, infiltration of inflammatory cells into synovial tissues, pannus formation, and cartilage and bone destruction were observed in the CIA mice. As expected, MTX significantly attenuated the pathological symptom of CIA in the knee and ankle joints. Consistently, astragalin also alleviated the histopathological arthritic damage in the CIA joints (**Figures 3A,B**). The 3D reconstruction of a micro-CT analysis of knee and ankle joints showed astragalin treatment markedly diminished bone destruction compared with untreated CIA control mice (**Figures 3C,D**).

Astragalin Reduced the Production of Pro-inflammatory Cytokines and the Expression of MMPs in Inflamed Joints of CIA Mice

Various cytokines regulate the pathogenesis of RA. The production of pro-inflammatory cytokines, particularly TNF-α, IL-1β, IL-6, and IL-8, may cause synovial inflammation and pain. MMP-1, MMP-3, and MMP-13 are essential factors for the degradation of joint cartilages. In the present experiment, the production of pro-inflammatory cytokines in the serum of CIA mice was determined using the ELISA kit, and the expression of MMPs in knee and ankle joint tissues were measured by immune-histochemical staining. As shown in **Figure 4A**, the serum levels of TNF-α, IL-1β, IL-6, and IL-8 were significantly lower in the astragalin-treated group than those in CIA-Veh group. The expression levels of MMP-1, MMP-3, and MMP-13 in cartilages and synovial tissues notably increased in CIA mice and reduced after the treatment of astragalin and MTX (**Figure 4B**).

Astragalin Suppressed the mRNA and Protein Expression of MMPs in TNF-α-Induced MH7A Cells

MMPs are mainly secreted by FLSs, which play a critical role in the destruction of joint cartilage (Burrage et al., 2006; Araki and Mimura, 2017; Malemud, 2017). Stimulating FLSs with TNF-α or IL-1 *in vitro* increases MMPs production. To assess the potential cytotoxicity of astragalin, cell viability was evaluated by the CCK-8 assay. As shown in **Figure 5A**, astragalin did not affect cell viability, even at a concentration as high as 250 μM after 72 h incubation. Therefore, we decided to set the highest concentration of astragalin at the 200 μM for the following experiments. To determine the protective effect of astragalin on MMPs expression, MH7A cells were incubated for 24 h

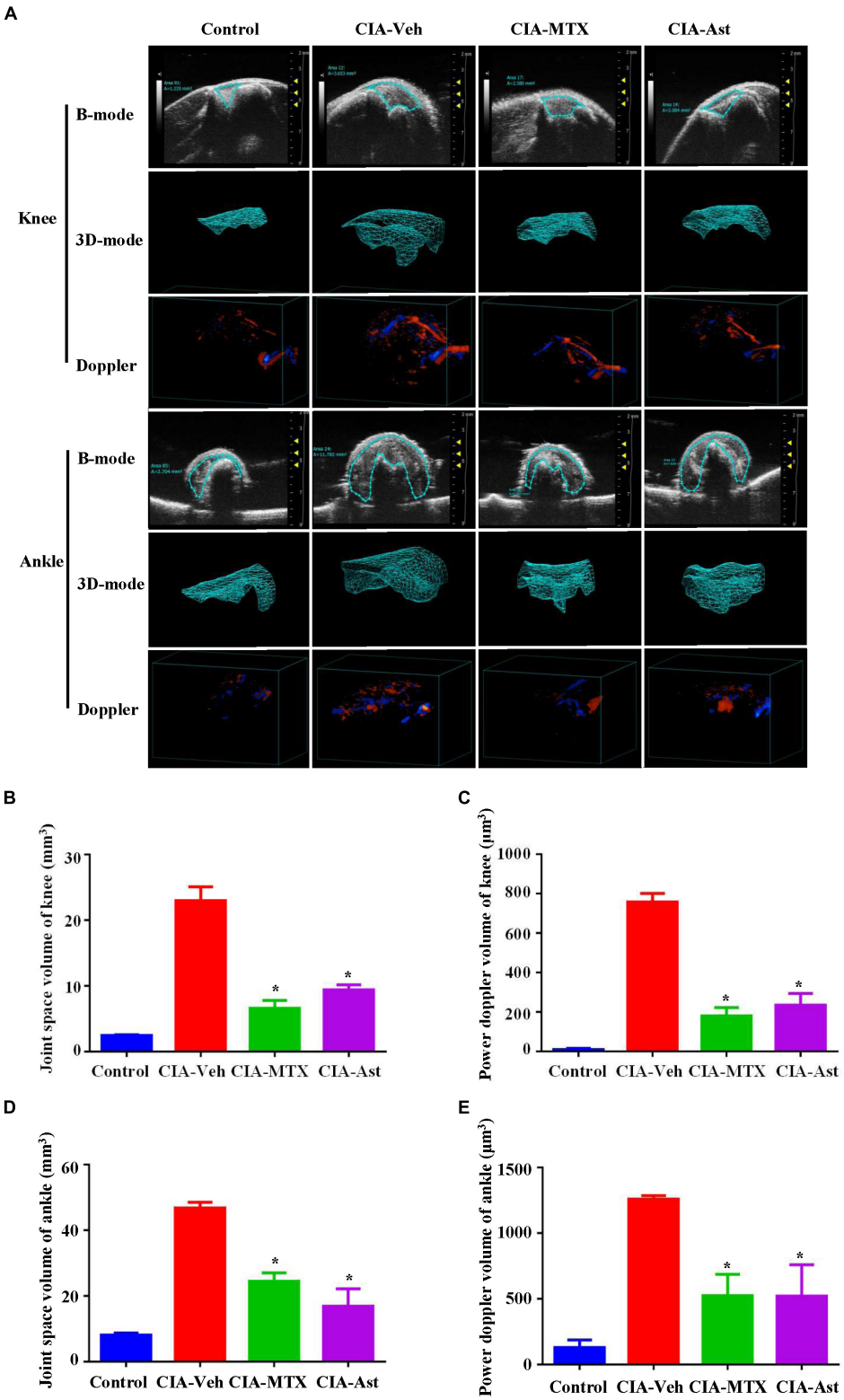

FIGURE 2 | Astragalin inhibited joint space widening and synovial vascularity. After 7 weeks of treatment, the knee joints and the ankle joints **(A)** were analyzed using the Vevo 2100 imaging system in both B mode and color doppler mode. 3D reconstructions of knee and ankle joints using the Vevo LAB software, which allowed for accurate volume measurement of the joint space widening and synovial vascularity **(B–E)**. Data are shown as mean ± SEM of four mice per group, $^{*}p < 0.05$, $^{**}p < 0.01$ versus CIA-Veh group.

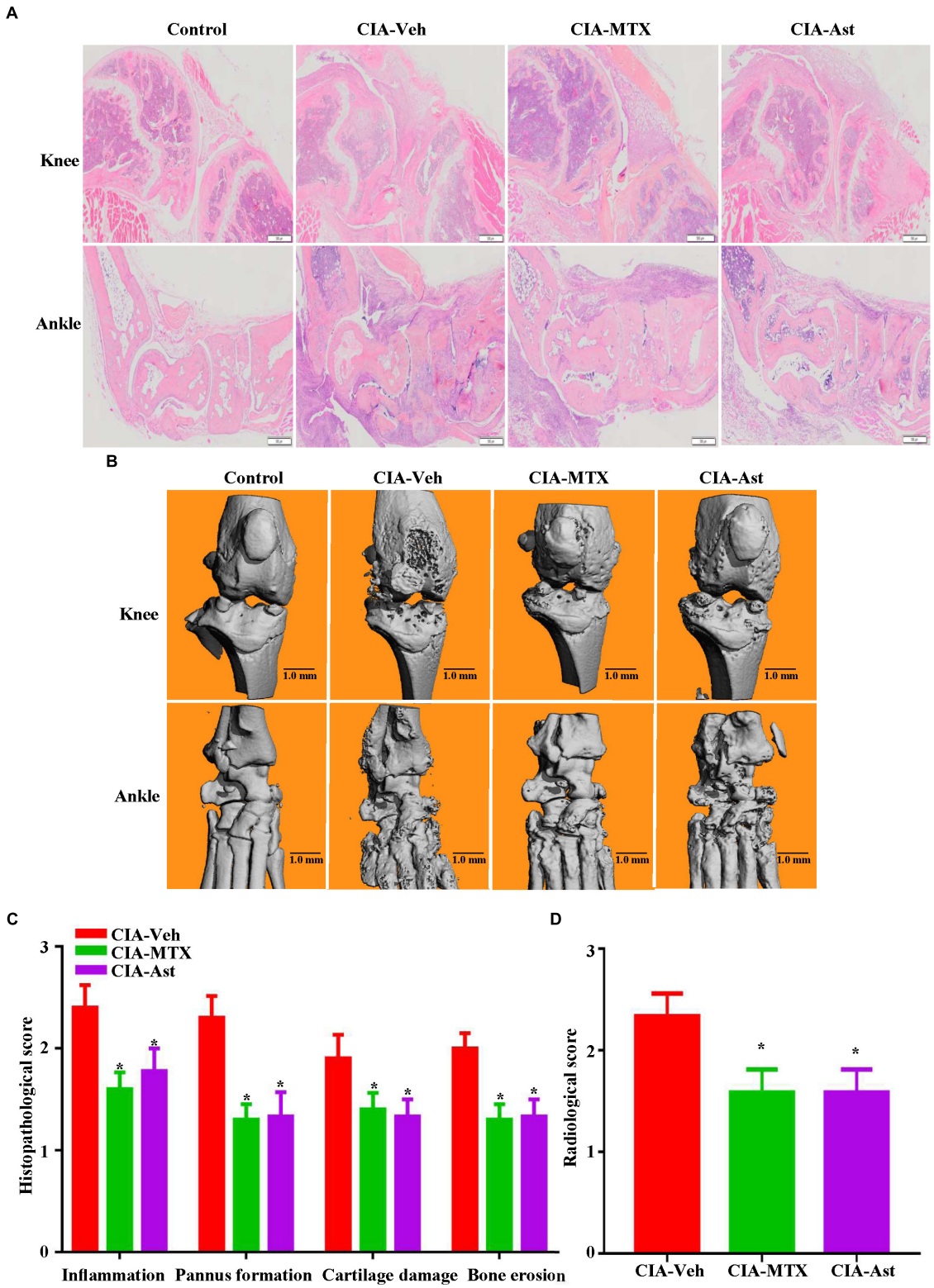

FIGURE 3 | Astragalin alleviated synovial inflammation and joint destruction. On day 50, mice were sacrificed, the knee and ankle joints stained with hematoxylin and eosin **(A)**. 3D reconstructions of the micro-CT analysis from CIA mice using the SCANCO proprietary software **(B)**. The histopathological severity was assessed and calculated **(C)**. Bone radiological destruction scores were attributed from the micro-CT analysis **(D)**. Data are shown as mean ± SEM of ten mice per group, $^{*}p < 0.05$ versus CIA-Veh group.

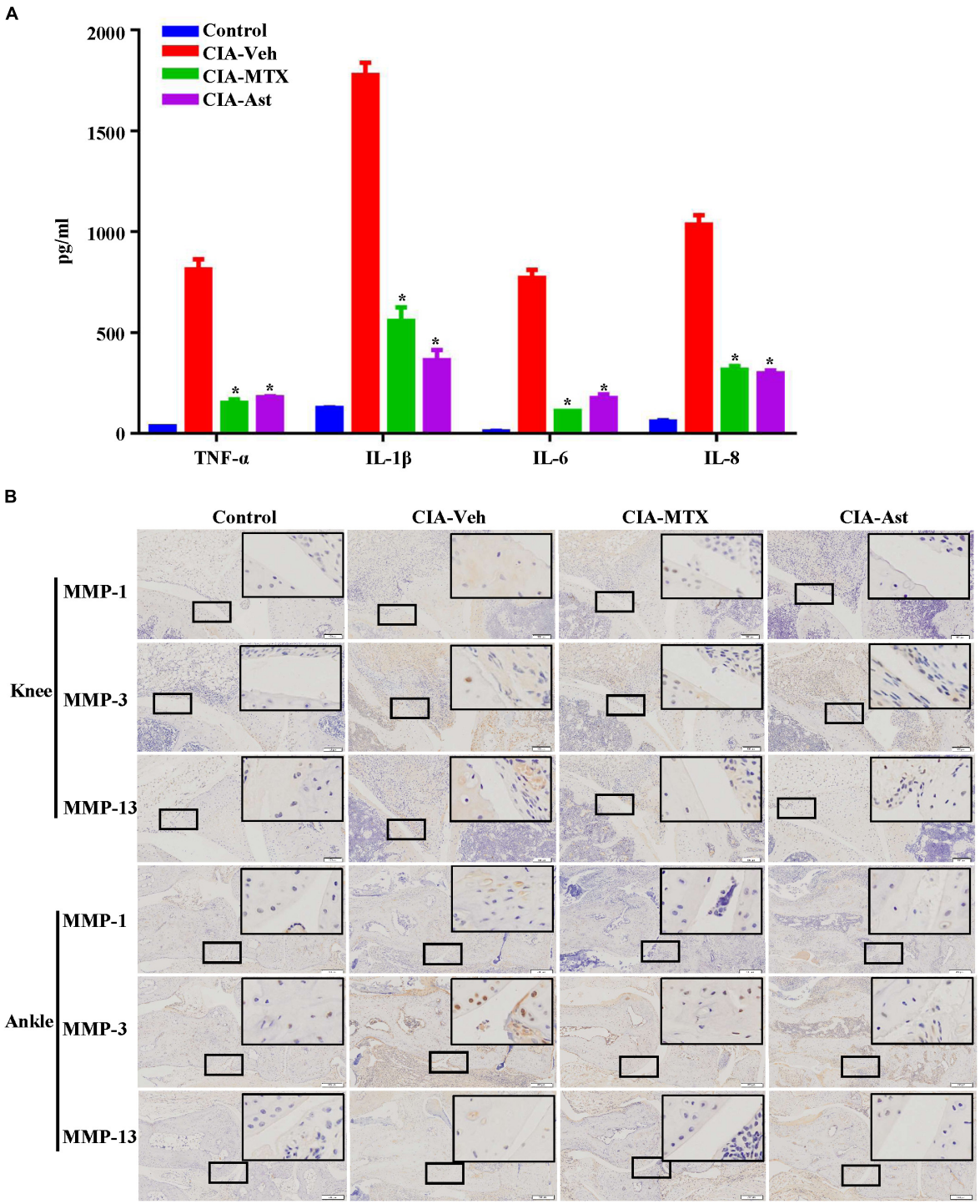

FIGURE 4 | Astragalin reduced the production of pro-inflammatory cytokines and the expression of MMPs in inflamed joints of CIA mice. The serum level of TNF-α, IL-1β, IL-6, and IL-8 were determined using the ELISA assay **(A)**. Expression of MMP-1, MMP-3 (100×) and MMP-13 at the sites of cartilages and synovial tissues in the knee joints and the ankle joints **(B)** were measured by immune-histological staining. Representative images are shown from three independent experiments. Data are shown as mean ± SEM of ten mice per group, $*p < 0.05$, $**p < 0.01$ versus CIA-Veh group.

with TNF-α. As shown in **Figures 5B–D**, TNF-α significantly increased MMPs expression both in mRNA and protein levels. Astragalin significantly and dose-dependently decreased the mRNA and protein expression of MMP-1, MMP-3, and MMP-13 in TNF-α-induced MH7A cells compared to the group treated with TNF-α alone.

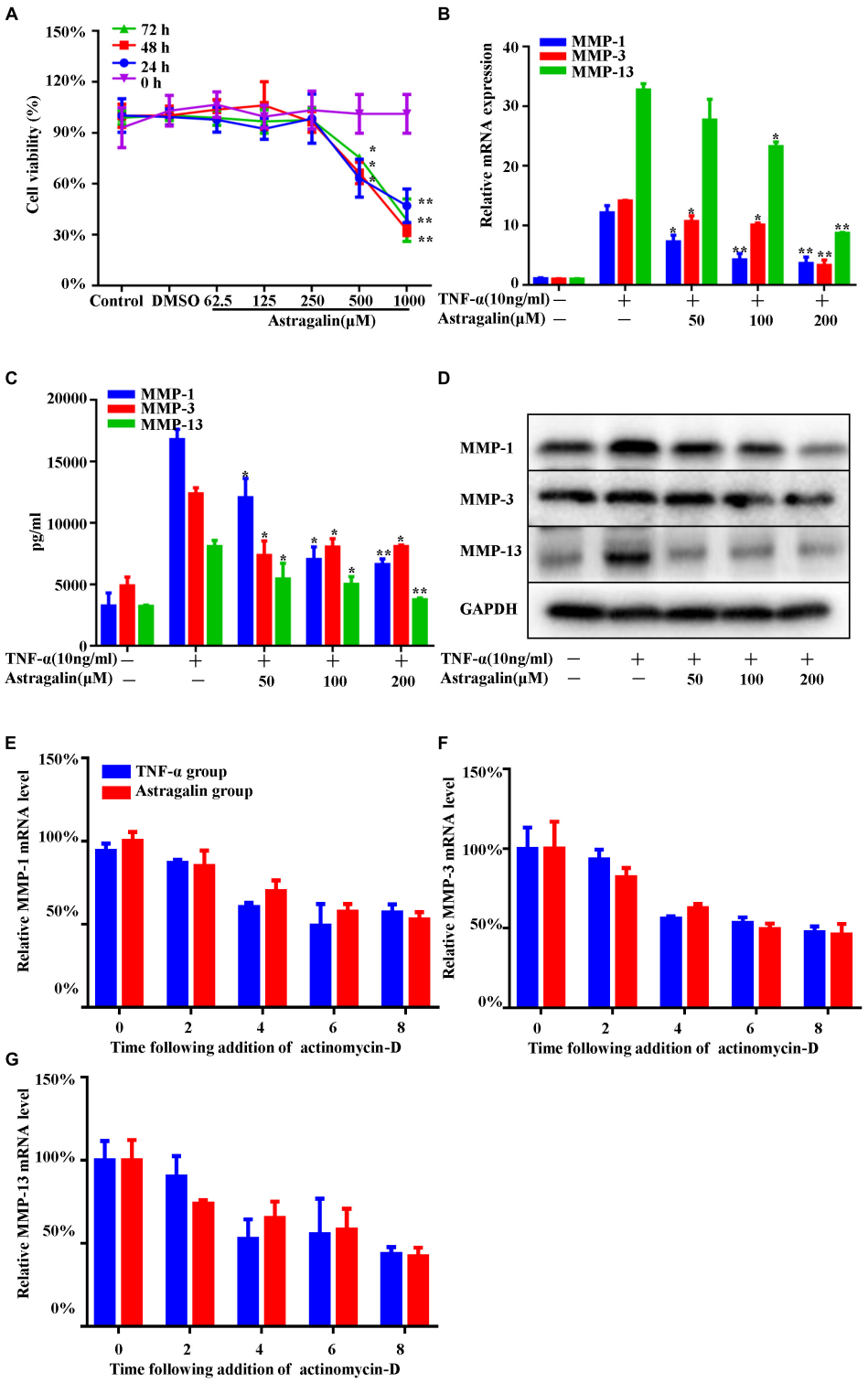

FIGURE 5 | Astragalin suppressed the mRNA and protein expression of MMPs in TNF-α-induced MH7A cells. MH7A cells were treated with different concentrations of astragalin for 0, 24, 48, and 72 h, and cell viability was measured by CCK-8 assay **(A)**. MH7A cells pretreated for 2 h with various concentrations of astragalin (0, 50, 100, 200 μM), and then exposure to TNF-α (10 ng/ml) for 24 h, the mRNA and protein levels of MMP-1, MMP-3, and MMP-13 were determined using the RT-PCR, ELISA assay, and western blot analysis **(B–D)**. MH7A cells were pretreated with astragalin (200 μM) or not for 2 h, then stimulated with TNF-α for 6 h. Actinomycin D (4 μg/mL) was added to the cells and then mRNA was isolated at time point 0, 2, 4, 6, and 8 h. The levels of MMPs mRNA stability were detected by RT-PCR **(E–G)**. Data are shown as mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with TNF-α stimulation alone.

In addition, the expression of MMPs is affected by transcriptional regulation and mRNA stability. To clarify the effect of astragalin on the mRNA stability of MMPs, we performed mRNA stability assays. MH7A cells were pretreated with astragalin (200 μM) or not for 2 h, then stimulated with TNF-α for 6 h. Actinomycin D (4 μg/mL) was added to the cells and then mRNA was isolated at time point 0, 2, 4, 6, and 8 h. The levels of MMPs mRNA stability were detected by RT-PCR. We found that astragalin did not alter the mRNA stability of MMPs (**Figures 5E–G**).

Astragalin Inhibited TNFα-Induced Activation of MAPK and AP-1 Pathways

Previous studies have shown that the MAPK and AP-1 mediated pathways can regulate the expression of MMPs synthesis and inflammatory cytokines (Schett et al., 2000). To further investigate the mechanisms of astragalin inhibiting production of MMPs, we examined the effect of astragalin on the MAPK and AP-1 mediated pathways in TNF-α-induced MH7A cells. MH7A cells were pretreated with various concentrations of astragalin for 2 h, stimulated with TNF-α (10 ng/ml) for 30 min. Then, the levels of the phosphorylation of MAPK (ERK, JNK, and p38), and the levels of c-Fos and c-Jun (a component of AP-1) were detected using western blot. Astragalin treatment significantly inhibited the phosphorylation of JNK and p38, but not ERK, in a dose-dependent manner (**Figures 6A–C**). The phosphorylation of c-Jun was decreased in TNF-α-induced cells by astragalin in a dose-dependent manner. However, it did not affect the phosphorylation of c-Fos (**Figures 6D,E**). In addition, the immunofluorescence analysis revealed that astragalin reduced the TNF-α-induced p-c-Jun protein expression and its translocation to the nucleus (**Figures 6F,G**).

DISCUSSION

Astragalin is an extract separated from leaves of persimmon and green tea seeds, which possess potent biological effects including anti-inflammatory, antioxidant and anticarcinogenic activities (Riaz and Rasul, 2018). It shows ability to inhibit the production of inflammatory mediators, such as IL-1β, IL-6, and TNF-α in macrophages and in mice with allergic asthma (Nhiem et al., 2011; Li et al., 2013; Liu et al., 2015). These studies suggested benefits of astragalin for the treatment of inflammatory disorders. However, to our knowledge, no study has investigated its anti-rheumatic arthritis effect on animal models or humans. In the present study, we demonstrated for the first time that astragalin effectively inhibited the worsening of synovial inflammation and joint destruction in mice with CIA. These beneficial effects may occur via inhibiting the destructive behaviors of rheumatoid FLSs. RA is a chronic inflammatory joint disease, which is characterized by synovial hyperplasia, infiltration of inflammatory cells into synovial tissue as well as the subsequent erosion of cartilage and bone (Smolen et al., 2016). CIA model is the most commonly used autoimmune model of RA (Brand et al., 2007). The main pathological features of CIA include synovium hyperplasia with infiltration of inflammatory cells, pannus formation, cartilage degradation, and erosion of bone. In this study, we found that astragalin markedly attenuated arthritis symptom in CIA mice. At the dose of 5 mg/kg/day, a strong anti-arthritic property of astragalin was manifested by a decrease in CIA-induced paw edema and swelling as observed in arthritis score. US has proven to be a valuable method for evaluating arthritic lesions in DBA/1J mice (Clavel et al., 2008). In the present study, ultrasound and color Doppler analysis suggested that the increase of joint space volume and vascularity in the knee and ankle joints were significantly attenuated by astragalin treatment. The results of the histological examination and micro-CT scan of the knee and ankle joints further confirmed that synovial hyperplasia, pannus formation, cartilage damage, and bone erosion were significantly attenuated by astragalin.

Destruction in articular cartilage and bone hallmarks the presence of RA (Myasoedova et al., 2010). Osteoclasts are major effectors of the destruction of cartilage and bone, but recent studies suggested that the destruction is primarily caused by FLSs (Huber et al., 2006; Bartok and Firestein, 2010). FLSs from the intimal lining are major inducers of cartilage destruction in RA for that they can produce enormous degradative enzymes. Among the various classes of proteinases produced by the inflamed synovium, MMPs are particularly significant (Boissier, 2011; Araki and Mimura, 2017). MMPs are a large group of enzymes that play critical roles in the destruction of articular cartilage and bone due to their ability to degrade a wide variety of extracellular matrix components (Burrage et al., 2006; Aikawa et al., 2008). Collagenases (MMP-1, MMP-13) and stromelysins (MMP-3) are especially important in RA (Green et al., 2003; Iwamoto et al., 2008; Araki et al., 2016). Their synthesis and activation are motivated by various factors, such as pro-inflammatory cytokines, growth factors, matrix proteins, and reactive oxygen species. Activated RA-FLSs are the major source of MMPs and inflammatory mediators in the synovial tissue. Therefore, the inhibition of MMPs can significantly reduce RA-FLSs cartilage invasiveness. Regulation of MMPs gene expression and activation is a good parameter for assessing disease activity in humans and evaluates the effects of various agents on inflammatory conditions, both *in vitro* and *in vivo* experiments. In the present study, we found that CIA mice treated with astragalin had lower histopathology scores, inflammation, synovial hyperplasia, articular cartilage, and bone destruction. At the same time, astragalin significantly down-regulated the expression levels of MMP-1, MMP-3, and MMP-13 in cartilages and synovial tissues in CIA mice. Consistent with our *in vivo* findings, astragalin also profoundly inhibited TNFα-induced production of MMP-1, MMP-3, and MMP-13 in cultured MH7A cells. Taken together, these findings suggested that the joint-protective properties of astragalin may be mainly attributed to the down-regulation of MMPs expression.

Stimulation of RA-FLSs with TNF-α or IL-1 *in vitro* increases MMPs production via transcriptional activation (Burrage et al., 2006). AP-1 binding sites have been found in the promoter region of all MMPs and AP-1 appears to play a crucial role in the transcriptional activity of MMPs (Shiozawa et al., 1997; Han et al., 2001; Sweeney et al., 2005). c-Jun and c-Fos are two major components of AP-1 in the stimulated RA-FLSs and mediate the

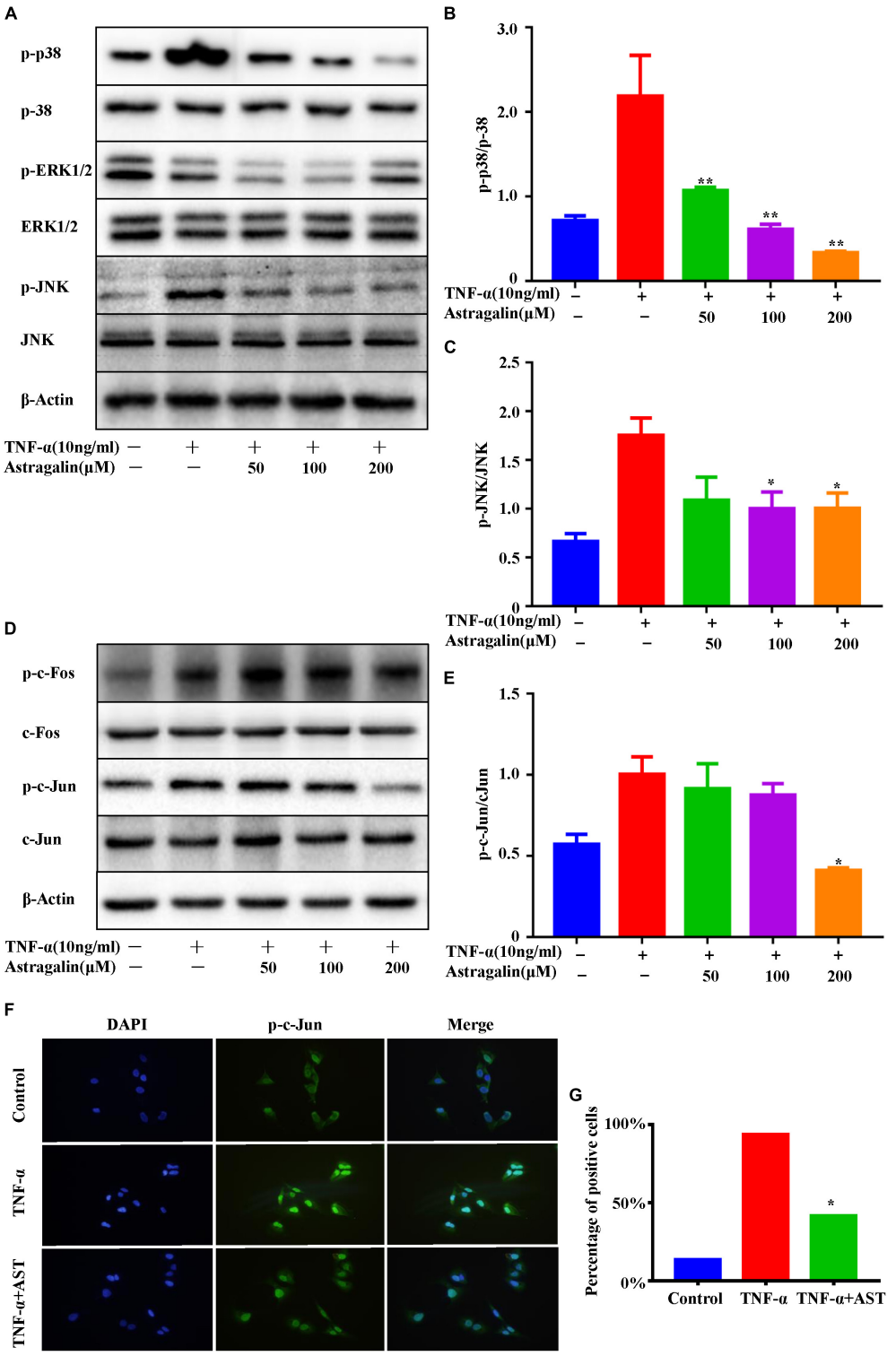

FIGURE 6 | Astragalin inhibited TNFα-induced activation of MAPK and AP-1 pathways. MH7A cells pretreated for 2 h with various concentrations of astragalin (0, 50, 100, 200 μM), and then exposure to TNF-α (10 ng/ml) for 0.5 h, the total and phosphorylated levels of JNK, ERK1/2, and p38 were determined by Western blot analysis **(A–C)**. The total and phosphorylated levels of c-Jun, c-Fos were analyzed by Western blot analysis **(D,E)**. MH7A cells pretreated with astragalin (200 μM) or not for 2 h, and then exposure to TNF-α (10 ng/ml) for 0.5 h. Immunofluorescence analysis was used to detect intracellular localization of p-c-Jun. Representative 400× images **(F)**. More than 200 cells from each sample were measured **(G)**. Data are shown as mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with TNF-α stimulation alone.

function of AP-1 (Han et al., 1998). Recent evidence suggests that interfering with AP-1 decoy oligonucleotides or using c-Fos/AP-1 inhibitors reduced the severity of CIA characterized by inflammatory cytokine overproduction and MMP synthesis (Aikawa et al., 2008), thus validating the crucial role of AP-1 in joint inflammation. Here, we demonstrated that astragalin significantly inhibited the activation of c-Jun, but not the c-Fos in TNF-α-induced MH7A cells. Therefore, we speculated that the joint-protective properties of astragalin are related to interfering with the AP-1 pathway.

AP-1 belongs to the class of basic leucine zipper transcription factors. Its activity can be markedly increased in synoviocytes as a response to the stimulation of cytokines, such as IL-1β and TNF-α, through MAPK, AKT, and other signaling pathways (Yamanishi and Firestein, 2001). Activation of p38 and JNK is commonly responsible for AP-1 activation through the phosphorylation of c-Jun. Next, we explored the effects of astragalin on the MAPK signaling pathway.

The MAPK pathway is central to many host responses and is one of the major signaling pathway-transmitting signals to immediate early genes implicated in the regulation of cytokine responses (Hammaker et al., 2003). Regulation of cytokine production in RA was directly associated with activation of MAPKs. Of the three MAPK families, JNK and p38 are highly active in RA-FLSs and also involved in the regulation of MMPs expression (Schett et al., 2000; Yan and Boyd, 2007; Yoshizawa et al., 2008; Guma and Firestein, 2012). Moreover, in RA-FLSs, TNF-α can bind to its surface receptors and activate the MAPK pathways, leading to the expression of multiple cytokines and chemokines, as well as the secretion of MMPs that contribute to tissue destruction (Li et al., 2017; Huang et al., 2018). As the upstream of AP-1, MAPK signaling may also be influenced by astragalin. Our results demonstrated that astragalin treatment effectively hindered the TNF-α-induced phosphorylation of MAPK in RA-FLSs, including p38 and JNK, but not ERK, in a dose-dependent manner. These findings suggested that the decreased activations of AP-1 and MAPK might account for the beneficial effects of astragalin in RA.

In summary, our study demonstrated that astragalin markedly ameliorated synovial inflammation and joint destruction in CIA mice. The joint-protective properties of astragalin could be mainly attributed to the down-regulation of MMPs expression by inhibiting the JNK/p38/AP-1 pathways. Taken together, astragalin may serve as an effective therapeutic drug for RA.

AUTHOR CONTRIBUTIONS

QL, QS, and YW conceived and designed the experiments. QJ and TW performed the research. HX analyzed the result. XW and YL wrote the manuscript draft. All authors have read and approved the submitted version.

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Subantimicrobial Dose Doxycycline Worsens Chronic Arthritis-Induced Bone Microarchitectural Alterations in a Mouse Model: Role of Matrix Metalloproteinases?

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Background: Rheumatoid arthritis (RA) is a chronic inflammatory joint disease hallmarked by irreversible damage of cartilage and bone. Matrix metalloproteinases (MMPs) involved in connective tissue remodeling play an important role in this process. Numerous MMPs have been examined in humans and animals, but their functions are still not fully understood. Therefore, we investigated the role of MMPs in the K/BxN serum-transfer model of RA with the broad-spectrum MMP inhibitor subantimicrobial dose doxycycline (SDD) using complex *in vivo* and *in vitro* methodolgy.

Methods: Chronic arthritis was induced by repetitive i.p. injections of K/BxN serum in C57BL/6J mice. SDD was administered daily in acidified drinking water (0.5 mg/mL, 80 mg/kg) during the 30 days experimental period. Mechanonociceptive threshold of the paw was evaluated by aesthesiometry, grasping ability by grid test, arthritis severity by scoring, neutrophil myeloperoxidase activity by luminescence, vascular hyperpermeability and MMP activity by fluorescence *in vivo* imaging and the latter also by gelatin zymography, bone structure by micro-computed tomography (micro-CT). Plasma concentrations of doxycycline were determined by liquid chromatography-mass spectrometry analysis.

Results: K/BxN serum induced significant inflammatory signs, mechanical hyperalgesia, joint function impairment, increased myeloperoxidase activity and vascular hyperpermeability. Significant increase of MMP activity was also observed both *in vivo* and *ex vivo* with elevation of the 57–60, 75, and 92 kDa gelatinolytic isoforms in the arthritic ankle joints, but neither MMP activity nor any above described functional parameters were influenced by SDD. Most importantly, SDD significantly reduced bone mineral density in the distal tibia and enhanced the Euler number in the ankle. Arthritis-induced microarchitectural alterations demonstrating increased irregularity and cancellous bone remodeling, such as increased Euler number was significantly elevated by SDD in both regions.

Conclusion: We showed increase of various MMP activities in the joints by *in vivo* fluorescence imaging together with *ex vivo* zymography, and investigated their functional significance using the broad-spectrum MMP inhibitor SDD in the translational RA model. This is the first demonstration that SDD worsens arthritis-induced bone microarchitectural alterations, but it appears to be independent of MMP inhibition.

Keywords: rheumatoid arthritis, matrix metalloproteinases, K/BxN serum-transfer arthritis, subantimicrobial dose doxycycline, bone homeostasis, *in vivo* optical imaging, micro-CT, gelatin zymography

INTRODUCTION

Rheumatoid arthritis (RA) is a progressive, chronic inflammatory joint disease leading to irreversible articular cartilage and bone destruction. It is one of the most common musculoskeletal disorder causing physical disability with a worldwide prevalence of approximately 1% (Gibofsky, 2012). Despite the therapeutic revolution in the last decades, the treatment of RA is not fully resolved. Although the novel biologics can significantly reduce synovitis and structural progression, they are far from being ideal drugs due to their high costs, ineffectiveness for chronic pain and sometimes serious side effects resulting from immunosuppression (Smolen et al., 2016; McWilliams and Walsh, 2017). Therefore, further research is needed to precisely explore its pathophysiological mechanisms, identify crucial mediators, and find new potential drug targets. These may include matrix metalloproteinases (MMPs), which are important players of joint damage in arthritic conditions, most importantly in RA (Rose and Kooyman, 2016).

MMPs are secreted or membrane-bound enzymes involved in the family of calcium- and zinc-dependent endopeptidases. Their major function is degrading the extracellular matrix, but they are also capable of cleaving certain non-matrix peptides (e.g., cytokines, chemokines, growth factors, cell surface receptors etc.) (Van Lint and Libert, 2007; Fingleton, 2017). They have crucial roles in physiological regulation of embryonic development, tissue remodeling and wound healing. Furthermore, they are involved in several patho-physiological processes, mainly in "collagenolytic" diseases associated with connective tissue destruction (e.g., arthritic diseases, cancer, atherosclerosis, pulmonary emphysema, chronic inflammatory skin diseases etc.) (Tokito and Jougasaki, 2016; Amar et al., 2017).

The most investigated MMPs in RA are collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), MMP-3 from stromelysins and MMP-14 from membrane-type (MT) MMPs (Rose and Kooyman, 2016). MMP-1 (interstitial collagenase or human fibroblast collagenase) is a ubiquitously expressed collagenase, which is the earliest MMP excessively produced under several pathological circumstances (Burrage, 2006; Shi et al., 2012). In RA it is originated from the synovium and the cartilage, and together with MMP-3 (stromelysin-1) they have been considered to be useful biomarkers for early diagnosis, disease activity and therapeutic efficacy (Green et al., 2003; Fiedorczyk et al., 2006). MMP-8 (collagenase-2 or neutrophil collagenase) is produced mainly by neutrophils, but is also expressed in chondrocytes and synovial fibroblasts. Surprisingly, its role is clearly protective in arthritic tissues confirmed by studies using MMP-8-deficient mice (Cox et al., 2010; Garcia et al., 2010). MMP-13 (collagenase-3) is expressed predominantly in the chondrocytes and cleaves most efficiently the type II collagen, which is the main matrix component of the articular cartilage. Therefore, it is not surprising to be a potential therapeutic target in RA and osteoarthritis (OA) (Singh et al., 2013). Expression of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are also elevated in arthritis (Dreier et al., 2004; Duerr et al., 2004), but interestingly they have distinct roles in RA. MMP-2 knockout mice showed significantly increased, while MMP-9-deficient ones significantly reduced severity of arthritis in comparison with their wildtypes, suggesting a protective

Abbreviations: ANOVA, analysis of variance; CIA, collagen-induced arthritis; CT, computed tomography; Dkk-1, Dickkopf-related protein 1; I.p., intraperitoneal; LC-MS, liquid chromatography-mass spectrometry; MMP, matrix metalloproteinase; MPO, myeloperoxidase; MT, membrane-type; OA, osteoarthritis; PGE2, prostaglandin E2; PLA2, phospholipase A2; RA, rheumatoid arthritis; ROI, region of interests; SDD, subantimicrobial dose doxycycline; SF, synovial fluid; ST, synovial tissue.

and a deleterious role of these enzymes in collagen antibody-induced arthritis, respectively (Itoh et al., 2002). Among the MT-MMPs, MMP-14 (MT1-MMP) plays predominant role in joint disorders. It is overexpressed in arthritic cartilage, fibroblasts and osteoclasts activating also proMMP-2 and proMMP-13, mediating bone resorption and promoting proinflammatory gene expression in macrophages (Burrage, 2006; Rose and Kooyman, 2016; Fingleton, 2017).

Although the role of the abovementioned MMPs has already been examined in several experimental arthritis models, surprisingly there are only two studies focusing on MMPs (MMP-8 and -13) in the K/BxN serum-transfer murine arthritis, which is one of the most translational RA model (Garcia et al., 2010; Singh et al., 2013). This is a commonly used inducible model of RA, in which transient polyarthritis is evoked in healthy recipients by passive transfer of arthritogenic serum originating from a spontaneously arthritic transgenic mouse strain. The serum contains primarily anti-glucose-6-phosphate isomerase antibodies, which form immune complexes and trigger RA-like joint inflammation and destruction (Korganow et al., 1999). The main advantages of this model are that it is suitable for studying the B- and T-cell independent immunological mechanisms and in case of repeated serum injection the RA-associated chronic pain with neuropathic components (Korganow et al., 1999; Christianson et al., 2010). Although the pharmacological interventions with MMP inhibitors are also valuable tools to examine the roles of MMPs, in this model their functions had only been studied using specific knockout mice.

Therefore, we investigated the activity and roles of MMPs in the K/BxN serum-transfer arthritis model with the non-selective MMP inhibitor subantimicrobial dose doxycycline (SDD) using a complex *in vivo* and *in vitro* methodolgy. In the present study we showed the increase of various MMP activities in the joints by *in vivo* fluorescence imaging together with *ex vivo* zymography, as well as demonstrated for the first time that SDD worsens arthritis-induced bone microarchitectural alterations most probably independently of MMP inhibition.

MATERIALS AND METHODS

Animals

Experiments were carried out on 12–20 weeks old male C57BL/6J mice weighing 20–30 g. They were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy, University of Pécs in 325 × 170 × 140 mm sized cages under a 12 h light/dark cycle at 24–25 °C, provided with standard mouse chow and water *ad libitum*. The total number of animals used in the experiments were 33 (17 in the 30 days and 16 in the 16 days experimental series).

Ethics Statement

All experiments were performed according to European legislation (Directive 2010/63/EU) and Hungarian Government regulation (40/2013., II. 14.) on the protection of animals used for scientific purposes, complied with the recommendations of the International Association for the Study of Pain. The studies were approved by the Ethics Committee on Animal Research of University of Pécs (license No.: BA 02/2000–2/2012).

Induction of the Arthritis

Arthritis was induced by repeated intraperitoneal (i.p.) injection of 150 μL of arthritogenic K/BxN serum on the days 0, 3, 10, and 20. Repeated administration was applied to evoke more persistent, long-lasting arthritis. K/BxN sera were obtained from the spontaneously arthritic transgenic K/BxN mice bred and kept in the Animal House of the Department of Physiology, Semmelweis University, Budapest, Hungary. Control animals received non-arthritogenic BxN serum from healthy BxN littermates of K/BxN animals following the same protocols (Borbély et al., 2015).

Preparation of Doxycycline-Treated Drinking Water

Doxycycline was administered in subantimicrobial dose (below 100 mg/kg), therefore 100 mg doxycycline hyclate (Sigma-Aldrich, St. Louis, MO, United States) was dissolved in 200 mL acidified water (0.5 mg/mL). Since there are no data for chronic doxycycline use in arthritis, the dosage and concentration were based on earlier publications related to other issues (Prall et al., 2002; Marx et al., 2014). The estimated daily consumption of an adult mouse was 5 mL resulting in an approximately 80–85 mg/kg oral dose. To avoid the precipitate formation and reach the expected doxycycline plasma concentration, the water was acidified to pH of 3.2 with 37% hydrochloric acid (Merck, Darmstadt, Germany). The pH of the drinking water was measured by Radelkis Laboratory Digital OP-211 pH meter (Radelkis Ltd., Budapest, Hungary). Mice provided with acidified tap water were used for controls. The water was maintained in standard, clear mouse water bottles (250 mL; Acéllabor Ltd., Vecsés, Hungary) placed in complete mouse cage setups and covered with aluminum foil to prevent the photolysis of doxycycline. All water bottles were changed every other day by the research staff.

Experimental Design

The study was performed in two series as outlined in **Figure 1**. Daily doxycycline treatment (approximately 80 mg/kg, p.o.) was started on day 0 and continued until day 30 in the first and until day 16 in the second series. The mechanonociceptive threshold of the hind paws were evaluated on days 5, 9, 15, and 24, neutrophil myeloperoxidase (MPO) activity and plasma extravasation on days 2, 8, 14, and 21, MMP activity on day 4, changes of the periarticular bone structure on day 30, arthritis severity and joint function every day during the 30 days experimental period. Following the micro-computed tomography (micro-CT) analysis, animals were euthanized, blood samples were collected for plasma concentration analyis of doxycycline and ankle joints were removed for gelatin zymography. Water consumption was checked every other day, while body weight was measured every day.

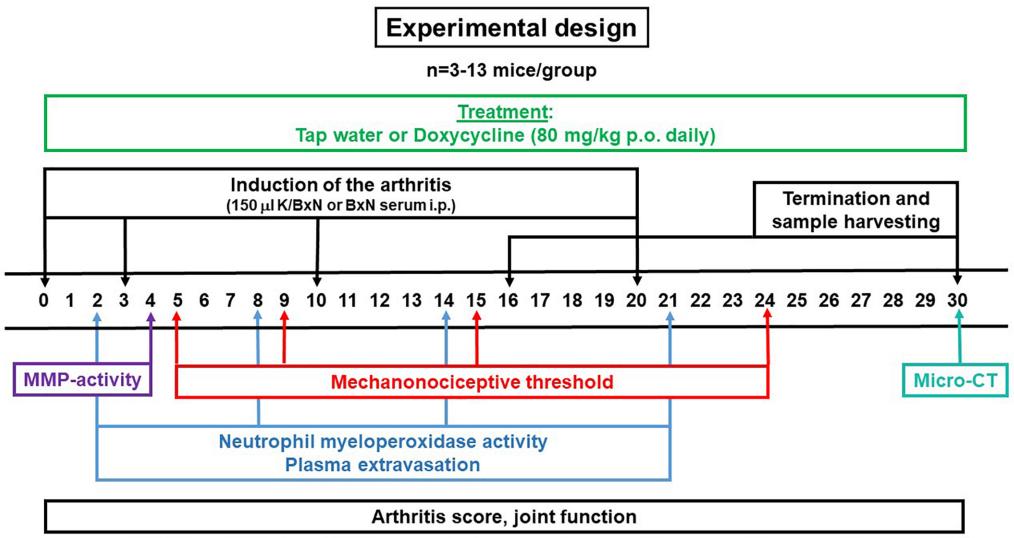

FIGURE 1 | Schematic drawing describing the experimental design of K/BxN serum-transfer arthritis model.

Evaluation of Disease Severity

Visible arthritic signs (hind limb edema and hyperemia) were semiquantitatively scored using a scale of 0–10 (0–1.5: healthy, 1.5–2.5: minimal signs referred to disease, 2.5–4: mild inflammation, 4–7: moderate inflammation, 7–10: severe inflammation) (Jakus et al., 2009; Horvath et al., 2017).

Evaluation of Mechanonociception

The dynamic plantar aesthesiometer (Ugo Basile 37400, Comerio, Italy) was used for the assessment of the mechanosensitivity of plantar surface of the hind paw. Mice were placed into acrylic glass boxes with wire grid floor, then after acclimation the plantar surface was touched with a straight metal filament lifting with increasing upward force (maximum force of 10 g with 4 s latency) until the animal withdrew his paw. Mechanical hyperalgesia was represented as a percentage decrease of the initial (before serum injection) withdrawal thresholds (Horvath et al., 2016).

Evaluation of Joint Function

The grasping ability correlating with joint function was determined using the grid test in the K/BxN serum-transfer arthritis model. Mice were placed on a horizontal wire grid, then it was turned over and the latency to fall was determined. The grid was maintained in horizontal position for a maximum of 20 s (Jakus et al., 2009).

In vivo Bioluminescence Imaging of Neutrophil MPO Activity

Neutrophil MPO-derived reactive oxygen species (ROS) production and the enzyme activity were assessed with luminol-derived bioluminescence. Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) sodium salt (150 mg/kg, Gold Biotechnology, Olivette, MO, United States) dissolved in sterile phosphate buffered saline (PBS, 30 mg/mL) was injected i.p. into anesthetized mice. They were anesthetized using ketamine (120 mg/kg i.p.; Calypsol, Gedeon Richter Plc., Budapest, Hungary) and xylazine (6 mg/kg i.p.; Sedaxylan, Eurovet Animal Health B.V., Bladel, Netherlands). Bioluminescence imaging was performed 10 min post-injection using the IVIS Lumina II (PerkinElmer, Waltham, MA, United States; 120 s acquisition, Binning = 8, F/Stop = 1). Identical Region of Interests (ROIs) were applied around the ankles and luminescence was expressed as total radiance (total photon flux/s) (Botz et al., 2014).

In vivo Fluorescence Imaging of Plasma Extravasation

Plasma extravasation was visualized by IR-676-based fluorescence imaging. IR-676 vascular fluorescent dye (0.5 mg/kg, Spectrum-Info Ltd., Kyiv, Ukraine) dissolved in 5% (v/v) aqueous solution of Kolliphor HS 15 (polyethylene-glycol-15-hydroxystearate; Sigma-Aldrich, St. Louis, MO, United States) was injected intravenously (i.v.) into anesthetized mice (120/6 mg/kg ketamine-xylazine i.p.). Fluorescence imaging was performed 20 min post-injection using the IVIS Lumina II (PerkinElmer, Waltham, MA, United States; auto acquisition time, Binning = 8, F/stop = 2, excitation/emission filter: 640/700 nm). Data were analyzed and ROIs were drawn around the ankle joints. Fluorescence was expressed as radiant efficiency ([photons/s/cm^2/sr]/[µW/cm^2]) (Botz et al., 2015).

In vivo Fluorescence Imaging of MMP Activity

MMP activity was assessed *in vivo* using MMPSense 750 FAST (PerkinElmer, Waltham, MA, United States), an activatable fluorescent imaging agent for MMP-2, -3, -7, -9, -12, and -13 according to the manufacturer's instructions (2 nmol/subject i.v.). Measurements were performed with IVIS Lumina II (PerkinElmer, Waltham, MA, United States; auto

acquisition time, Binning = 2, F/stop = 1, excitation/emission filter: 745/800 nm) 6 h later. ROIs were applied around the ankles and fluorescence was expressed as radiant efficiency ([photons/s/cm^2/sr]/[μW/cm^2]) (Borbély et al., 2015).

In vivo Micro-CT Analysis of the Region of Ankle Joints

Micro-CT imaging was performed in a self-control manner before and 30 days after the induction of arthritis. The quantitative values calculated from the pictures at the end of the study were compared to the initial images of the same mice before the experiment. The right ankle joints were scanned using a 17.5 μm voxel size by a SkyScan 1176 *in vivo* micro-CT (Bruker, Kontich, Belgium). After reconstruction of the scans the bone structural changes were evaluated using the CT Analyser® software. Standardized ROIs were drawn around the periarticular region of the distal tibia and fibula, as well as the ankle including both the tibio-tarsal and tarso-metatarsal joints. In these ROIs bone mineral density, bone surface density, number of pores, volume of open pores, percent increase of open pore volume, and the Euler number, a measure of trabecular connectedness were evaluated (Borbély et al., 2015).

Detection of MMP Activities by Gelatin Zymography

At the end of both series of the experiment, on days 16 and 30, mice were euthanized with sodium pentobarbital (100 mg/kg i.p.; Euthanimal, Alfasan Nederland B.V., Woerden, Netherlands) and ankle joints were removed to assess MMP activities. First the ankle joints were homogenized in a 4 times volume of homogenization buffer containing 50 mM Tris base (Merck, Darmstadt, Germany) and 1 mL 0.5% Triton (Sigma-Aldrich, St. Louis, MO, United States) dissolved in 500 mL distilled water for 2 × 10 s at 20,000 rpm with T25 digital ULTRA-TURRAX homogenizer (IKA-Werke GmbH&Co. KG, Staufen, Germany). Than the joint homogenates were centrifuged at 4°C for 10 min at 10,000 rpm and the supernatants were collected for gelatin zymography. Gelatinolytic activities of MMPs were examined as previously described (Bencsik et al., 2014, 2015). Briefly, 8% polyacrylamide gels were copolymerized with gelatin (2 mg/mL, type A from porcine skin, Sigma-Aldrich, St. Louis, MO, United States), and 25 μg of protein per lane was loaded. An internal standard (American Type Culture Collection, Manassas, VA, United States) was loaded into each gel to normalize activities between gels. After electrophoresis (90 V, 90 min), gels were washed with zymogram renaturation buffer (Novex, Carlsbad, CA, United States) for 40 min. Samples were incubated for 20 h at 37°C in zymogram development buffer (Novex, Carlsbad, CA, United States).

In a separate set of experiments, one sample from non-arthritic and arthritic doxycycline-free experimental groups was loaded into the gel in 4 replicates. After renaturation, the gel was cut into 4 pieces, which were separately incubated in development buffer containing doxycycline hyclate at 0, 0.05, 0.1, or 0.2 μg/mL concentrations, respectively, in accordance with the plasma levels of the doxycycline-treated animals. In another setup 3 pieces of the gel were separately incubated with 2, 20, or 200 μg/mL doxycycline hyclate in comparison with the previously applied 0 and 0.2 μg/mL concentrations to reveal whether these higher concentrations above the originally measured plasma levels are able to inhibit MMP activity.

Gels were then stained with 0.05% Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO, United States) in a mixture of methanol-acetic acid-water [2.5:1:6.5 (v/v)] and destained in aqueous 4% methanol-8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark-blue background. Gels were scanned in a transilluminator and band intensities were quantified by Quantity One software (BioRad, Hercules, CA, United States), and expressed as the ratio to the internal standard, and presented in arbitrary units. For positive controls, gelatinase zymography standard containing human MMP-2 and -9 (Chemicon Europe Ltd., Southampton, United Kingdom) was used. For negative control, lanes containing tissue samples were cut off after renaturation and were separately incubated for 20 h at 37°C in development buffer in the presence of the calcium chelator EGTA [ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; 10 mM]. Since no gelatinolytic activities could be seen at all, we concluded that the all visible bands derive from MMP activities (data not shown).

Measurement of Water Consumption and Plasma Concentration of Doxycycline

Mice were kept with a maximum of 8 mice/cage density. Water consumption was measured every other day by weighing the water bottles and measuring the volume of the residual water for each cage until day 30. At the end of both series of the experiment, on days 16 and 30, animals were euthanized with sodium pentobarbital (100 mg/kg i.p.) and blood was taken by cardiac puncture to analyze the plasma concentrations of doxycycline.

The plasma concentrations were determined by liquid chromatography-mass spectrometry (LC-MS) system (Ruz et al., 2004). Stock solutions of doxycycline hyclate, oxytetracycline hydrochloride (≥95%; Sigma-Aldrich, St. Louis, MO, United States) and calibration standards were prepared by weighing 10 mg of the reference standards. These were transferred to individual 10 mL volumetric flasks, diluted with solvent mixture (1% (v/v) acetic acid in methanol:water = 20:80) to obtain a concentration of 1 mg/mL, and stored at 2–8°C protected from light. Solutions of 5000, 2500, 250, 25, 2.5, 1.25, and 0.625 ng/mL concentrations served as calibration standards.

Plasma samples (300 μL) in Eppendorf tubes (2 mL) were measured by spiking with 2 μL of 50 ng/mL oxytetracycline internal standard solution. After mixing, 20 μL of 1 mol/L trichloroacetic acid (Fluka, Buchs, Switzerland) was added, vortex-mixed for 1 min and centrifuged for 15 min at 14 500 rpm. The supernatants were transferred to autosampler vials and 20 μL was injected into the LC–MS–MS system (Agilent LC-MSD-TRAP-XCT_plus, Santa Clara, CA, United States). Ionization parameters and ion optics voltages were optimized for the detection of the oxytetracycline and doxycycline standards (250 ng/L). The Agilent ChemStation and Agilent LC/MSD Trap softwares were applied. Linearity, precision, accuracy,

specificity and stability were validated, all results were within the acceptable range.

Measurement of Phospholipase A2 Activity and Prostaglandin E2 Level in the Joint Homogenates

Cytosolic phospholipase A2 (cPLA2) activity was measured by the colorimetric cPLA2 assay kit (Abcam, Cambridge, United Kingdom; ab133090; sensitivity: 3.5–42 nmol/min/mL) from the tibio-tarsal joint homogenates according to the manufacturer's protocol. Based on the results of pilot experiments, our undiluted samples were measured in duplicates. The absorbance was recorded at 410 nm using a plate reader (Fluostar Optima, BMG Labtech, Ironmass Consulting Ltd., Budapest, Hungary) and the enzyme activity values were calculated as nmol/min/mL.

Prostaglandin E2 (PGE2) levels from the same homogenates were measured by a colorimetric ELISA kit (antibodies-online GmbH, Aachen, Germany; ABIN365349; detection range: 0.4–80 pg/mL, sensitivity: 0.2 pg/mL) according to the recommendations of the manufacturer in duplicates at 450 nm using on the same plate reader. PGE2 concentration values were calculated as pg/mL by a double logarithmic depiction of the standard curve followed by regression analysis and fitting the samples' absorbance values to the best fit linear regression.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism. Results were expressed as means ± standard errors of means (S.E.M.). Arthritis severity, mechanical hyperalgesia, joint function, water consumption, change of body weight and *ex vivo* MMP activity were evaluated by two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test, *in vivo* luminescence and fluorescence imaging, *in vitro* MMP activity, PLA2 activity and PGE2 level measurements by one-way ANOVA followed by Bonferroni's multiple comparison test, micro-CT data by two-way ANOVA followed by Sidak's multiple comparison test. In all cases $p < 0.05$ was considered to be statistically significant.

RESULTS

Doxycycline Does Not Decrease Either *in vivo* or *ex vivo* MMP Activity in the Arthritic Ankle Joints

Arthritis induced a significant increase of fluorescent signal indicating *in vivo* MMP activity in the ankle joints on day 4, but significant difference did not occur between the tap water- and SDD-treated arthritic groups (**Figures 2A,B**). Sixteen days after arthritis induction 57–60 kDa MMP isoform, 75 kDa MMP-2 and 92 kDa MMP-9, but at 30 days only 57–60 kDa isoform increased significantly in the ankle joint homogenates of both arthritic groups as compared to the non-arthritic controls (**Figures 3A–I**). On day 30, interestingly, remarkably elevated activity of 92 kDa MMP-9 was observed not only in the tap water-consumed arthritic, but also in the non-arthritic group, which was significantly reduced by SDD in both groups (**Figure 3I**). Activity of 72 kDa isoform was similar in both non-arthritic and arthritic groups on both days 16 and 30 (**Figures 3C,G**).

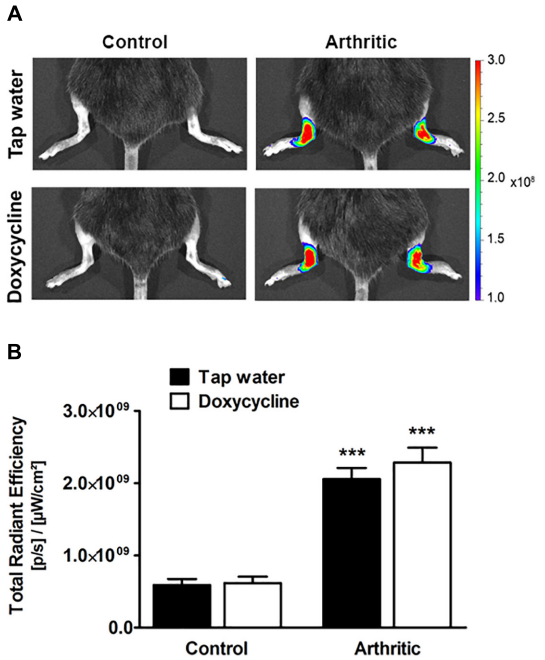

FIGURE 2 | Doxycycline does not inhibit arthritis-induced *in vivo* MMP activity in the ankle joints. **(A)** Representative fluorescence images illustrating *in vivo* MMP activity and **(B)** its quantitative analysis in the ankle joints of non-arthritic ($n = 3$) and arthritic ($n = 8$) mice treated with doxycycline (80 mg/kg p.o. every day during the 30 days experimental period) as compared to the non-arthritic ($n = 3$) and arthritic ($n = 8$) tap water-consumed controls 4 days after arthritis induction. Data are shown as means ± S.E.M. of $n = 3$–8 mice/group, $^{***}p < 0.001$ vs. respective non-arthritic controls (one-way ANOVA followed by Bonferroni's multiple comparison test).

Doxycycline Does Not Influence Arthritis-Induced Clinical Signs, Mechanical Hyperalgesia, and Joint Function Impairment

Considerable paw edema and hyperemia developed few days after serum injection in the arthritic groups, which reached its maximum on day 7. Then the severity of arthritis decreased slightly by the 2nd boost injection (day 10), which stabilized the disease symptoms. After day 17 the clinical score decreased steeply and despite the 3rd boost injection inflammation did not increase remarkably (**Figure 4A**). Mechanical hyperalgesia (tap water consuming arthritic group: from 8.81 ± 0.1 to 6.99 ± 0.36 g, doxycycline-treated arthritic group: from 8.93 ± 0.09 to 6.79 ± 0.25 g) and the time spent on the grid were significantly reduced 5 days after arthritis induction and remained unchanged during the 30 days experimental period (**Figures 4B,C**). SDD treatment did not influence any of the parameters, the severity of

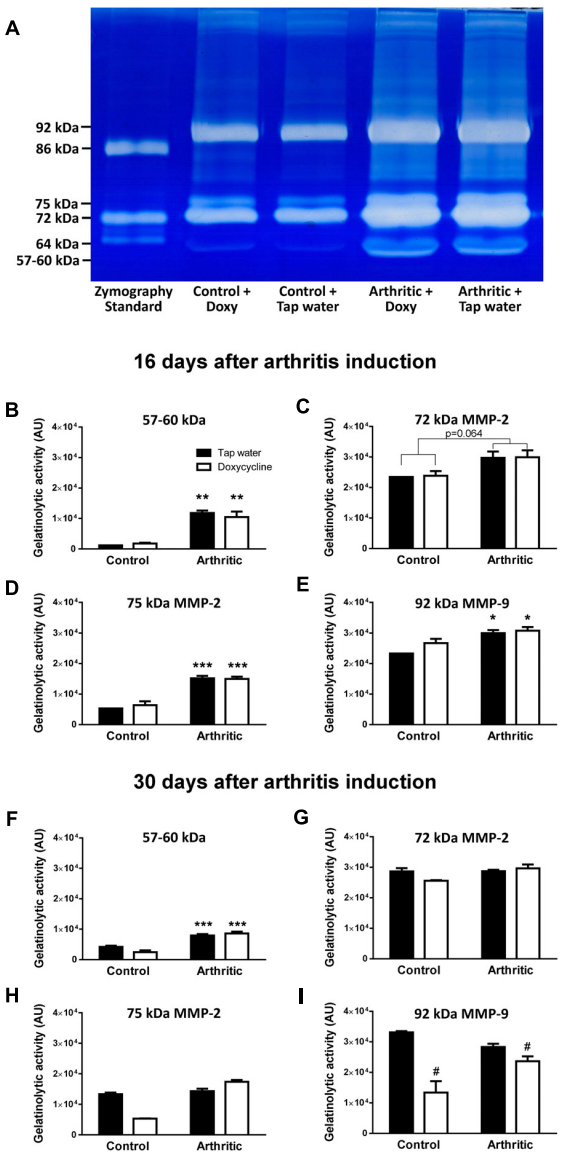

FIGURE 3 | Doxycycline does not inhibit arthritis-induced *ex vivo* MMP activity in the ankle joints. **(A)** Representative gel image illustrating *ex vivo* MMP activity from homogenized mouse ankle joints 16 days after arthritis induction. **(B–E)** Quantitative analysis of MMP activity in the ankle joints of non-arthritic control and arthritic mice treated with doxycycline (80 mg/kg p.o. every day during the 16 days experimental period) compared to the non-arthritic control and arthritic controls consuming tap water 16 days after arthritis induction. **(F–I)** Quantitative analysis of *ex vivo* MMP activity in the ankle joints of non-arthritic control and arthritic mice treated with doxycycline (80 mg/kg p.o. every day during the 30 days experimental period) compared to the non-arthritic control and arthritic controls consuming tap water 30 days after arthritis induction. Data are shown as means ± S.E.M of n = 2–5 mice/group, $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$ vs. respective non-arthritic controls; $^{\#}p < 0.01$ vs. respective tap water consuming mice (two-way ANOVA followed by Bonferroni's multiple comparison test).

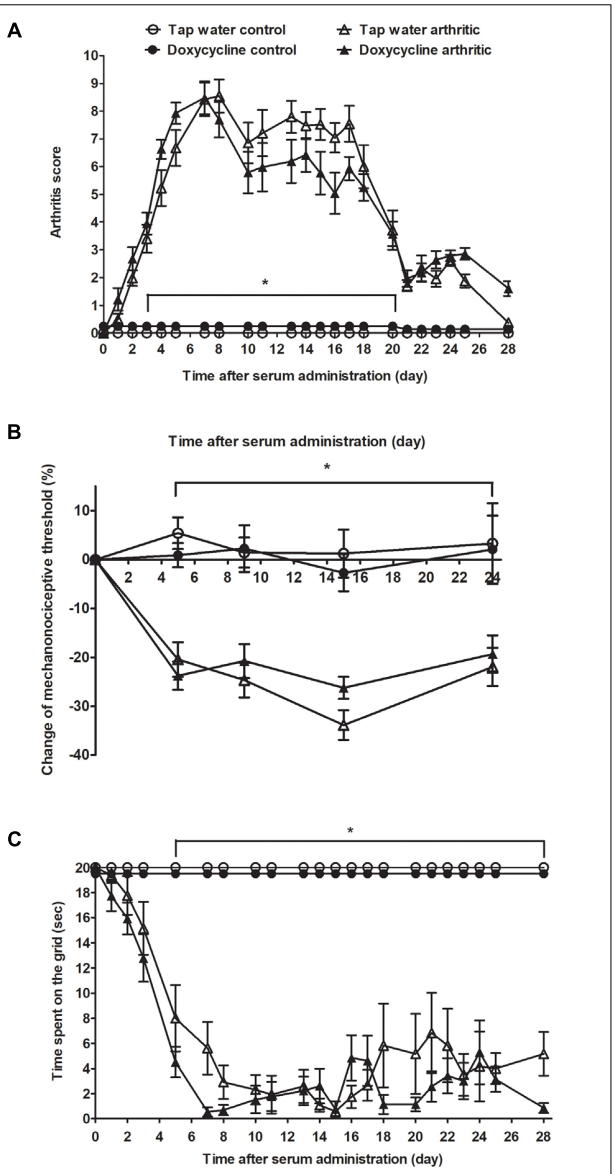

FIGURE 4 | Doxycycline has no effect on K/BxN serum-induced joint inflammation, mechanical hyperalgesia and grasping ability deterioration. Alterations of the **(A)** semiquantitative clinical score, **(B)** mechanonociceptive threshold, and **(C)** time spent on the grid in arthritic (n = 13) and non-arthritic (n = 5) mice treated with doxycycline (80 mg/kg p.o. every day during the 30 days experimental period) as compared to tap water consuming arthritic (n = 12) and non-arthritic animals (n = 3). Data are shown as means ± S.E.M. of n = 3–13 mice/group, $^{*}p < 0.05$ vs. respective non-arthritic controls (two-way ANOVA followed by Bonferroni's multiple comparison test).

clinical signs, the mechanical hyperalgesia and the joint function impairment were similar to the tap water consuming arthritic mice (**Figures 4A–C**).

Doxycycline Does Not Influence the Neutrophil MPO Activity and Plasma Extravasation in the Arthritic Ankle Joints

Both arthritic groups showed intensive luminol-derived bioluminescence signal in the ankle joints reaching the maximum on day 2, but SDD had no inhibitory effect at any

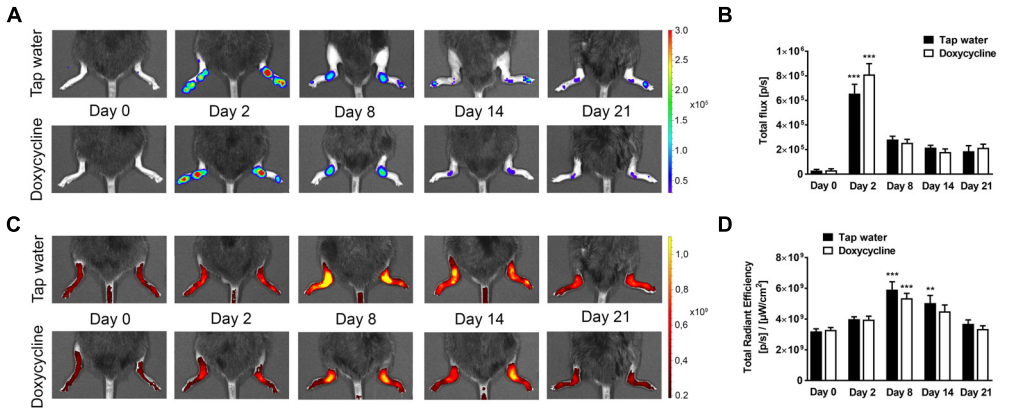

FIGURE 5 | Doxycycline does not alter arthritis-induced neutrophil MPO activity and plasma extravasation. **(A)** Representative bioluminescence and **(B)** fluorescence images illustrating MPO activity and plasma extravasation, respectively, and **(C,D)** their quantitative analysis in the ankle joints of arthritic mice treated with doxycycline (80 mg/kg p.o. every day during the 30 days experimental period; $n = 13$) as compared to the tap water consuming controls ($n = 12$) on days 0, 2, 8, 14, and 21. Data are shown as means ± S.E.M. of $n = 12$–13 mice/group, $^{**}p < 0.001$, $^{***}p < 0.001$ vs. respective day 0 controls (one-way ANOVA followed by Bonferroni's multiple comparison test).

of the time points (**Figures 5A,B**). Plasma extravasation was similarly high in the arthritic ankle joints of both groups in the early phase, which increased further until day 8, then decreased slightly until day 21. However, significant difference was also not observed between the groups (**Figures 5C,D**).

Doxycycline Significantly Increases Trabecular Connectivity and Reduces Bone Mineral Density in the Periarticular Region

The arthritis induced a marked irregularity of the ankle bones, narrowing of the tibio-tarsal joints, and widespread erosions, visually apparent on both CT slices and 3D reconstructions (**Figures 6A**, **7A**). This was reflected by the quantitative analysis, which revealed that the number and volume of open and all pores increased significantly due to arthritis regardless of treatment with SDD in the ankle region (**Figures 6B–D**). Open and total pore volume also increased in the distal tibia (**Figures 7D,E**). Bone mineral density decreased in the periarticular region in SDD-treated mice, but not in their tap water consuming controls (**Figure 7B**). Bone surface density increased in both groups in the distal tibia, highlighting the marked osteophyte formation (**Figure 7C**). The Euler number, a measure of bone structural connectedness was significantly greater in SDD-treated animals in both regions (**Figures 6E**, **7F**).

Chronic Oral SDD Administration Results in Subantimicrobial Concentration in the Systemic Circulation

The plasma concentrations of doxycycline ranged from 0.036 to 0.151 μg/mL in the mice drinking the 0.5 mg/mL solution for 16 or 30 days (**Table 1**). The water consumption did not differ between respective tap water consuming and SDD-treated groups, but it was significantly lower in the arthritic groups as compared to non-arthritic controls as a sign of general sickness behavior (**Supplementary Figure S1A**). The original body weight of the mice was not significantly different (average 29 g), but after day 7 significant 15–25% weight loss was observed in both arthritic groups as compared to the non-arthritic ones. Furthermore, the weight loss of SDD drinking arthritic mice was significantly greater than the tap-water consuming arthritic ones during the period of days 7–10 (**Supplementary Figure S1B**).

Increased MMP Activity in the Arthritic Ankle Joint Homogenates Was Not Influenced by Doxycycline

All examined MMP isoform activities were enhanced in the doxycycline-free arthritic joint homogenates, but none of them changed after incubation with 0.05, 0.1, and 0.2 μg/mL doxycycline, the concentration range measured in the mouse plasma (**Figures 8A–D**). Among higher concentrations only the 200 μg/mL concentration which is remarkably above both the subantimicrobial concentration and the maximal concentration detected in the plasma, was able to significantly inhibit MMP-9, but not MMP-2 activity (**Supplementary Figures S2A–D**).

Doxycycline Does Not Alter cPLA2 Activity and PGE2 Level in the Joints

Based on the observed worsening effect of SDD on arthritic bone structure deterioration and potential involvement of the prostanoid system in bone metabolism, we measured cPLA2 activity and PGE2 levels in the tibio-tarsal joint homogenates. On day 16 PLA2 activity, but not PGE2 concentration increased significantly in the arthritic joint homogenates of the tap water drinking group as compared to non-arthritic controls. In the late phase (day 30), neither cPLA2 activity, nor PGE2 concentrations were significantly elevated in the arthritic joints. SDD treatment did not influence these parameters at either timepoints (**Supplementary Figure S3**).

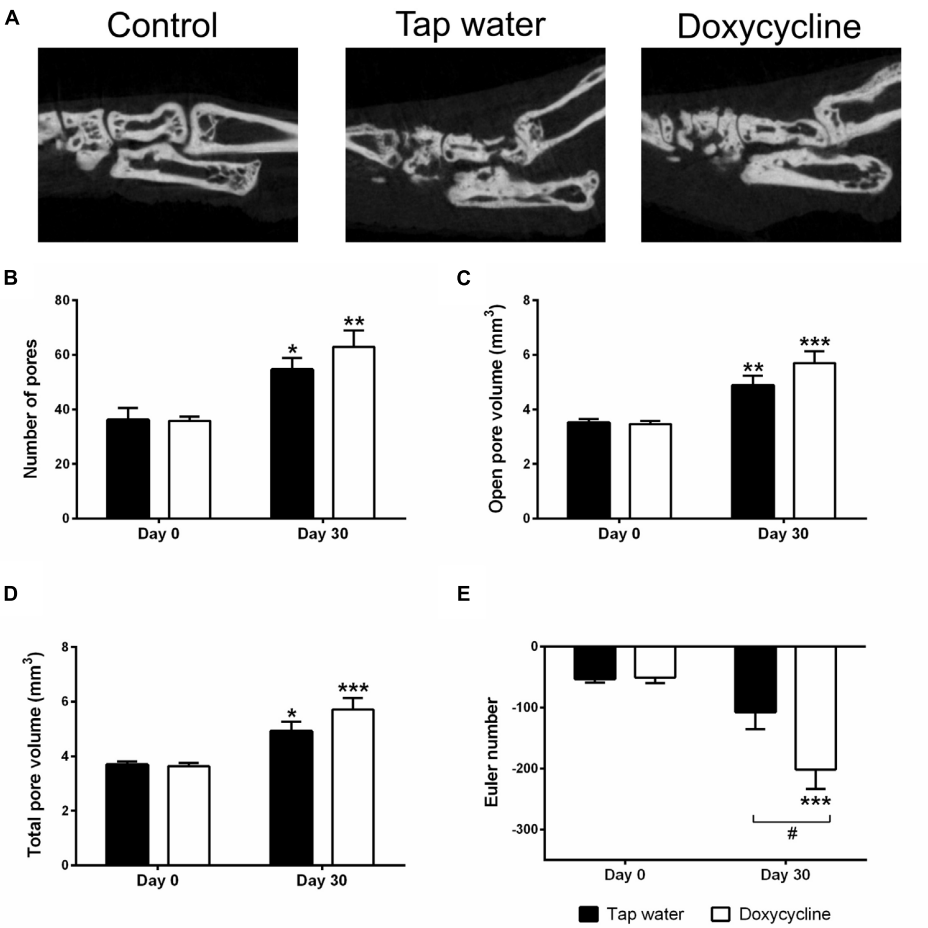

FIGURE 6 | Doxycycline treatment does not affect arthritis-induced bone erosions around the ankle, but increased cancellous bone connectivity. **(A)** Representative sagittal CT slices of the ankle joints, **(B)** change of the number of pores, **(C)** volume of open pores, **(D)** the total volume of pores, and **(E)** the Euler number. Data are shown as means ± S.E.M. of $n = 6–7$ mice/group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. respective day 0 control, $^{\#}p < 0.05$ vs. tap water consuming mice (two-way ANOVA followed by Sidak's multiple comparison test).

DISCUSSION

We showed here the increase of various MMP activities in the joints by *in vivo* fluorescence imaging together with *ex vivo* zymography, as well as investigated their functional significance using the broad spectrum MMP inhibitor SDD in the translational mouse model of RA. This is the first demonstration that SDD alters arthritic bone microarchitecture most probably independently of MMP inhibition.

Various MMP activities and their functional significance were examined in the K/BxN serum-transfer arthritis model, which is particularly appropriate to investigate the innate immune system-mediated effector phase and the chronic neuropathy-like pain component of arthritis (Korganow et al., 1999; Christianson et al., 2010). However, there are only two papers studying the role of specific MMPs in this model. The first paper shows that MMP-8 deficiency increases joint inflammation and bone erosion suggesting the protective role of MMP-8, while the second one demonstrates that the lack of MMP-13 decreases both clinical and histological severity of arthritis indicating the promoting function of MMP-13 in the disease (Garcia et al., 2010; Singh et al., 2013).

TABLE 1 | Water consumption (mL/day/mouse; mean ± S.E.M.; $n = 3–13$ mice/group) and plasma doxycycline concentration (μg/mL; mean ± S.E.M.).

Group	Treatment	Water consumption (mL/day/mouse)	Plasma doxycycline concentration (μg/mL)
Control	Tap water	8.05 ± 0.33	n.a.
	Doxycycline	6.97 ± 0.26	0.066 ± 0.002
Arthritic	Tap water	4.09 ± 0.27	n.a.
	Doxycycline	3.79 ± 0.21	0.071 ± 0.002

Consumption and plasma concentration data represent 3 mice in non-arthritic control, 12 mice in arthritic tap water-consumed group, 5 mice in non-arthritic control, 13 mice in arthritic doxycycline-treated group.

In order to obtain a deeper and more complex insight about the activity and roles of various MMPs in this model, we applied integrative *in vivo* and *in vitro* methodology. Significantly

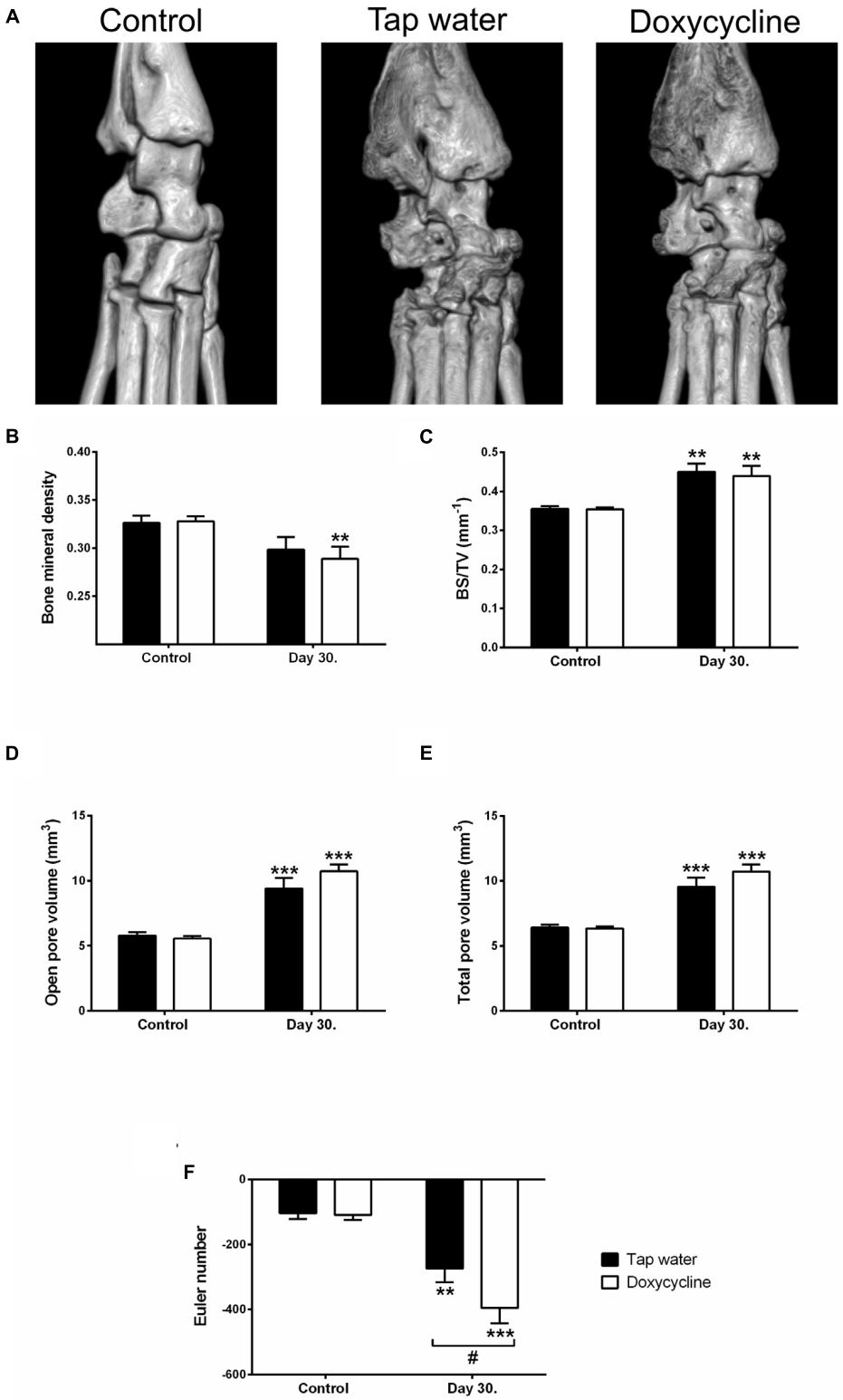

FIGURE 7 | Doxycyline treatment decreases bone mineral density in the periarticular region of the distal tibia, while increasing trabecular connectivity, but has no effect on the formation of arthritic bone erosions. **(A)** Representative 3D micro-CT reconstructions of the right ankle joints, **(B)** bone mineral density, **(C)** bone surface density, **(D,E)** open and total pore volume, and **(F)** Euler number. Data are shown as means ± S.E.M. of $n = 6–7$ mice/group, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. respective day 0 control, $^{\#}p < 0.05$ vs. tap water consuming mice (two-way ANOVA followed by Sidak's multiple comparison test).

increased MMP activity in the arthritic ankle joints was observed *in vivo* using a non-selective fluorescent imaging dye sensitive for MMP-2, -3, -7, -9, -12, and -13. In accordance with these findings, enhanced activities of a 57–60 kDa MMP, 75 kDa MMP-2, and 92 kDa MMP-9 isoforms were shown from the arthritic ankle joint homogenates by gelatin zymography *ex vivo*. The increased gelatinase activity at the band of 57–60 kDa was particularly surprising. There are three possible types of MMPs (proMMP-1, -3, and -13) that this can be related to, but the most likely candidates are proMMP-3 and proMMP-13. ProMMP-3 is secreted as a 57 kDa form, which can be glycosylated resulting in a 60 kDa protein (Wilhelm et al., 1987). Its presence is further suggested by the fact that it was also purified from human rheumatoid synovial fibroblasts (Okada et al., 1986). The molecular weight of the proMMP-13 is also 60–65 kDa. Its active form (MMP-13) is 50–55 kDa in size, but it is further cleaved into a final active form of 48 kDa (Freije et al., 1994; Knauper et al., 1996). MMP-13 is expressed by human chondrocytes (Blavier and Delaisse, 1995; Borden et al., 1996; Mitchell et al., 1996; Reboul et al., 1996), synovial membrane (Wernicke et al., 1996), synovial stroma (Lindy et al., 1997), and synovial fibroblasts (Westhoff et al., 1999). ProMMP-1 is also a potential candidate on the basis of the molecular weight, but its presence in the gel is unlikely, because MMP-1 cleaves gelatin about 40 times less effectively than MMP-13 (Knauper et al., 1996). It is worth mentioning that the mouse and human type MMP-1 are not the same, in mice the equivalent of human MMP-1 is MMP-1a, which is structurally similar, but differently expressed (Foley and Kuliopulos, 2014). Overall, we suggest that the 57–60 kDa gelatinase activity refers to proMMP-3 and/or proMMP-13 isoforms, although it could accurately be determined only by mass spectroscopy.

Several data support the relevance of these MMP isoforms in the pathogenesis of human and experimental RA. In RA patients, MMP-1 and MMP-3 in the serum, synovial tissue (ST) and fluid (SF), MMP-2 and MMP-7 in the SF, MMP-9 in the plasma and SF, MMP-12 and MMP-13 in the ST and SF have already been detected (Ahrens et al., 1996; Wernicke et al., 1996; Yoshihara et al., 2000; Moore et al., 2000; Tolboom et al., 2002; Green et al., 2003; Tchetverikov et al., 2003; Liu et al., 2004). In some cases, their distinct roles were also described in animal models. In a previous study, genetic ablation of MMP-2 resulted in an exacerbated level of collagen antibody-induced arthritis, while the lack of MMP-9 attenuated it compared to the wildtype mice indicating suppressive role of MMP-2 and pro-inflammatory role of MMP-9 in the process (Itoh et al., 2002). Interestingly, despite the convincing human data, the role of MMP-3 could not be confirmed in animal models, since disease severity was not altered in MMP-3-deficient mice in two antigen-induced arthritis models (Mudgett et al., 1998; van Meurs et al., 1999). In contrast, MMP-12-null mice showed more extensive articular inflammation and cartilage destruction associated with massive neutrophil infiltration in the collagen-induced arthritis (CIA) model suggesting the protective role of macrophage-driven MMP-12 in RA (Bellac et al., 2014). The deleterious role

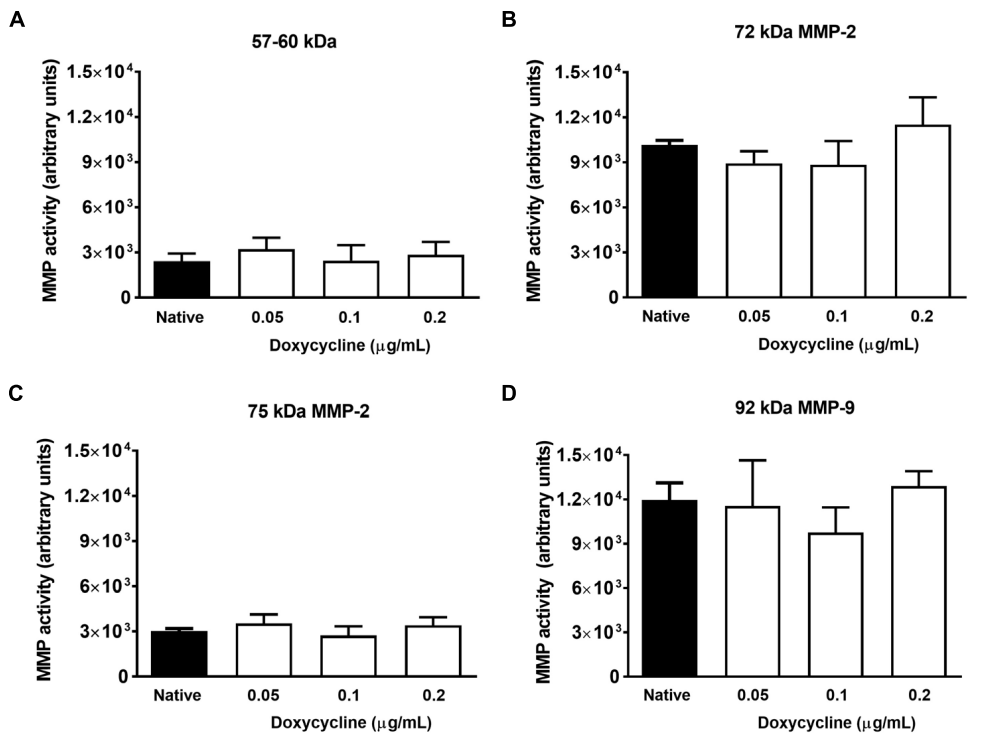

FIGURE 8 | Doxycycline does not inhibit *in vitro* MMP activity in the arthritic joint homogenates. Changes of *in vitro* gelatinolytic activity of **(A)** 57–60 kDa, **(B)** 72 kDa, **(C)** 75 kDa, and **(D)** 92 kDa MMP isoforms in the arthritic and the non-arthritic control joint homogenates incubated with 0.05, 0.1, and 0.2 μg/mL doxycycline compared to the native control. Data are shown as means ± S.E.M. of $n = 4$ samples/group (one-way ANOVA followed by Bonferroni's multiple comparison test).

of MMP-13 was also proven with gene-deficient mice and a highly selective inhibitor. MMP-13 deficiency in the K/BxN serum-transfer arthritis model, as well as selective MMP-13 inhibitor treatment in severe combined immunodeficiency and CIA models resulted in significantly reduced joint inflammation and cartilage destruction (Jungel et al., 2010; Singh et al., 2013).

Since we have detected significantly increased K/BxN serum-induced MMP activation with both *in vivo* optical imaging and gelatin zymography, we investigated their functional relevance in the model with the widely used non-selective inhibitor SDD. Doxycycline belongs to the tetracycline family, whose MMP-inhibitory effect in subantimicrobial dose has been known for three decades (Greenwald et al., 1987). After decades of research its precise inhibitory mechanisms were also identified, it blocks primarily MMP activity by the chelation of the catalytic zinc and altering enzyme conformation, but also suppresses their gene expression and the proteolytic activation (Golub et al., 1998; Smith et al., 1999). Although 50% inhibitory concentrations of tetracyclines ranging from 5 to 500 μM had been shown to inhibit MMP-1, -2, -8, -9, and -13 *in vitro*, only MMP-13 activity was reduced by 5 μM doxycycline bioavailable in the tissues after oral administration (Smith et al., 1999). Due to its unique feature, i.e., the lack of antimicrobial actions, SDD became one of the main pathways of the development of synthetic MMP inhibitor drugs. Moreover, it was already approved by the U.S. Food and Drug Administration for the treatment of chronic periodontitis and acne rosacea (Golub et al., 2016). There are only few literature data when tetracyclines including doxycycline were administered in arthritis models and there are no papers for its chronic use. Oral tetracycline treatment suppressed metalloproteinase activity in arthritic tissue, but even very high doses failed to exhibit substantial anti-inflammatory efficacy (reduce joint swelling or paw diameter) in adjuvant-induced arthritis of the rat (Greenwald et al., 1992). Furthermore, acute oral pretreatment with doxycycline (3, 10, and 30 mg/kg) in an acute antigen (modified Bovine Serum Albumin)-induced arthritis model of the mouse dose-dependently inhibited mechanical hyperalgesia (Pinto et al., 2010). Clinical trials also proves its efficacy in arthritis (Greenwald, 2011; Golub et al., 2016), but primarily due to the lack of selectivity it did not become a widely used drug in RA therapy. However, it is a valuable tool to investigate the roles of arthritis-related MMPs in preclinical research.

In our study, despite the predictable daily water consumption of the mice and reaching subantimicrobial plasma concentration (**Table 1**), SDD did not inhibit either the *in vivo* MMP activity or the *ex vivo* activity of gelatinases derived from the arthritic ankle. The latter result has been confirmed by incubating the arthritic joint homogenates with three different doxycycline concentrations according to the plasma levels measured from the SDD-treated animals. When testing higher, antimicrobial concentrations of doxycycline much above the highest plasma concentration measured in our study, only the highest concentration was able to significantly inhibit MMP-9, but not MMP-2 activity. These results are in good accordance with similar data, which we obtained in lung and heart derived from mice chronically exposed to cigarette smoke, where SDD treatment did not alter MMP activity *ex vivo*. However, *in vitro* treatment only with the highest plasma concentration measured from SDD-treated animals (0.24 μg/mL) was able to significantly decrease MMP-9, but not MMP-2 activity in the lung. Meanwhile, in the heart, none of the doxycycline concentrations decreased MMP-2 activity similarly to the joints (unpublished data). Although plasma doxycycline concentrations have been reliably determined, one limitation of this study is that we could not measure the tissue concentration of doxycycline in the joint homogenates due to technical difficulties. In accordance with MMP activity results none of the K/BxN serum-induced mechanical hyperalgesia, clinical signs, joint function impairment, neutrophil MPO activity and vascular hyperpermeability were altered by the SDD treatment.

Although characteristic functional symptoms accompanied by significantly increased MMP activity in the joints cannot be inhibited by SDD treatment in our chronic arthritis model, it has profound effects on inflammatory homeostatic imbalance of the bones. In our study significant decrease of bone mineral density, which is also a hallmark clinical feature of human RA, was only demonstrated in the region of the distal tibia of SDD-treated mice. Open porosity representing bone erosions increased in K/BxN serum-transfer arthritis model similarly in both SDD and tap water consuming animals. Bone surface density only increased significantly in the distal tibia, which can be explained by the osteophyte-formation in this region. Arthritis was accompanied by an increased trabecular connectivity in both distal tibia and ankle that is likely to reflect bone neoformation due to inflammation. SDD treatment further significantly enhanced this parameter in both regions of arthritic mice as compared to their tap water consuming controls suggesting that SDD treatment results in detrimental overall effect as shown by aggravated periarticular bone resorption and reactive bone remodeling.

Previous studies have shown no effects of SDD on bone architecture under healthy conditions (Fowlkes et al., 2015), but profoundly improved bone homeostasis in ovariectomy-induced osteopenia by inhibiting not only MMPs, but other collagenases involved in bone resorption (Pytlik et al., 2004). Furthermore, doxycycline can shift the bone homeostasis toward the osteoblastic pathway by directly inhibiting osteoclasts and facilitating their apoptosis via inhibiting the Dickkopf-related protein 1 (Dkk-1) pathway (Gomes et al., 2017). Since Dkk-1 overexpressing mice display an osteopenic phenotype, and anti-Dkk-1 antibody treatment prevents bone loss in experimental OA, interference with these signaling pathways is likely to be involved in the increased trabecular connectivity observed in SDD-treated mice in our experiment (Funck-Brentano et al., 2014). Another potential mechanism of SDD on arthritic bone structure deterioration may be PLA2 inhibition and the decreased level of PGE2, which plays an important role in bone metabolism (Lisowska et al., 2018). It is well-known that tetracyclines inhibit PLA2 activity and can theoretically decrease PGE2 level in bones (Pruzanski et al., 1992, 1998), but our present results did not confirm this concept. PLA2 activity, but not PGE2 concentration increased significantly in the arthritic joint homogenates of the tap water drinking group on day 16, when stable inflammatory symptoms such edema and hyperemia were present as compared

to non-arthritic controls. In the late phase (day 30), when the inflammation was over and the arthritic bone structure deterioration was observed, neither PLA2 activity, nor PGE2 levels elevated. However, SDD administration did not influence these parameters at either timepoint.

CONCLUSION

In conclusion, K/BxN serum-transfer arthritis model is characterized by significantly increased MMP levels, but the widely used non-selective MMP inhibitor SDD is not likely to inhibit MMP activity in the joints. SDD clearly worsens the chronic arthritis-induced bone microarchitectural alterations in a complex manner by simultaneously decreasing mineralization and increasing the trabecular connectivity.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

ÁH performed the evaluation of mechanical hyperalgesia, arthritis severity and joint function impairment, the preparation of doxycycline-treated drinking water, the measurement of water consumption, and wrote the manuscript. BB and ÁH carried out the *in vivo* bioluminescence and fluorescence imaging and assisted in data analysis, in study design and in writing the manuscript. TK and BB performed the micro-CT imaging, analyzed the data, and drafted the paper. KC helped to prepare the doxycycline-treated drinking water, participated in study design, and revised the manuscript. IK and AF performed the measurement of plasma concentration of doxycycline and drafted the manuscript. TS, ÉK, and PB carried out the gelatin zymography, PLA2 activity and PGE2 concentration measurements, helped to design the study, and to write the manuscript. AM provided K/BxN and BxN sera and revised the paper. PF and ZH designed the experiments, assisted in data analysis, and in writing the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00233/full#supplementary-material

FIGURE S1 | (A) Water consumption (mL/day/mouse) and **(B)** percentage change of body weight (%). Data are shown as means ± S.E.M. of n = 3–13 mice/group, $^{*}p < 0.05$ vs. respective non-arhritic control mice, $^{\#\#\#}p < 0.001$ vs. tap water consuming arthritic mice (two-way ANOVA followed by Bonferroni's multiple comparison test).

FIGURE S2 | Doxycycline in high concentration (200 μg/mL), but not in lower ones inhibits *in vitro* MMP-9 activity in the arthritic joint homogenates. Changes of *in vitro* gelatinolytic activity of **(A)** 57–60 kDa, **(B)** 72 kDa, **(C)** 75 kDa, and **(D)** 92 kDa MMP isoforms in the arthritic and the non-arthritic control joint homogenates incubated with 0.2, 2, 20, and 200 μg/mL doxycycline compared to the native control. Data are shown as means ± S.E.M. of n = 4 samples/group, $^{*}p < 0.05$ vs. native control (one-way ANOVA followed by Bonferroni's multiple comparison test).

FIGURE S3 | (A) Cytosolic phospholipase A2 activity and **(B)** prostaglandin E2 levels of tibio-tarsal joint homogenates. Data are shown as mean ± S.E.M. of n = 3–7/group, $^{**}p < 0.01$ vs. respective non-arthritic controls (one-way ANOVA followed by Bonferroni's multiple comparison test).

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frontiers in Pharmacology

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Paradoxical Skin Reactions to Biologics in Patients With Rheumatologic Disorders

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Targeted immune-modulating treatment with biological agents has revolutionized the management of immune-mediated inflammatory diseases, including rheumatologic conditions. The efficacy and tolerability of biological agents, from the initial tumour necrosis factor (TNF)-α inhibitors to the new anti-cytokine monoclonal antibodies, have dramatically changed the natural history of debilitating conditions such as rheumatoid arthritis and seronegative spondyloarthropathies. The widening use of biologics across several rheumatologic diseases has been associated with a new class of adverse events, the so-called paradoxical reactions. These events are inflammatory immune-mediated tissue reactions, developing paradoxically during treatment of rheumatologic conditions with targeted biologics that are commonly used for treating the idiopathic counterparts of these drug-induced reactions. The skin is frequently involved, and, even if considered rare to uncommon, these cutaneous manifestations are an important cause of biologic agent discontinuation. TNF-α antagonist-induced psoriasis, which can manifest *de novo* or as exacerbation of a pre-existing form, is the prototypic and most frequent paradoxical skin reaction to biologics while other reactions, such as eczematous and lichenoid eruptions, hidradenitis suppurativa, pyoderma gangrenosum, Sweet's syndrome and granulomatous skin diseases, occur much more rarely. Management of these reactions consists of topical or systemic skin-directed therapies, depending on the severity and extension of the cutaneous picture, and it is generally associated with switching over to other disease-modifying regimens for treating the underlying rheumatologic condition. Here, we review in detail the current concepts and controversies on classification, pathogenesis and clinical management of this new class of cutaneous adverse events induced by biologics in rheumatologic patients.

Keywords: paradoxical skin reactions, biologics, rheumatological disorders, psoriasis, TNFα-inhibitors

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Abbreviations: IFN, Interferon; IFN type-1, interferon-type 1; IL, interleukin; IL-10, interleukin 10; ILC, innate lymphoid cell; iTreg, induced regulatory T-cells; NDs, neutrophilic dermatoses; TGF-β, transforming growth factor-beta; Th, T helper cell; TNF-α, tumor necrosis factor-alpha; TNFR2, tumor necrosis factor receptor 2; Tregs, regulatory T-cells.

INTRODUCTION

Targeted biological agents have dramatically changed the treatment landscape of immune-mediated inflammatory diseases (IMIDs) with rheumatological conditions being at the front of this revolution. The efficacy and tolerability of targeted biological agents have determined a paradigm shift in the treatment of several rheumatologic conditions, modifying the natural history of progressive, invalidating disease, such as rheumatoid arthritis (RA) and seronegative spondyloarthropathies (SpA). While biological agents (BA) have a superior safety and tolerability profile compared to conventional disease-modifying anti-rheumatic drugs (DMARDs), they may cause different cutaneous adverse events, either of infectious, inflammatory or neoplastic origin (Hernandez et al., 2013). Furthermore, targeted treatment with BA is increasingly associated with a new class of adverse events, the so-called paradoxical immune-mediated inflammatory reactions. Paradoxical reactions (PR) are defined by the development of inflammatory immune-mediated tissue manifestations in IMID patients treated with targeted biological agents. The skin is frequently involved by this paradoxical inflammation, as in the case of plaque psoriasis developing in a rheumatological patient during treatment with TNF-α inhibitors (TNFi) (Viguier et al., 2009).

Cutaneous PR have been described as a class-effect of targeted BA, especially TNFi, first in rheumatologic patients and subsequently across all other indications, such as psoriasis and inflammatory bowel-disease (IBD). Reports of different, organ-specific PR are constantly increasing, as long-term use of new anti-interleukin (anti-IL-6, -IL-17,-IL-12/23) and first-to-second generation TNFi biosimilars is growing (Toussirot and Aubin, 2016). Furthermore, cutaneous PR represent an intriguing immunological and clinical dilemma, whose unraveling may improve our knowledge of the pathogenesis of chronic inflammatory diseases. These puzzling cutaneous eruptions may also represent a new type of adverse drug reactions in the era of precision medicine, resulting from the interaction between targeted manipulation of cytokine-molecular networks by BA and patient's genetic predisposition (Cabaleiro et al., 2016). We review the clinical spectrum of paradoxical cutaneous inflammation induced by targeted BA in patients with rheumatological disorders, discussing the current controversies on classification, pathogenesis and clinical management.

Cutaneous Paradoxical Reactions: Definition and Scope of the Problem

Psoriasis, and its clinical variants, represents the prototypical cutaneous PR, as this was the first paradoxical reaction pattern described in rheumatologic patients treated with the first-generation BA, namely the TNFi. Therefore, most clinical and experimental studies on PR have focused on paradoxical psoriasis, providing the conceptual framework for the other cutaneous PR. The literature on epidemiology of PRs in patients treated with BA is scarce, as most of the clinical evidence derives from retrospective studies, case series and reports. The estimated prevalence of cutaneous PR ranges from 0.6 to 5.3% in patients treated with TNFi (Sfikakis et al., 2005; Fouache et al., 2009; Ko et al., 2009; Famenini and Wu, 2013; Bae et al., 2018). In a registry-based observational study, the incidence of paradoxical psoriasis in RA patients treated with TNFi has been estimated in 1.04 per 1000 persons/years. Patients treated with TNFi presented an incidence rate ratio (IRR) of 2.0–5.94 for the onset of paradoxical psoriasis compared to patients treated with conventional DMARDs (Hernandez et al., 2013). In RA patients, the incidence of paradoxical psoriasis has been estimated in one new case for every 550 patients treated with adalimumab per year (Harrison et al., 2009). In the context of adverse drug reaction, cutaneous PRs could be classified as uncommon-to rare events. Typically, cutaneous PRs, such as paradoxical psoriasis, can be induced *de novo* in rheumatologic patients without a history of cutaneous inflammatory disease during treatment with a BA. On the other hand, cutaneous PR can be an exacerbation, with or without a change in clinical morphology, of a pre-existing cutaneous inflammatory disease in a genetically-predisposed patient. This is the case of paradoxical palmoplantar pustular psoriasis developing during TNFi in a RA patient with a history of plaque psoriasis. Key features supporting the causal relationship between a skin PR and therapy with a BA are: (a) the temporal association and (b) clinical outcome of the PR after BA withdrawal. Cutaneous PR can occur at any time during treatment with a BA, but more than 60% of cases of paradoxical psoriasis has been reported to develop within the first year of treatment (Brown et al., 2017). As observed in cutaneous drug reactions, the cessation of the triggering "culprit" BA determines clinical resolution or improvement of the skin PR. Re-treatment, or drug re-challenge, with the same BA or related BA-class has been associated with the relapse of paradoxical skin inflammation.

CLASSIFICATION OF CUTANEOUS PARADOXICAL REACTIONS

In rheumatologic patients, cutaneous PRs induced by BA can present with different clinical aspects and extent, involving the skin, its appendages and transitional epithelial surfaces. Cutaneous PRs, as defined previously, encompass a variety of inflammatory manifestations/conditions, which can be both treated and triggered with the same cytokine-targeted BA. Cutaneous PRs reported in rheumatologic patients are summarized in **Figure 1** and include psoriasis and its spectrum of clinical phenotypes (plaque, pustular, generalized, palmoplantar, scalp, guttate, and inverse), hidradenitis suppurativa (HS), neutrophilic dermatosis (the prototypical forms pyoderma gangrenosum and Sweet's syndrome) and granulomatous skin disease (granuloma annulare, interstitial granulomatous dermatitis, necrobiosis lipoidica, and sarcoidosis). Other BA-inducible cutaneous inflammatory conditions, such as atopic dermatitis, cutaneous vasculitis, drug-induced lupus erythematosus and other allergic and hypersensitivity reactions are not strictly considered "paradoxical" because their idiopathic counterparts are not generally treated with these agents.

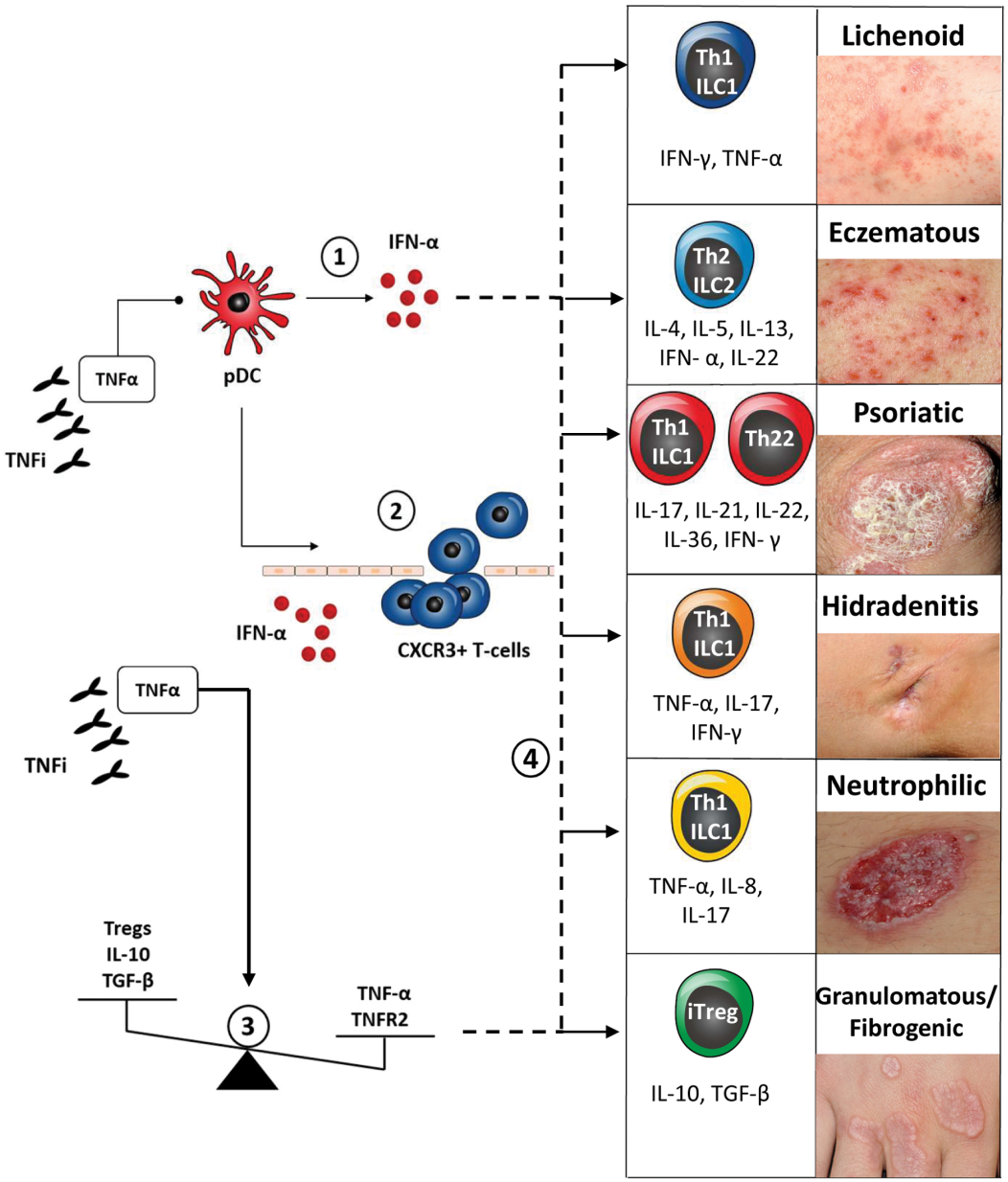

FIGURE 1 | Classification and pathogenesis of cutaneous paradoxical reactions to biologic agents (*) and their related key immune-response/cytokine patterns.
(1) TNF-α/type-1 IFN cytokine imbalance: pharmacological blockade of TNF-α by TNF-inhibitors (TNFi) determines uncontrolled activation of plasmacytoid dendritic cells (pDCs), with sustained production of IFN-α. IFN-α drives paradoxical skin inflammation via downstream effectors cytokines, thus eliciting key cutaneous immune response patterns → clinical correlation: paradoxical psoriasis and psoriasiform eruptions.
(2) IFN-α induced spatial shift of activated innate immune cells and chemokine (C-X-C motif) receptor 3 (CXCR3) positive T-cells from extra-cutaneous tissue compartments to the skin → clinical correlation: *de novo* induction of psoriasiform eruptions and hidradenitis suppurativa.
(3) TNF-α/IL-10 cytokine imbalance and Tregs and TNFR2 imbalance → clinical correlation: paradoxical cutaneous sarcoidosis and granulomatous disease.
(4) Shift in cutaneous immune response pattern → clinical correlation: *de novo* induction of lichenoid, eczematous PR or change in psoriatic morphology (plaque to pustular).
(*) Based on expert opinion and on current available clinical and experimental evidence; cutaneous immune response patterns adapted from Eyerich and Eyerich (2018).

Classification of inflammatory skin disease is traditionally based on clinical morphology of primary and secondary skin lesions in combination with a histological description of epidermal-dermal tissue involvement and underlying pathomechanisms (Dainichi et al., 2014). Adverse cutaneous drug reactions also share similar classification systems, with a combination of clinical descriptors of lesion morphology (psoriasiform, bullous etc.) histological pattern (spongiotic,

lichenoid/interface dermatitis, etc.) and underlying predominant immunologic/hypersensitivity mechanism (type I-IV reaction) (Isabwe et al., 2017). A recent trend in the classification of inflammatory skin disease is to integrate clinicopathological data with molecular-immunologic information, such as predominant disease cell-subset, cytokine expression patterns and molecular biomarkers (Inkeles et al., 2015; Garzorz-Stark and Lauffer, 2017) Recently, Eyerich and Eyerich (2018) summarized the cutaneous immune-response patterns (Th1-, Th2-, Th17/Th22- and Treg-cells and related cytokines) associated with specific cutaneous-tissue response patterns (lichenoid, eczematous/blistering, psoriatic, fibrogenic/granulomatous), providing a molecular-pathophysiological approach to the traditional, complex dermatological nosology. This conceptual classification can be used for the description of cutaneous PR, along with its prevalently associated clinical and immune-response patterns, according to currently published data (refer to **Figure 1**). According to its initial descriptions, cutaneous PRs can be considered almost identical to its corresponding, "classic" inflammatory skin disease in terms of clinical, histological and immunological presentation. In the following sections we will discuss some limitations of this concept, based on recent clinical and experimental studies.

In BA-treated patients, the clinical picture of cutaneous PR may vary from typical inflammatory skin lesions - clinically and histological identical to its correspondent primary, non-BA induced skin disease – to atypical inflammatory skin manifestations, with "overlapping" clinical and histological features. For example, paradoxical TNFi-induced psoriasis may present with a wide clinical spectrum, with typical erythematous-squamous or pustular lesions, clinically indistinguishable from conventional plaque or pustular psoriasis, to atypical papulo-squamous eruptions with "psoriasiform," "eczematous" or "lichenoid" lesion morphology (Succaria and Bhawan, 2017). Correlation with histological aspects of lesional skin is crucial for diagnosis of the PR type and differentiation with other cutaneous adverse events. Moreover, in most published series the spectrum of histological changes of a "psoriasiform" paradoxical eruption is quite variable, ranging from typical psoriatic pattern to lichenoid/interface dermatitis, pustular folliculitis and eosinophil-rich perivascular dermatitis pattern. Psoriatic alopecia has been recently described as a distinct clinical phenotype of anti-TNF-α-induced, paradoxical psoriasis, presenting with patchy, non-cicatricial alopecia due to marked inflammatory involvement of the scalp skin and hair follicles (**Figure 1**; Osório et al., 2012; George et al., 2015). Histologically, features of both conventional scalp psoriasis and alopecia areata have been observed (Doyle et al., 2011).

Lichen planus (LP)-like or lichenoid skin eruptions are characterized by an interface dermatitis histological pattern and prominent Th1/ILC1 (type 1 innate lymphoid cell)-IFN (interferon)-γ-biased immune-response. This skin-directed cytotoxic reaction can be triggered by microbial antigens, xenobiotics and drugs. Paradoxical, lichenoid eruptions have been reported in RA and psoriatic arthritis (PsA) patients during treatment with TNFi, variably involving the skin, oral/genital mucosa, nails and hair-follicles (Vergara et al., 2002; Asarch et al., 2009; Garcovich et al., 2010).

Hidradenitis suppurativa is a chronic, inflammatory disease of the follicular epithelium, presenting with suppurative lesions (nodules, abscesses, pustules, fistulas, sinus-tracts, and hypertrophic scars) affecting the skin folds and anogenital area. The cutaneous immune response pattern of primary HS is characterized by Th17/ILC3 lymphocyte subset, with strong IL-1β, TNF-α, IL-17 cytokine-signature, and peripheral recruitment of IL-17-producing neutrophils and Th17-cells. Paradoxical HS has been recently reported in patients with RA or spondyloarthropathies (PsA, AS, SAPHO) during long-term treatment (mean 25 months) with TNFi and other BA (tocilizumab, rituximab) (Delobeau et al., 2016; Faivre et al., 2016). Most patients presented known risk factors for HS (smoking, overweight), but the relapse of paradoxical HS after re-treatment with involved BA supports causality.

Pustular psoriasis and neutrophilic dermatoses (pyoderma gangrenosum, Sweet syndrome) present a sterile pustule as hallmark cutaneous lesion of neutrophilic inflammation. Both "neutrophilic" inflammatory conditions share common downstream inflammatory mediators, such as TNF-α, IL-8, IL-17, IL12/23 and IL-36, which promote activation and migration of neutrophils in the skin. Pyoderma gangrenosum, the prototypical form of hypodermal neutrophilic, can be both treated and paradoxically triggered by almost all the TNFi (etanercept, adalimumab, infliximab, golimumab) (Vandevyvere et al., 2007; Brunasso et al., 2010; Kowalzick et al., 2013; Marzano et al., 2018; Skalkou et al., 2018).

Granulomatous skin conditions are a heterogenous group of chronic inflammatory diseases, which include also reactive or drug-induced processes. Reactive granulomatous skin eruptions have a wide spectrum of clinical morphologies with several clinical entities, such as interstitial granulomatous dermatitis (IGD) and palisaded neutrophilic and granulomatous dermatitis (PNGD). These reactive conditions can be triggered by systemic inflammatory conditions, such as connective tissue disease and the rheumatic disease, and by several drugs, such as TNFi (Deng et al., 2006). Localized and generalized forms of granuloma annulare have been reported in rheumatologic patients during treatment with TNFi (Voulgari et al., 2008). Histological evidence of dermal non-caseating granulomas is a hallmark of cutaneous sarcoidosis, which can present several clinical-morphological variants (Amber et al., 2015; Rosenbach and English, 2015). Paradoxical development of sarcoidosis-like skin lesions has been reported in rheumatologic patients treated with TNFi, especially with etanercept (Dhaille et al., 2010; Massara et al., 2010; Robicheaux Clementine et al., 2010; Lamrock and Brown, 2012; Decock et al., 2017). In sum, cutaneous PR presents a wide spectrum of clinical-histological reaction patterns.

PATHOGENESIS OF CUTANEOUS PARADOXICAL INFLAMMATION

Since the initial descriptions, cutaneous inflammatory disease presenting *de novo* in rheumatologic patients during treatment

with potent, cytokine-targeted BA represented a clinical and immunological conundrum. Considering the molecular taxonomy of inflammatory skin disease, cutaneous PR can be explained by the complex interplay between host-specific factors (genetic predisposition) and BA-induced specific shifts in cytokine and cellular immune-response patterns (Palucka et al., 2005; Grine et al., 2015; Verwoerd et al., 2016). According to current experimental data, cutaneous paradoxical inflammation may result from different putative immune-pathogenetic mechanisms (summarized in **Figure 1**), leading to different types of clinical reactions. BA- induced immune-pathogenesis of cutaneous PRs include one or more of the following mechanisms as *primum movens*: (a) a cytokine imbalance; (b) a shift in cutaneous immune response pattern; (c) a spatial shift of activated innate or adaptive immune cells to the skin; (d) imbalance or dysfunction of regulatory T-cells.

In the case of paradoxical psoriasis, a TNFi-induced cytokine imbalance between TNF-α and type 1-Interferons (IFN-α) has been reported as key-pathogenetic factor (Marzano et al., 2014). Lesional skin of psoriasiform PR displayed an increased tissue-expression of MxA (myxovirus-resistance protein A), i.e., type-1 interferon activity (de Gannes et al., 2007). Notably, systemic treatment with recombinant IFN-α and topical application of IFN-α inducers (imiquimod) are both able to elicit psoriatic skin lesions in clinical and experimental models (Gilliet et al., 2004). Continuous therapeutic TNF-α blockade thus cause a specific, cutaneous cytokine imbalance, favoring development of inflammatory plasmacytoid dendritic cells (pDCs) and increased type-1 IFN production (Conrad et al., 2018). Increased IFN-α-activity in turn determines abnormal trafficking and homing of pDCs and innate immune cells to the skin, in an inflammatory loop. Furthermore, in RA patients, treatment with TNFi has been associated with increased expression of the chemokine receptor CXCR3 on peripheral T-cells, potentially favoring trafficking of activated T-cells to the skin (Aeberli et al., 2005). This dysregulated, paradoxical innate immune response may then translate clinically to paradoxical psoriasis in genetically susceptible subjects.

Recent experimental studies raised several controversies on the true nature of cutaneous PR, differentiating its immune pathogenesis from their "classical," non-paradoxical counterparts. Stoffel et al. (2018) reported both psoriasiform and eczematous PR to have a distinct lesional immune response pattern, with a common strong Th1- and type-1 IFN pattern, differing, respectively, from "classic" psoriasis and eczema controls. In the same study, psoriasiform PR presented an increased expression of type 1 IFN (IFN-α, IFN-γ) and pro-inflammatory cytokines (IL-36, IL-19, IL-20), whereas eczematous PR were associated with up-regulated Th-2 cytokines (IL-13, IL-5) and IL-22 expression. In psoriasiform PRs, the involvement of IL-1-(IL-36) and IL-17 (IL-17A) cytokine families, sustaining a pro-inflammatory loop mechanism, has been also reported in patients with IBD and psoriasis (Tillack et al., 2014; Deubelbeiss et al., 2018). Finally, the group of Gilliet et al. (2004) designed an experimental murine model for paradoxical skin inflammation to support the differences between paradoxical psoriasis and "classic" psoriasis. In this model, induction of the "paradoxical" psoriasiform phenotype is mainly driven by type 1 IFN expression and cutaneous infiltration of pDCs due to temporal TNF-α/IFN imbalance. The resulting paradoxical psoriasiform skin inflammation is mostly independent from T-cells, i.e., from adaptive immune responses, which is in contrast with "classical" psoriasis (Conrad et al., 2018). BA-induced shifts of cutaneous immune response patterns may then interact with host-specific genetic risk variants for inflammatory cutaneous disease, promoting the development of PRs. Indeed, preliminary studies support the role of specific IL-23 receptor (IL-23R) gene polymorphisms to be linked to anti-TNF-α-induced paradoxical psoriasis (Sherlock et al., 2013).

CLINICAL IMPLICATIONS OF PARADOXICAL REACTIONS TO BIOLOGICAL AGENTS

Cutaneous PR occurring during treatment with BA represents a clinical challenge in terms of differential diagnosis and management. Clinical management should be aimed at treating the cutaneous signs (eruption) and symptoms (pain, pruritus etc.) while maintaining control of the underlying rheumatologic condition. As discussed previously, the modification of the anti-rheumatic treatment regimen, i.e., a treatment suspension/withdrawal or therapeutic switch, is associated in most cases with an improvement or resolution of the cutaneous PR. Therefore, when approaching the rheumatologic patient with a PR, the clinician should take into account several factors, including: (a) the extent and severity of skin involved by the PR, (b) the severity and activity of the background rheumatologic condition, (c) the patient's quality of life and comorbidities, (d) the possible loss of efficacy of the culprit BA in the case of cessation/retreatment, (e) the availability of alternative treatment options for the rheumatologic condition. Dose-reduction and discontinuation strategies of BA in rheumatologic patients should be evaluated on a per-case basis by the treating rheumatologist, as there are no definitive guidelines for the management of cutaneous PR (van Herwaarden et al., 2014). In the case of anti-TNF-α induced psoriasis, a practical treatment algorithm has been initially proposed by Collamer et al. (2008) and this can be adapted to other cases of cutaneous PRs as well.

In a BA-treated rheumatologic patient developing inflammatory skin lesion a high-grade of suspicion for PR should be taken. Interdisciplinary care should necessary include evaluation by a dermatologist and lesional skin biopsy, to aid clinical-histological correlations and differential diagnosis with other cutaneous adverse events. The severity of cutaneous PR can be graded with simple assessments, such as the extent of body surface area (BSA) involved, symptom-based scores (pruritus/pain intensity scores) and patient-reported outcomes (dermatology life quality index [DLQI]). The addition of a skin-directed therapy (topical or systemic) is a reasonable initial strategy to manage the cutaneous PR and to maintain the patient on treatment with the BA. Topical skin-directed therapies (topical steroids, keratolytic agents, immunomodulators, vitamin

D analogs) are a viable option for PR with mild (BSA < 5%) to moderate (BSA 5–10%) skin involvement, such as localized plaque psoriasis, lichenoid reactions or granulomatous lesions. In the case of PR with moderate-to severe skin involvement (>10% BSA), progressive course and/or high symptom-burden (QoL impairment) treatment can be escalated with the addition of UV-phototherapy or traditional systemic agents, such as methotrexate, retinoids (acitretin), cyclosporine and systemic steroids. Combination treatment regimens with systemic, skin-directed agents and ongoing BA (for example TNFi) should be carefully managed in close collaboration by the rheumatologist and the dermatology consultant.

In the case of severe PR with extensive (>10% BSA), unstable disease and/or high disease-burden, treatment with the BA should be discontinued. For example, severe plaque psoriasis, erythrodermic psoriasis, generalized pustular psoriasis or highly pruritic lichenoid or eczematous eruption would necessarily lead to discontinuation of anti-rheumatic treatment with a TNFi. According to published studies, almost 50% of patients will present an improvement or resolution of paradoxical skin lesions, following withdrawal of the BA. Another 45% of patients with anti-TNF-α induced psoriasis may present persistent or recurring cutaneous lesions, despite BA discontinuation. The more severe PR, such as generalized pustular psoriasis or psoriatic alopecia, can run a persistent course, only with partial improvement, after discontinuing the BA (Brown et al., 2017). Re-treatment with the same BA, after cessation of the cutaneous PR, should be evaluated on the basis of concomitant rheumatologic condition and availability of alternative treatment options. There is a substantial risk of recurrence of the cutaneous PR after re-treatment with the same BA, but there is no strong evidence in published studies (Wollina et al., 2008). Therapeutic switch of the PR-triggering BA with another BA of the same class (i.e., alternative TNFi) or of different class can be considered in moderate-to severe cutaneous PR, to control the underlying rheumatologic condition. Therapeutic switch to another BA is also indicated in the severe, paradoxical psoriasis subtypes, as in the case of generalized pustular psoriasis. Switching to a new BA-class, for example from a TNFi to anti-IL6 treatment (tocilizumab), is a common strategy in the management of RA and has been also reported in the case of paradoxical psoriasis (Rueda-Gotor et al., 2012; Shimizu et al., 2015; Cantini et al., 2017).

CLINICAL IMPLICATIONS OF PARADOXICAL REACTIONS TO BIOLOGICAL AGENTS

The unexpected occurrence of paradoxical inflammation during treatment with BA has emerged as a new type of drug-related adverse event, with a complex pathophysiology. The skin is one of the main organs affected by these reactions, presenting with a wide spectrum of clinical and pathological aspects. In rheumatologic patients, cutaneous PRs are frequent and clinically relevant. Adequate clinical management of these reactions is paramount to maintain control of background rheumatologic disease and to improve drug survival of BA. In some cases, therapeutic switch to another class of BA or to new, small-molecule-based disease modifying drugs is warranted to oppose paradoxical inflammation. The understanding of these new types of adverse reactions will hopefully shed light on the complex interactions between host-specific factors (genetic predisposition), immune-mediated comorbidities, immune-regulatory mechanisms and targeted immune-modulation.

AUTHOR CONTRIBUTIONS

SG, CS, EB, MC, and AM designed and reviewed the manuscript and contributed in drafting the manuscript. GG contributed in drafting and reviewing the manuscript. All the authors approved the final version of the manuscript.

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Long-Term Retention Rate of Anakinra in Adult Onset Still's Disease and Predictive Factors for Treatment Response

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Background: Anakinra (ANA) is an effective treatment choice in patients with adult onset Still's disease (AOSD). Variables affecting treatment survival include loss of efficacy or adverse events, but also the decision to discontinue treatment after long-term clinical remission.

Objectives: Aims of this study were: (i) to assess the drug retention rate (DRR) of ANA during a long-term follow-up looking for any difference related to the line of biologic treatment, the concomitant use of conventional disease modifying anti-rheumatic drugs

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(cDMARDs) and the different type of AOSD (systemic versus chronic articular); (ii) to identify predictive factors of lack of efficacy, loss of efficacy, and ANA withdrawal owing to long-term remission.

Methods: AOSD patients classified according with Yamaguchi criteria and treated with ANA were retrospectively enrolled in 18 Italian tertiary Centers. Demographic, laboratory, clinical and therapeutic data related to the start of ANA (*baseline*), the 3-month assessment and the last follow-up visit while on ANA treatment were retrospectively collected and statistically analyzed.

Results: One hundred and forty-one AOSD patients (48 males, 93 females) treated with ANA for a mean period of 35.96 ± 36.05 months were enrolled. The overall DRR of ANA was 44.6 and 30.5% at the 60- and 120-month assessments, respectively, with no significant differences between: (i) biologic naïve patients and those previously treated with other biologics (log-rank $p = 0.97$); (ii) monotherapy and concomitant use of cDMARDs (log-rank $p = 0.45$); (iii) systemic and chronic articular types of AOSD (log-rank $p = 0.67$). No variables collected at *baseline* could predict primary inefficacy, while the number of swollen joints at baseline was significantly associated with secondary inefficacy ($p = 0.01$, OR = 1.194, C.I. 1.043–1.367). The typical AOSD skin rash was negatively related with ANA withdrawal owing to long-term remission ($p = 0.03$, OR = 0.224, C.I. 0.058–0.863).

Conclusion: Long-term DRR of ANA has been found excellent and is not affected by different lines of biologic treatment, concomitant use of cDMARDs, or type of AOSD. The risk of losing ANA efficacy increases along with the number of swollen joints at the start of therapy, while the typical skin rash is a negative predictor of ANA withdrawal related to sustained remission.

Keywords: autoinflammatory diseases, systemic onset juvenile idiopathic arthritis, personalized medicine, canakinumab, innovative biotechnologies, interleukin-1

INTRODUCTION

Adult onset Still's disease (AOSD) is a systemic inflammatory disorder characterized by daily high-spiking fevers, evanescent salmon-colored maculopapular rash, sore throat, serositis, hepatosplenomegaly, lymphadenopathy, myalgia, arthritis, and/or arthralgia. Laboratory investigations usually reveal leukocytosis with neutrophil predominance, increased acute-phase reactants and high levels of serum ferritin, while serum liver enzymes may be often elevated (Pouchot et al., 1991). This condition is frequently considered as the adult counterpart of systemic onset juvenile idiopathic arthritis (SOJIA) (Uppal et al., 1995; Luthi et al., 2002; Martini, 2012).

Clinical presentation of AOSD can be distinguished into two main phenotypes: a "systemic type" characterized by predominantly systemic features including fever, rash, serositis, and organomegaly, and a "chronic articular type" with patients suffering from articular manifestations mimicking rheumatoid arthritis with a polyarticular symmetric pattern. The systemic type can be distinguished into a monocyclic and polycyclic course. A monocyclic AOSD is defined as a single flare lasting from 2 months to 1 year; conversely, the polycyclic course is characterized by recurrent systemic flares with remissions between attacks (Cush et al., 1987).

In the absence of a definitive diagnostic test, diagnosis of AOSD is clinical and requires the exclusion of infectious, neoplastic, autoimmune and other autoinflammatory diseases. Different classification criteria have been proposed for AOSD during the last decades, with the Yamaguchi's criteria being the most sensitive and the Fautrel's Criteria the most specific (Yamaguchi et al., 1992; Fautrel et al., 2002). However, Fautrel's set of criteria includes measurement of glycosylated ferritin, which is not routinely available in many health care facilities.

Abbreviations: ANA, anakinra; AOSD, adult onset Still's disease; cDMARDs, conventional disease modifying anti-rheumatic drugs; CI, confidence interval; CRP, C-reactive protein; CT, computed tomography; DAS28-CRP, disease activity score in 28 Joints-C-reactive protein; DRR, drug retention rate; ESR, eritrocyte sedimentation rate; IL, interleukin; IVIGs, intravenous immunoglobulins; MAS, macrophage activation syndrome; MRI, magnetic resonance imaging; NSAIDs, non-steroidal anti-inflammatory drugs; SOJIA, systemic onset juvenile idiopathic arthritis.

Disease severity is often determined by using the Pouchot's score modified by Rau et al. (Pouchot et al., 1991; Rau et al., 2010). This score ranges from 0 to 12 according to the presence or absence of 12 AOSD-related manifestations, each scoring one point.

The pathogenesis of AOSD is already widely unclear with both innate and acquired immunity involved. Nevertheless, based on clinical features and laboratory evidence, this disease has been recently included among polygenic multifactorial autoinflammatory disorders (Hayem, 2009; Rossi-Semerano and Koné-Paut, 2012; Rigante, 2017). In this regard, as for monogenic autoinflammatory diseases, interleukin(IL)-1 blockade has proven to induce a dramatic response in AOSD patients with clinical and laboratory manifestations disappearing within a few days from the start of treatment (Nordström et al., 2008; Giampietro et al., 2013; Cavalli et al., 2015; Ortiz-Sanjuán et al., 2015). On this basis, the IL-1β inhibitor canakinumab has been approved for the treatment of AOSD previously unresponsive to non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. However, most of data on the therapeutic role of IL-1 inhibition in AOSD patients currently relates to the recombinant IL-1 receptor antagonist Anakinra (ANA), associated or not with conventional disease modifying anti-rheumatic drugs (cDMARDs). Sustained and complete efficacy of ANA may also allow a decrease in the frequency of injections until complete withdrawal in some cases (Kong et al., 2010). Nevertheless, beyond the dramatic efficacy reported in most patients, lack and loss of efficacy to ANA have been frequently described (Giampietro et al., 2013; Ortiz-Sanjuán et al., 2015; Rossi-Semerano et al., 2015). Hence, the present study is aimed at assessing the long-term drug retention rate (DRR) of ANA, taking into account the impact of lack and loss of efficacy, adverse events and withdrawal owing to treatment-induced long-term remission. In addition, predictive factors related to lack or loss of efficacy or withdrawal because of long-term remission will be sought among demographic, clinical and laboratory features collected at the start of ANA treatment.

MATERIALS AND METHODS

Patients and Data Collection

Patients enrolled in the present study are almost overlapping with those previously presented in a recent observational study aimed at providing information about efficacy and safety of ANA and Canakinumab when administered in AOSD patients (Colafrancesco et al., 2017).

Demographic, clinical and laboratory data were retrospectively collected from patients suffering from AOSD treated with ANA and attending 18 Italian tertiary Centers. All patients were diagnosed with AOSD according to the Yamaguchi's criteria (Yamaguchi et al., 1992).

Before starting ANA, patients had undergone a careful laboratory and radiologic screening in order to rule out infections, neoplasms, and other rheumatologic disorders. Patients were closely monitored with weekly clinical and laboratory evaluations during the first month of treatment and then every 3 months or in case of clinical need (disease relapse or safety concerns).

The primary aims of the study were: (i) to assess the long-term DRR of ANA; (ii) to identify any demographic or clinical variable capable to predict the lack or the loss of efficacy to ANA treatment; (iii) to search for predictors of treatment withdrawal owing to sustained remission while on ANA administration. Secondary aims of the study were: (i) to identify any impact on the DRR of ANA by the concomitant use of cDMARDs on the DRR of ANA and by the different biologic line of ANA therapy; (ii) to assess any difference on ANA retention rate according with the different type (systemic versus chronic articular) of AOSD; (iii) to evaluate the long-term cumulative risk for loss of ANA efficacy; (iv) to assess variables related with the treatment duration of ANA; (v) to clarify which AOSD manifestations are more frequently persistent in patients suspending ANA because of lack of efficacy.

The primary endpoints were represented by: (i) the Kaplan–Meier survival estimates obtained during a 120-month follow-up period; (ii) clinical and laboratory variables significantly associated with lack of efficacy, loss of efficacy and ANA withdrawal due to long-term AOSD remission at regression analysis. Secondary endpoints were represented by statistically significant differences in the Kaplan–Meier survival curves between patients treated with ANA as first biologic agent and those previously treated with other biologics; patients concomitantly treated with cDMARDs and those undergoing ANA as monotherapy; patients with systemic AOSD and patients presenting chronic articular disease. Additional secondary endpoints were represented by: the estimates of cumulative probability of loss of ANA efficacy at the reverse Kaplan–Meier analysis; the identification of clinical and laboratory AOSD manifestations significantly more frequent at the 3-month assessment (or at the last ANA administration) in subjects experiencing lack of efficacy compared to the other patients; a statistically significant correlation between treatment duration and the following variables recorded at the start of ANA treatment (*baseline*): age at disease onset, disease duration at the start of ANA, Pouchot score, disease activity score in 28 Joints-C reactive protein (DAS28-CRP), serum ferritin level, and physician's global assessment.

The demographic, clinical and laboratory variables collected at *baseline* and at the 3-month assessment were: age at AOSD onset, age at diagnosis, disease duration at the start of ANA treatment, number of tender joints, number of swollen joints, DAS28-CRP, previous cDMARDs, previous biologics, concomitant cDMARDs, concomitant corticosteroids, Pouchot score, serum ferritin level, physician's global assessment of disease activity on a visual analog scale. In addition, the presence or absence of the following clinical and laboratory manifestations were collected: fever, salmon-colored maculopapular skin rash, pleuritis, pneumonia, pericarditis, lymphadenopathy, pharyngodynia, myalgia, arthritis, hepatomegaly, macrophage activation syndrome (MAS), increased liver enzymes levels, leukocytosis, increased erythrocyte sedimentation rate (ESR) and raised CRP. At the last follow-up visit, treatment duration and specific causes leading to ANA withdrawal (primary inefficacy,

secondary inefficacy, long-term AOSD remission, safety issues, and loss of compliance) were recorded.

The study protocol was conformed to the tenets of the Declaration of Helsinki and was approved by the local Ethics Committee of the University of Florence (Reference No. 364-16OCT2013). Informed consent was obtained from each patient.

Definition of Clinical and Laboratory Criteria

The disease was considered "chronic articular" when involvement was prevalently polyarticular with erosive damage and low levels of inflammatory markers. Conversely, AOSD was "systemic" if the patient showed increased inflammatory markers, hyperferritinemia and multi-organ involvement. Disease severity was determined using the modified Pouchot's score as proposed by Pouchot et al. (1991) and Rau et al. (2010). Systemic AOSD was not distinguished into monocyclic and polycyclic course, as this distinction has been found irrelevant with respect to the prognostic stratification (Cush et al., 1987).

Fever was defined by a temperature higher than 39°C. Diagnosis of pleuritis, pericarditis and pneumonia was based on echographic-radiological documentations; similarly, lymphoadenopathy was confirmed by ultrasound and/or computed tomography (CT) scan in at least two different sites. Hepatomegaly was identified by ultrasound, CT scan or magnetic resonance imaging (MRI). With regard to laboratory evaluations, leukocytosis was defined as a white cell count higher than 15,000/mm^3 and hyperferritinemia consisted in a serum ferritin level higher than 3,000 ng/ml; ESR and CRP were considered elevated in accordance to each laboratory reference limit.

A *lack of efficacy* (or *primary inefficacy*) was considered as no satisfactory improvement of clinical manifestations during the first weeks of ANA treatment according to physician's judgment. A *loss of efficacy* (or *secondary inefficacy*) was defined as persistent reappearance of AOSD manifestations leading to ANA withdrawal after a persistent complete response (at least 3 months). Withdrawal of ANA owing to *long-term clinical remission* was based on physician's judgment; however, a completely asymptomatic period of at least 6 months had been observed in all cases discontinuing ANA due to persistent clinical remission. *Follow-up duration* was defined as the time between the start of ANA and time of ANA withdrawal or the last visit while on ANA treatment.

The study was carried out in accordance with the tenets of the Declaration of Helsinki and the recommendations of the local Ethical Committee (AOUS, Siena, Italy) rules.

Statistical Analysis

Descriptive statistics included sample size, percentages, means, and standard deviations. After having assessed normality distribution with Shapiro–Wilk test, pair wise comparisons of quantitative data were performed by using unpaired two-tailed *t*-test or Mann–Whitney two tailed *U*-test, as appropriate; Fisher exact test was used for analyzing categorical variables. Correlation analysis was performed using the Spearman test and the Pearson test, as required. Drug survival rates were analyzed by using the Kaplan–Meier plot with "time 0" corresponding to the start of treatment and the "event" being the discontinuation of therapy. Log-rank (Mantel–Cox) test was used to compare different survival curves. The cumulative risk for loss of efficacy was assessed by reverse Kaplan–Meier plot with "time 0" being the start of ANA and the "event" corresponding to treatment withdrawal due to secondary inefficacy.

Binary regression analysis was performed using the backward stepwise to identify baseline clinical and laboratory features associated with lack of efficacy, secondary inefficacy and long-term remission leading to ANA withdrawal as dependent variables. The demographic, clinical and laboratory data collected at *baseline* as listed above were used as independent variables.

Binary regression analysis aimed at identifying variables related to ANA withdrawal due to long-term remission was performed on patients suffering from active AOSD for at least 12 months in order to exclude subjects with a monocyclic disease course.

Odds ratio (OR), its statistical significance and corresponding 95% CI were evaluated for independent variables significantly associated with dependent variables at regression analysis.

The SPSS software, version 24, was used for all statistical computations, always considering a significance level of 95% (p-value < 0.05).

RESULTS

One hundred and forty-one patients (48 males, 93 females) treated with ANA because of AOSD were enrolled in the study. Their demographic and clinical data are summarized in **Table 1**, while **Table 2** provides information about previous and concomitant treatments.

ANA was administered for a mean period of 35.96 ± 36.05 months (median period of 23 months). Withdrawal of ANA was observed in 20 patients (14.2%) because of long-term treatment-induced remission, 16 cases (11.3%) due to primary inefficacy and in 11 (7.8%) cases because of secondary inefficacy. Other 25 (17.7%) patients discontinued ANA because of side effects, as reported in **Table 3**.

Seventeen out of 20 patients suspending ANA due to long-term remission had suffered from active AOSD for at least 12 months. These patients were treated with ANA for a mean period of 35.6 ± 35.4 months. All but two patients experiencing primary inefficacy continued ANA up to the 3-month follow-up visit; secondary inefficacy was observed after a mean period of 35.8 ± 36.1 months of treatment. **Figure 1** shows the cumulative risk for loss of efficacy over time, which was 3.4% during the first 12 months, 13.5% at the 60-month assessment, and 17.5% after 120 months.

Regarding dosages employed, 128 (90.8%) patients were administered with standard posology (100 mg/day), 4 (2.8%) patients with higher dosages (200 mg/day), and 9 (6.4%) with lower than standard dosages (100 mg every other day or less).

TABLE 1 | Demographic, clinical, and laboratory variables referred to the start of Anakinra in the entire cohort of patients enrolled with AOSD.

Demographic features and disease patterns	
Age at disease onset, years (mean ± SD)	35.3 ± 17.1
Age at diagnosis, years	37.32 ± 16.95
Disease duration before starting ANA, months	50.4 ± 81.6
Systemic disease pattern	105 (74.5%)
Chronic articular pattern	36 (25.5%)
Clinical manifestations, *n* (%)	
Number of tender joints	6.6 ± 6.1
Number of swollen joints	3.0 ± 4.2
DAS28-CRP	4.5 ± 1.5
Pouchot score	5.58 ± 1.92
Body temperature ≥ 39°C	136 (96.5)
Salmon-like skin rash	104 (73.8)
Pleuritis	21 (14.9)
Pericarditis	26 (18.4)
Pneumonia	10 (7.1)
Lymphoadenitis	73 (51.8)
Hepatomegaly	66 (46.8)
Pharingodynia	76 (53.9)
Arthritis	99 (70.2)
MAS during clinical history	12 (8.5)
Altered laboratory markers, n (%)	
Increased ESR	120 (85.1)
Increased CRP	129 (91.5)
Leukocytosis	99 (70.2)
Increased serum ferritin	95 (67.4)
Increase liver enzymes	47 (33.3)

ANA, anakinra; CRP, C-reactive protein; DAS28-CRP, disease activity score in 28 Joints-C-reactive protein; ESR, erythrocyte sedimentation rate; MAS, macrophage activation syndrome; SD, standard deviation.

The overall DRR of ANA was 44.6 and 30.5% at the 60- and 120-month assessments, respectively. After having excluded patients suspending ANA due to long-term remission, the DRR of ANA was 55.2 and 39.5% at the 60- and 120-month assessments, respectively. After excluding also patients discontinuing ANA because of adverse events, the DRR of ANA was 68.2 and 54.6% at the 60- and 120-month evaluations, respectively. These data are illustrated in **Figure 2** as Kaplan–Meier survival curves during a 120-month follow-up period.

As represented in **Figure 3**, no differences were found in the DRR of ANA between patients undergoing their first biologic agent and those previously treated with other biologics (log-rank $p = 0.97$). Similarly, no differences were highlighted in the DRR of ANA between patients treated with IL-1 inhibition as monotherapy and those co-administered with cDMARDs both at baseline (log-rank $p = 0.45$) and at the last follow-up (log-rank $p = 0.28$). These results are graphically represented in **Figure 4**. The lack of statistical significance was maintained when patients suspending ANA due to clinical remission were excluded (log-rank $p = 0.62$ at baseline and log-rank $p = 0.24$ at the last follow-up). No statistically significant differences were identified in the DRR of ANA between the systemic and the chronic articular type (log-rank $p = 0.67$) of AOSD, as also reported in **Figure 5**.

TABLE 2 | Information about treatment approaches preceding and accompanying treatment with Anakinra (ANA).

	Number of patients (%)
Previous treatments	
NSAIDs	97 (68.8)
Corticosteroids	138 (97.9)
cDMARDs	120 (85.1)
Methotrexate	91 (64.5)
Cyclosporine	50 (35.5)
Hydroxychloroquine	30 (21.3)
Colchicine	12 (8.5)
Azathioprine	9 (6.4)
Salazopyrine	8 (5.7)
Leflunomide	5 (3.5)
Gold salts	1 (0.7)
IVIGs	1 (0.7)
Biologic agents	29 (20.6)
Etanercept	20 (14.9)
Infliximab	10 (7.1)
Adalimumab	6 (4.3)
Golimumab	2 (1.4)
Tocilizumab	2 (1.4)
Abatacept	2 (1.4)
Rituximab	2 (1.4)
Certolizumab	1 (0.7)
Concomitant treatments at baseline	
cDMARDs	87 (61.7)
Methotrexate	63 (44.7)
Cyclosporine	15 (10.6)
Hydroxychloroquine	12 (8.5)
Colchicine	4 (2.8)
Leflunomide	2 (1.4)
Salazopyrine	2 (1.4)
Corticosteroids at the last follow-up	58 (41.1)
cDMARDs at the last follow-up	72 (51.1)

cDMARDs, conventional disease modifying anti-rheumatic drugs; IVIGs, intravenous immunoglobulins; NSAIDs, non-steroidal anti-inflammatory drugs.

TABLE 3 | Adverse events inducing Anakinra withdrawal during the whole follow-up period.

Adverse events	Frequency, n (%)
In-site reactions	10 (7.1)
Generalized urticarial rash	6 (6.3)
Leukopenia/decreased platelet count	2 (1.4)
Angioedema	2 (1.4)
Macrophage activation syndrome	2 (1.4)
Infections	1 (0.7)
Lymphoproliferative disorders	1 (0.7)
Eosinophilia	1 (0.7)

No significant correlations were identified between treatment duration and age at disease onset ($\rho = -0.11$, $p = 0.24$), disease duration at the start of ANA ($\rho = 0.10$, $p = 0.31$), baseline Pouchot score ($\rho = 0.03$, $p = 0.77$), DAS28-CRP ($\rho = 0.06$, $p = 0.56$),

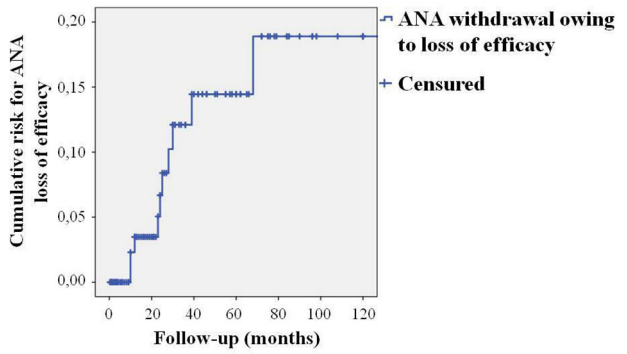

FIGURE 1 | Reverse Kaplan–Meier survival curve showing the cumulative risk for loss of efficacy to Anakinra treatment over time. The "time 0" is represented by the start of ANA treatment; the "event" corresponds to treatment withdrawal due to secondary inefficacy.

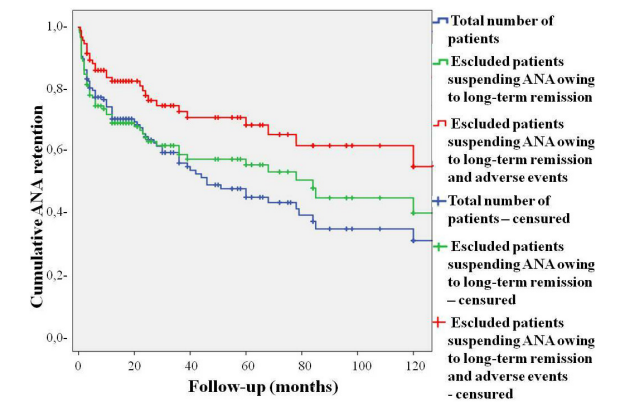

FIGURE 2 | Kaplan–Meier survival curves describing the cumulative retention of Anakinra over the whole follow-up period taking into account all causes of treatment withdrawal (blue line), only adverse events and lack/loss of efficacy (green line), and only lack and loss of efficacy (red line). The "time 0" is represented by the start of ANA treatment; the "event" corresponds to treatment withdrawal.

serum ferritin levels ($\rho = 0.09$, $p = 0.34$), and physician's global assessment ($\rho = -0.008$, $p = 0.94$).

At the binary stepwise regression analysis no variables collected at *baseline* were found to predict primary inefficacy. Conversely, the number of swollen joints at baseline accounted for the only variable capable to predict secondary inefficacy ($p = 0.01$, OR = 1.194, C.I. 1.043–1.367), while neither tender joints nor DAS28-CRP could be included in a predictive model ($p = 0.30$ and $p = 0.29$, respectively).

Binary stepwise regression analysis applied on data collected at *baseline* identified skin rash as the only variable negatively associated with ANA discontinuation due to long-term remission over time ($p = 0.03$, OR = 0.224, C.I. 0.058–0.863).

Among AOSD clinical manifestations, fever ($p = 0.006$), pharyngodynia ($p = 0.02$), tender ($p = 0.02$), and swollen ($p = 0.021$) joints were the clinical manifestations significantly associated with primary inefficacy at the 3-month follow-up visit

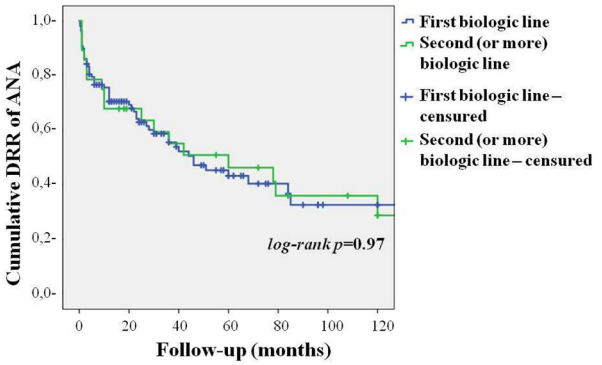

FIGURE 3 | Cumulative DRR of Anakinra in patients with AOSD undergoing their first biologic agent and those previously treated with other biologics.

(or at the last ANA administration). Among laboratory findings, leukocytosis ($p = 0.004$) and increased ESR ($p = 0.006$) observed at the 3-month assessment were significantly associated with primary inefficacy, while CRP ($p = 0.10$) and serum ferritin ($p = 0.14$) did not.

DISCUSSION

Different clinical and basic research have uncovered the key-role of IL-1 in an extended spectrum of immune dysregulatory conditions, and after showing the dramatic success and safety profile of IL-1 inhibitors in the treatment of cryopyrin-associated periodic syndrome, a complex disease spectrum caused by excessive release of the proinflammatory cytokine IL-1 (Cantarini et al., 2011), many clinicians have been encouraged to a wider use of these drugs in other disorders, including AOSD. During the last decade, ANA has proven to induce a rapid and dramatic improvement of all clinical and laboratory AOSD manifestations, resulting in tapering and discontinuation of concomitant therapy with corticosteroids, NSAIDs, and cDMARDs (Lequerré et al., 2008; Nordström et al., 2008; Giampietro et al., 2013; Ortiz-Sanjuán et al., 2015; Colafrancesco et al., 2017). The efficacy of ANA may also allow a decrease in the frequency of injections until complete withdrawal in some cases (Kong et al., 2010; Colafrancesco et al., 2017). However, lack and loss of efficacy are not rare and require a switch to other treatment approaches, including different IL-1 blockers or IL-6 inhibition (Giampietro et al., 2013; Cavalli et al., 2015; Ortiz-Sanjuán et al., 2015; Rossi-Semerano et al., 2015). Therefore, the present study has been designed to identify any baseline predictor of different outcomes to ANA treatment and evaluate the effectiveness of ANA over time by assessing the long-term DRR with regard to the different causes which might affect survival.

A remarkable DRR was identified during a 10-year study period with 30.5% of patients continuing ANA at the 120-month assessment, when reasons for discontinuation were considered as a whole. This percentage increased to 39.5% when considering only lack/loss of efficacy and adverse events, while more than half of patients continued ANA when the sole lack and loss of efficacy

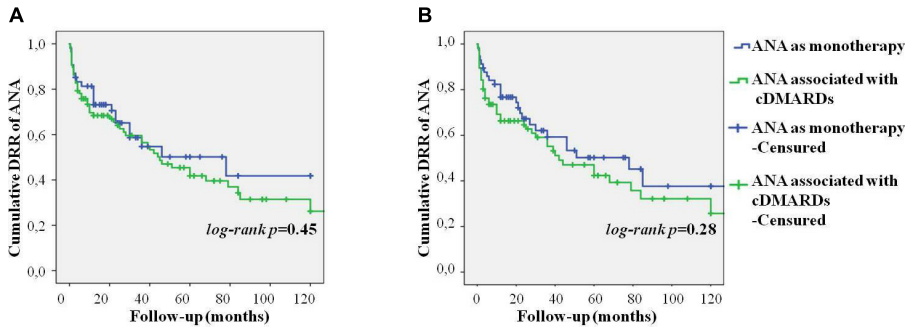

FIGURE 4 | Cumulative DRR of Anakinra in patients with AOSD concomitantly administered with cDMARDs and those treated with IL-1 blockade as monotherapy at the start of ANA treatment **(A)** and at the last follow-up visit **(B)**.

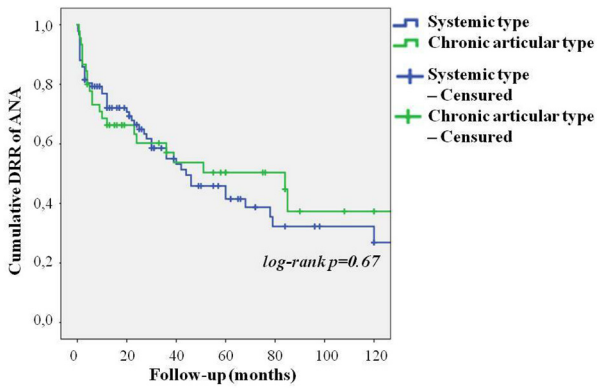

FIGURE 5 | Cumulative DRR of Anakinra in patients with AOSD in both systemic and chronic articular type.

were taken into account. Notably, the risk for loss of efficacy was considerably low with a less than 4% of cumulative risk identified during the first follow-up year. Likewise, the cumulative risk for loss of efficacy increased to only 13.5% during a 5-year period and affected about 1/6 patients during the entire follow-up. As a whole, these results confirm the excellent efficacy of ANA in a higher number of AOSD patients during a substantially long observational period.

In this study the occurrence of adverse events accounts for a leading reason capable to affect the DRR of ANA. However, as also highlighted by Colafrancesco et al. (2017), in our cohort of patients the frequency of adverse events was higher than that reported in previous studies. This could be related to different variables including the longer follow-up period and the relatively low percentage of patients concomitantly treated with cDMARDs. In this regard, Rossi-Semerano et al. (2015) found that background cDMARDs treatment was associated with lower odds of adverse events among patients administered with ANA because of different indications, most of which were represented by AOSD and SOJIA. In addition, the high percentage of MAS cases might be indicative of particular disease severity in our cohort. To the best of our knowledge, this is the first study assessing the DRR of ANA in AOSD patients. However, Woerner et al. (2015) analyzed the DRR of ANA in 51 SOJIA patients undergoing their first biologic agent and in eight cases treated with ANA as second or third biologic option. The estimate of ANA continuation was about 35% at the 100-month assessment in biologic naïve patients when adverse events, ineffectiveness of treatment, loss of response, convenience of use, and patient's choice were included in the statistical computation. Conversely, the DRR of ANA was strikingly lower among the eight patients previously treated with other biologics, running by 33% at 24 months. A further study proposed by Otten et al. (2013) described a 65% retention of ANA at 12-month follow-up in patients previously exposed to the tumor necrosis factor-α inhibitor etanercept. As inferred from **Figure 3**, our findings are in line with the results reported by Otten et al. (2013). Similarly, the long-term DRR of ANA obtained from our AOSD patients is very similar to that reported by Woerner et al. (2015) on biologic naïve SOJIA subjects. Nevertheless, the differences identified in the DRR of ANA between patients undergoing their first biologic agent and those previously treated with other biologics are almost unremarkable in our cohort of patients. This discrepancy could be explained by the higher number of patients enrolled in our study.

Noteworthy, the DRR of ANA was not affected by the concomitant use of cDMARDs suggesting that long-term outcome does not differ between monotherapy and combination treatment. However, although other studies have shown similar results (Nigrovic et al., 2011; Giampietro et al., 2013), to date there is no clear evidence that using concomitant cDMARDs does not influence ANA efficacy in SOJIA or AOSD patients and the need to combine ANA with other immunosuppressive drugs should further be explored.

The stepwise binary regression analysis performed on demographic, clinical and laboratory data collected at the start of treatment did not identify any variable capable to anticipate the lack of ANA efficacy; conversely, the number of swollen joints at baseline was the only variable capable to predict secondary inefficacy, while the presence of the typical salmon-like skin rash represented the sole variable associated with the lack of ANA withdrawal owing to sustained clinical remission. **Figures 6**, **7** show joint involvement and maculopapular skin rash in a patient with AOSD.

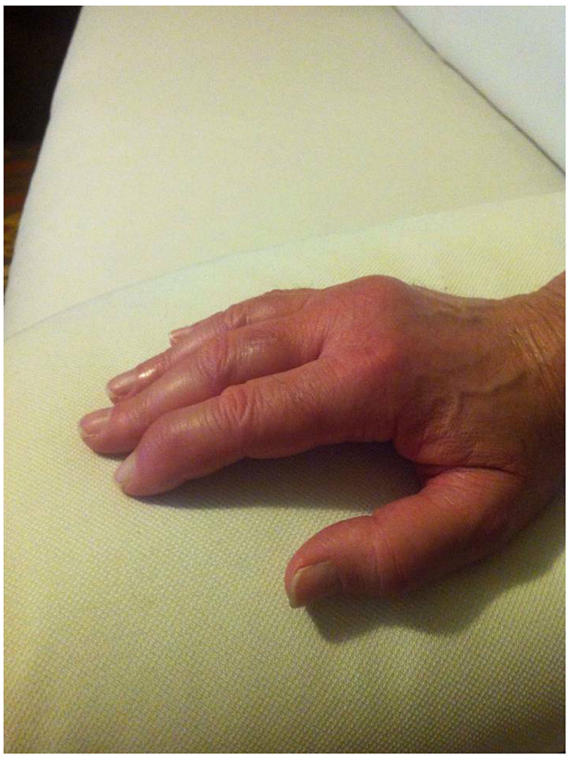

FIGURE 6 | Arthritis involving the right hand in a male patient with AOSD. In particular, the second and third metacarpophalangeal joints, as well as the distal interphalangeal joint of the index finger and the proximal interphalangeal joints of the middle and annular fingers are swollen.

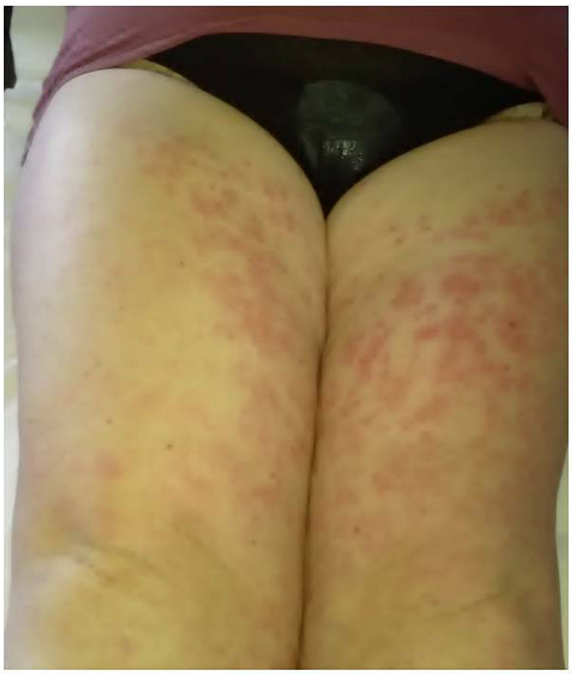

FIGURE 7 | Maculopapular skin rash involving the lower limbs in a female patient suffering from AOSD.

According to published evidences, articular involvement has been frequently described as less responsive than AOSD systemic features (Cavalli et al., 2015; Ortiz-Sanjuán et al., 2015). More in detail, although the frequency of joint symptoms decreases during ANA treatment, articular involvement remains more frequent than other AOSD manifestations (Ortiz-Sanjuán et al., 2015). In addition, articular involvement seems to resolve slowly (within 1 or 2 years), while joint damage could also progress (Lequerré et al., 2008; Lahiri and Teng, 2010; Giampietro et al., 2013). A similar experience has been highlighted also in SOJIA patients: according with Gattorno et al. (2008), subjects with complete response to ANA showed a significantly lower number of active joints compared with patients experiencing incomplete advantage. As a whole, these data seem to corroborate our results on the predictive value of the baseline number of swollen joints on a later loss of efficacy.

Noteworthy, the presence of skin rash at the start of ANA treatment turned out to be a negative predictor of long-term remission leading to ANA withdrawal. This finding could be explained by an even higher IL-1 overproduction in AOSD patients presenting with skin manifestations. In support of this, the persistence of skin lesions has been found to correlate with systemic disease activity, onset of complications, and a more severe prognosis (Fortna et al., 2010; Lee et al., 2012; Yamamoto, 2012; Kikuchi et al., 2014; Sarkar et al., 2014; Cozzi et al., 2016). In this regard, a possible role of the innate immune system of the skin has been suggested in maintaining the degree of systemic inflammation in AOSD (Ruscitti et al., 2016a), while tissue IL-1 levels and other IL-1 family members have proved to be expressed largely in the skin of AOSD patients (Chen et al., 2004; Ruscitti et al., 2016a; Han et al., 2017).

Interestingly, no statistically significant results have been obtained in relationship with AOSD duration before starting ANA treatment. Indeed, according with previous experiences on SOJA patients a shorter time from disease onset to receiving ANA was significantly associated with a favorable response (Nigrovic et al., 2011; Pardeo et al., 2015). On this basis, some authors have speculated about a "window of opportunity" after disease onset during which an early treatment with IL-1 inhibition might have the highest advantage (Nigrovic et al., 2011; Vastert et al., 2014). However, according with our results, disease duration before starting ANA does not predict any outcome and is not correlated with treatment duration in adults with AOSD. Furthermore, no differences were identified in the DRR of ANA on the basis of the different lines of biologic treatment.

Of note, no predictive values were identified neither for the baseline number of tender joints nor for the DAS28-CRP, that is a very useful disease activity score to make an objective, reproducible and comparable evaluation of arthritis activity. Also, no significant differences were identified in the DRR of ANA between AOSD patients suffering from systemic type and those presenting a chronic articular type, while baseline articular involvement did not represent a risk factor for primary inefficacy. On this basis, although the risk for secondary inefficacy increases

along with the number of swollen joints at the start of treatment, the presence of arthritis does not affect the short- and long-term response to ANA. Conversely, both systemic and articular features persisted at ANA withdrawal in the subset of patients experiencing lack of efficacy. In particular, fever, pharyngodynia, tender and swollen joints, leukocytosis and increased ESR proved to be the significantly more frequent in the case of ANA failure compared to other patients at the 3-month assessment (or at the last ANA administration).

When we looked for baseline variables capable to correlate with treatment duration, no significant findings were identified among demographic, laboratory or clinimetric data. In particular, neither the Pouchot score, nor the DAS28-CRP nor the physician's global assessment correlated with the treatment duration. Similarly, no correlations were identified with baseline serum ferritin levels. These findings are of any importance as both the Pouchot score and serum ferritin levels have been correlated with AOSD activity, clinical severity, and prognosis (Efthimiou et al., 2014; Ruscitti et al., 2016b), but do not seem to anticipate the response to ANA treatment.

The main limit of this study is represented by its retrospective nature that prevented to include some interesting quantitative variables for statistical computation. In particular, white cell count, liver enzymes levels, ESR and CRP at baseline were only available as qualitative data (increased/not increased). Consequently, they could not be correlated with treatment duration and were computed as binary information in the stepwise regression analysis. However, all the variables analyzed showed a very low percentage of missing values (<5%) in the data set. In addition, this study is based on a real-life experience and data recorded reflect the everyday management of a wide number of AOSD patients enrolled in 18 different Italian tertiary Centers. This is especially important in reducing recruitment and withdrawal biases, as no defined criteria are currently applicable for starting or stopping ANA in AOSD.

CONCLUSION

The present study highlights an excellent long-term DRR of ANA with no significant differences according with the different line of biologic treatment, concomitant use of cDMARDs, or type of AOSD (systemic versus chronic articular). In addition, while no variables have been found to predict primary inefficacy of ANA, the risk for a loss of efficacy increases along with the number of swollen joints at the start of treatment; similarly, the presence of the typical salmon-like maculopapular skin rash at the start of ANA is the only clinical manifestation negatively associated with sustained remission leading to ANA withdrawal over time.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AV and LC conceived and designed the study and wrote the first draft of the manuscript. AV performed the statistical analysis. All authors have critically reviewed the draft manuscript and approved the submitted version.

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Aminaphtone Efficacy in Primary and Secondary Raynaud's Phenomenon: A Feasibility Study

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Objectives: The aim of this six-month open feasibility study was to evaluate skin blood perfusion and clinical symptom changes during aminaphtone treatment in patients with either primary or secondary Raynaud's phenomenon to systemic sclerosis.

Methods: Ninety-two patients referring for Raynaud's phenomenon have been enrolled in November during routine clinical assessment, after informed consent. Aminaphtone was administered 75 mg twice daily in addition to current treatments to forty-six patients. Skin blood perfusion was measured by Laser Speckle Contrast Analysis (LASCA) at the level of fingertips, periungual areas, dorsum and palm of hands, and face at baseline (W0), after one (W1), four (W4), twelve (W12) and twenty-four (W24) weeks of treatment. Raynaud's condition score (RCS) and both frequency and duration of Raynaud's attacks were assessed at the same time.

Results: Compared with the control group, despite colder period of the year, aminaphtone treated patients showed a progressive statistically significant increase of blood perfusion, as well as a decrease of RCS, frequency of Raynaud's attacks/day and their duration, from W0 to W12 in all skin areas. From W12 to W24 no further increase of blood perfusion was observed. The results were similar in both primary and secondary Raynaud's phenomenon patients. Five weeks after aminaphtone discontinuation blood perfusion values were significantly higher than those at baseline in the majority of skin areas.

Conclusion: This study demonstrates that aminaphtone treatment increases skin blood perfusion and improves Raynaud's phenomenon clinical symptoms, with sustained efficacy up to 6 months, even in patients with systemic sclerosis. A randomized, blind, controlled, clinical trial including a larger number of subjects is advisable to confirm these early results.

Keywords: Raynaud phenomenon, aminaphtone, blood perfusion, systemic sclerosis, laser speckle contrast analysis, microcirculation, clinical symptoms, Raynaud condition score

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INTRODUCTION

Raynaud's phenomenon (RP) is a vasospastic disorder causing discoloration of fingers, toes, and occasionally other areas like nose and tongue, with classic triphasic expression: pallor (ischaemic phase), followed by cyanosis (cyanotic phase) and lastly redness (reactive hyperemic phase) (Herrick, 2012; Hughes and Herrick, 2016; Wigley and Flavahan, 2016). The pathogenesis of

RP is still not entirely clear or understood, but recent insights into the pathogenic mechanisms underlying RP include vascular, neuronal and intravascular abnormalities which may identify crucial key points and potential targets for therapeutic intervention (Herrick, 2012; Hughes and Herrick, 2016; Wigley and Flavahan, 2016).

Raynaud's phenomenon is classified as primary (idiopathic, not associated with any disease), or secondary to several clinical conditions, such as connective tissue diseases, in particular systemic sclerosis (SSc) (Bernero et al., 2013; Park et al., 2015).

Systemic sclerosis is characterized by a deregulation of the vascular tone, in which RP is the most frequent clinical manifestation, associated with structural damage and intimal thickening of the vascular wall, which leads to reduced blood flow and chronic tissue ischemia (Gabrielli et al., 2009; Cutolo et al., 2010b; Kanno et al., 2017). In addition, endothelial cell dysfunction, characterized by an imbalance between vasoconstrictor and vasodilator mediators, is a primary event in the pathogenesis of SSc, followed by fibrosis (Gabrielli et al., 2009; Sulli et al., 2009; Corallo et al., 2016; Hughes and Herrick, 2016).

In the last years, several clinical trials and observational studies on RP have been published, reflecting the increased awareness of the disease burden (Wigley et al., 1994, 1998; Fries et al., 2005; Thompson and Pope, 2005; Gliddon et al., 2007; Cutolo et al., 2014; Corallo et al., 2016). Pharmacological therapies for treatment and prevention of RP include calcium channel blockers, antiplatelet and anticoagulant drugs, endothelin receptor antagonist, phosphodiesterase inhibitors, iloprost, and statins (Wigley et al., 1994, 1998; Fries et al., 2005; Thompson and Pope, 2005; Gliddon et al., 2007; Abou-Raya et al., 2008; Herrick, 2013; Cutolo et al., 2014). However, current treatments for RP have limited efficacy, which was mainly demonstrated by physician/patient reported outcomes (PROs) (Wigley et al., 1994, 1998; Fries et al., 2005; Thompson and Pope, 2005; Gliddon et al., 2007; Abou-Raya et al., 2008).

Aminaphtone is a synthetic derivative of 4-aminobenzoic acid (2-hydroxy-3-methyl-1,4 apthohydroquinone-2-p-aminobenzoatate), which has been used for more than 40 years in some European and South American countries in the treatment of clinical consequences of microvascular impairment (e.g., chronic venous insufficiency of the lower limbs, ulcers of legs, and microangiopathy in diabetes) (De Anna et al., 1989; Pereira de Godoy, 2010; Belczak et al., 2014; Romano et al., 2014; Martinez-Zapata et al., 2016; Felice et al., 2018). Recently, aminaphtone was reported to improve the symptoms associated with RP, as well as to reduce endothelin-1 production on cultured human endothelial cells (Scorza et al., 2008a; Parisi et al., 2015).

Laser speckle contrast analysis (LASCA) is a validated technique that quantifies skin blood perfusion over an area (Ruaro et al., 2014, 2016; Sulli et al., 2014; Lambrecht et al., 2016).

The aim of this longitudinal six-month open feasibility study was to evaluate skin blood perfusion changes by LASCA and RP-related clinical symptoms by PROs during aminaphtone treatment, in patients with either primary RP or secondary RP to SSc.

MATERIALS AND METHODS

Patients

Recruitment of all patients was performed in November 2016, during routine clinical assessment, at the outpatient clinic of the Division of Rheumatology of the University of Genova. Patients were enrolled in November to carry out the study during the six colder months of the year, in order to avoid that seasonal variations of temperature could influence skin blood perfusion assessment and study results. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice, and all patients provided written informed consent.

Forty-six consecutive patients with active RP, asking for treatment during standard clinical assessments, were recruited: 11 primary RP (mean age 49 $\pm$ 19 SD years, mean RP duration 6 $\pm$ 3 years) and 35 secondary RP to SSc according to the ACR/EULAR 2013 criteria (mean age 61 $\pm$ 17 SD years, mean RP duration 11 $\pm$ 9 years) (van den Hoogen et al., 2013; Wigley and Flavahan, 2016).

In patients with secondary RP, SSc duration was determined by onset of first non-Raynaud symptom clearly attributable to SSc (LeRoy et al., 1988; LeRoy and Medsger, 2001; Cutolo et al., 2007b). Furthermore, SSc patients were categorized as having limited (lcSSc) or diffuse cutaneous SSc (dcSSc) according to LeRoy criteria, as well as included into the proper pattern of microangiopathy by nailfold videocapillaroscopy and Cutolo's criteria (13 patients showing the "Early," 13 "Active," and 9 "Late" pattern of microvascular damage) (LeRoy et al., 1988; Cutolo et al., 2000, 2004).

Patients with glucose-6-phosphate-dehydrogenase deficiency or other clinical conditions contraindicating the use of aminaphtone were excluded from the study.

Further 46 patients with RP (10 primary RP, mean age 56 $\pm$ 12 SD years, mean RP duration 8 $\pm$ 4 years; 36 secondary RP to SSc, mean age 63 $\pm$ 11 SD years, mean RP duration 12 $\pm$ 10 years) were also enrolled as a control group (nailfold videocapillaroscopy patterns: 14 "Early," 13 "Active," and 9 Late").

Aminaphtone Treatment and Concomitant Medications

Aminaphtone was administered 75 mg twice daily in addition to current treatments, as our usual clinical practice in RP patients, due to its ability to ameliorate the capillary resistance and permeability and to inhibit erythrocyte aggregation at microcirculation level (as reported inside technical sheet). The posology was in agreement with that reported inside the technical data sheet of the drug.

The exclusion criteria were: treatment with drugs that could potentially influence peripheral blood perfusion (iloprost, calcium channel blockers) and presence of recent digital ulcers requiring bosentan administration.

The inclusion criteria were: patients on stable drug regimen since at least 2 months prior to study entry, no changes made during the follow-up, treatment free period of at least 2 months from prostanoids and endothelin-1 receptor antagonists.

Concomitant treatments in patients treated with aminaphtone were: aspirin (average dosage 100 mg/die, used by 34 patients), proton pump inhibitors (30 patients), antihypertensive drugs (4 patients), cyclosporine (average dosage 150 mg/die, 6 patients), methotrexate (average dosage 10 mg/die, 4 patients). Concomitant treatments in control group were: aspirin (average dosage 100 mg/die, used by 34 patients), proton pump inhibitors (31 patients), antihypertensive drugs (6 patients), cyclosporine (average dosage 150 mg/die, 5 patients), methotrexate (average dosage 10 mg/die, 3 patients).

Evaluation of Skin Blood Perfusion by Laser Speckle Contrast Analysis (LASCA)

Blood perfusion was measured as perfusion units (PU) in all patients by LASCA technique (PeriCam PSI, Perimed, Sweden), as previously reported (Ruaro et al., 2014, 2016; Sulli et al., 2014; Lambrecht et al., 2016), at the level of dorsal and palmar aspect of hands and face, at baseline (W0), after one (W1), four (W4), twelve (W12) and twenty-four (W24) weeks of aminaphtone treatment, and after one (W25) and five (W29) weeks since treatment discontinuation. Raynaud's condition score (RCS) and both frequency and duration of Raynaud's attacks were assessed at the same time (see below). For acclimatization, each patient stayed inside the building for a minimum of 15 min before the blood perfusion was examined, at room temperature of about 23°C.

After image recording, different regions of interest (ROIs) were drawn at the fingertip level, periungual areas, dorsum and palm of hands, tip of noise, and whole face (Sulli et al., 2014; Ruaro et al., 2016). Blood perfusion was measured inside the ROIs (see example in **Figure 1**). The evaluator was blind to both patient treatment and time of visit. For each anatomic area, the average BP was calculated by summing the perfusion values of the two sides, left and right.

All instrumental technical parameters were standardized for all patients and used at follow-up visits.

Clinical Evaluation of Raynaud's Symptoms

The clinical efficacy of aminaphtone on RP symptoms was evaluated at baseline (W0), after one (W1), four (W4), twelve (W12) and twenty-four (W24) weeks of treatment, and after one (W25) and five (W29) weeks since treatment discontinuation. The Raynaud condition score (RCS) that evaluates the limitation of daily activity on a scale from 1 to 10 (10 represents the total inability to do any activity) was used (Pope, 2011; Bose et al., 2015). Furthermore, the frequency (average number of event during the day) and duration (in minutes) of Raynaud's attacks were also recorded.

Nailfold Videocapillaroscopy (NVC)

All patients were assessed by nailfold videocapillaroscope (NVC), equipped with a 200× contact lens, connected to image analysis software (Videocap, DS MediGroup, Milan, Italy). Severity of microangiopathy was detected according to the proper pattern of microvascular damage ("Early," "Active," or "Late"), as previously reported (Cutolo et al., 2000, 2004; Sulli et al., 2008).

Statistical Analysis

Statistical analysis was carried out by non-parametric tests. The Wilcoxon signed-rank test was used to compare paired groups of variables, and Mann–Whitney *U* test to compare unpaired groups of variables. Kruskal–Wallis test was used to compare continuous variables with nominal variables with more than two

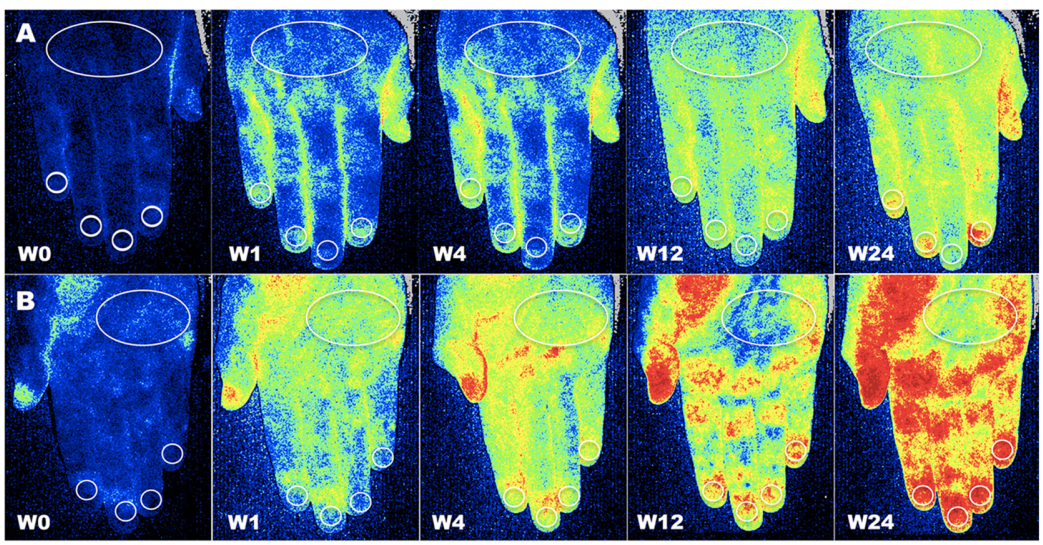

FIGURE 1 | Assessment of blood perfusion. Example of evaluation of blood perfusion by LASCA technique at the level of dorsal **(A)** and palmar **(B)** aspect of hands, respectively, at baseline (W0), after one (W1), four (W4), twelve (W12) and twenty-four (W24) weeks of treatment with aminaphtone in patient F.B. (Blue color = low blood perfusion, yellow color = intermediate blood perfusion, red color = high blood perfusion (White circles = regions of interest for perfusion measuring at the level of dorsum, periungual, palm and fingertip areas).

levels. Friedman test was employed to detect differences across multiple related comparisons. The *p* values lower than 0.05 were considered statistically significant.

RESULTS

Clinical characteristics of enrolled patients are reported in **Table 1**.

Despite colder period of the year, progressive statistically significant increase of blood perfusion was observed from W0 to W12 in all skin areas of RP patients ($p < 0.001$ for all skin areas) (see **Figure 2** and **Table 2A** for perfusion values and statistical significance at single times). From W12 to W24 no further increase of blood perfusion was observed (**Table 2A**). Noteworthy was the fact that all patients on aminaphtone treatment had an increase of blood perfusion from W0 to W1; likewise 38/44 patients from W1 to W4, 36/43 from W4 to W12 and 8/43 patients from W12 to W24 had a further increase of blood perfusion.

A progressive statistically significant decrease of RCS, frequency of Raynaud's attacks/day and their duration was also recorded from W0 to W12 ($p < 0.0001$ for all) (see **Figure 2** and **Table 3A** for clinical values and statistical significance at single times). From W12 to W24 clinical symptoms did not change significantly (**Table 2A**).

TABLE 1 | Clinical findings in patients with Raynaud's phenomenon.

		Total RP patients	Age (years)	Gender Female/Male	Weight (kg) PRP	RP duration (years)		NVC patterns Early/Active/Late	lcSSc/dcSSc
						PRP	SRP-SSc		
AMI	No. of patients	46		40/6		11	35	13/13/9	24/11
	mean ± SD		58 ± 11		65.1 ± 5.9	6 ± 3	11 ± 9		
CNT	No. of patients	46		40/6		10	36	14/13/9	25/11
	mean ± SD		60 ± 11		64.8 ± 6.3	7 ± 4	11 ± 10		

AMI = aminaphtone treated group; CNT = control untreated group; RP = Raynaud's phenomenon; PRP = primary Raynaud's phenomenon; SRP = secondary Raynaud's phenomenon; SSc = systemic sclerosis; NVC = nailfold videocapillaroscopy; Early, Active, Late = patterns of microangiopathy; lcSSc = limited cutaneous SSc; dcSSc = diffuse cutaneous SSc. AMI vs. CNT: p = not significant for all results.

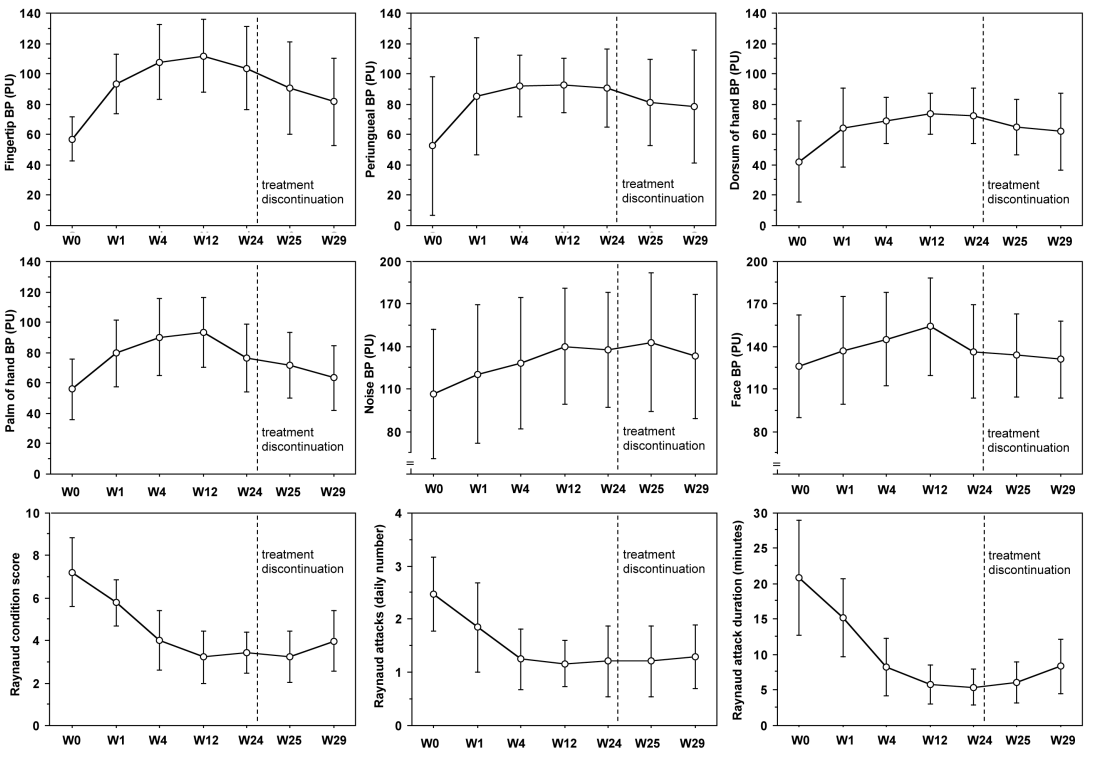

FIGURE 2 | Blood perfusion and clinical trends during aminaphtone treatment. Variation of blood perfusion (BP) in six skin areas from baseline (W0) to twenty-four (W24) weeks during aminaphtone treatment, and after one (W25) and five (W29) weeks since treatment discontinuation, in all patients with Raynaud's phenomenon. Variation of Raynaud Condition Score (RCS), Raynaud's frequency (number of daily attacks) and duration (minutes) are also reported. Charts report means along with standard deviation values (PU = perfusion units). See **Table 2** for statistical significances.

TABLE 2 | Variation of blood perfusion and clinical symptoms in RP patients.

		Fingertip BP PU mean ± SD	Periungueal BP PU mean ± SD	Palm BP PU mean ± SD	Dorsum BP PU mean ± SD	Noise BP PU mean ± SD	Face BP PU mean ± SD	RCS score 0–10 mean ± SD	RP frequency No. of daily attacks mean ± SD	RP duration minutes mean ± SD
A. Cumulative RP patients treated with aminaphtone (46 patients)										
timing	**Week 0**	57.1 ± 14.8	52.4 ± 35.6	56.1 ± 19.9	42.3 ± 26.7	106.5 ± 45.6	126.2 ± 36.1	7.22 ± 1.62	2.48 ± 0.69	20.83 ± 8.14
	Week 1	93.2 ± 19.4	85.0 ± 28.6	79.7 ± 21.9	64.5 ± 25.9	120.6 ± 48.7	137.1 ± 38.0	5.78 ± 1.09	1.85 ± 0.84	15.20 ± 5.52
	Week 4	107.7 ± 24.7	91.9 ± 20.2	90.2 ± 25.2	69.3 ± 15.1	128.4 ± 46.2	145.2 ± 33.2	4.00 ± 1.40	1.25 ± 0.58	8.25 ± 4.04
	Week 12	111.9 ± 24.3	92.3 ± 17.6	93.4 ± 23.0	73.8 ± 13.8	140.2 ± 41.1	154.0 ± 34.7	3.23 ± 1.23	1.16 ± 0.43	5.81 ± 2.79
	Week 24	103.81 ± 27.6	90.6 ± 25.7	76.7 ± 22.3	72.2 ± 18.4	137.5 ± 40.8	136.5 ± 32.9	3.42 ± 0.96	1.21 ± 0.67	5.40 ± 2.56
	Week 25	90.7 ± 30.3	81.2 ± 28.3	71.7 ± 21.9	64.7 ± 18.3	143.0 ± 49.2	134.0 ± 29.4	3.23 ± 1.19	1.21 ± 0.67	6.09 ± 2.98
	Week 29	81.7 ± 28.8	78.2 ± 37.2	63.3 ± 21.1	62.0 ± 25.3	133.1 ± 43.7	130.0 ± 27.4	3.98 ± 1.42	1.30 ± 0.60	8.37 ± 3.86
Statistical significance	W0 vs. W1	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0301$	$p = 0.0142$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W4	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0061$	$p = 0.0009$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W12	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W24	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0016$	$p = 0.0549$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W25	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0012$	$p = 0.0564$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W29	$p < 0.0001$	$p < 0.0001$	$p = 0.0554$	$p < 0.0001$	$p = 0.0068$	p = ns	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W4	$p < 0.0001$	$p < 0.0001$	$p = 0.0016$	$p < 0.0001$	$p = 0.0007$	$p = 0.0007$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W12	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W24	$p = 0.0305$	p = ns	p = ns	$p = 0.0361$	$p = 0.0247$	p = ns	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W25	p = ns	p = ns	p = ns	p = ns	$p = 0.0137$	p = ns	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W29	$p = 0.0032$	p = ns	$p = 0.0010$	p = ns	p = ns	p = ns	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W4 vs. W12	$p = 0.0005$	$p = 0.0188$	$p = 0.0519$	$p = 0.0002$	$p = 0.0031$	$p = 0.0011$	$p < 0.0001$	$p = 0.0441$	$p < 0.0001$
	W4 vs. W24	p = ns	p = ns	$p = 0.0014$	p = ns	p = ns	p = ns	$p = 0.0059$	p = ns	$p < 0.0001$
	W4 vs. W25	$p = 0.0002$	$p = 0.0469$	$p = 0.0005$	p = ns	p = ns	$p = 0.0175$	$p < 0.0001$	p = ns	$p = 0.0004$
	W4 vs. W29	$p < 0.0001$	$p = 0.0439$	$p < 0.0001$	p = ns	p = ns	$p = 0.0062$	p = ns	p = ns	p = ns
	W12 vs. W24	p = ns	p = ns	$p < 0.0001$	p = ns	p = ns	$p = 0.0050$	p = ns	p = ns	p = ns
	W12 vs. W25	$p < 0.0001$	$p = 0.0194$	$p < 0.0001$	$p = 0.0075$	p = ns	$p = 0.0002$	p = ns	p = ns	p = ns
	W12 vs. W29	$p < 0.0001$	$p = 0.0241$	$p < 0.0001$	$p = 0.0079$	p = ns	$p < 0.0001$	$p = 0.0001$	$p = 0.0125$	$p < 0.0001$
	W24 vs. W25	$p < 0.0001$	$p = 0.0030$	p = ns	$p = 0.0123$	p = ns	p = ns	p = ns	p = ns	$p = 0.0548$
	W24 vs. W29	$p < 0.0001$	$p = 0.0217$	$p < 0.0001$	$p = 0.0139$	p = ns	p = ns	$p = 0.0125$	p = ns	$p < 0.0001$
	W25 vs. W29	$p < 0.0001$	p = ns	$p = 0.0020$	p = ns	$p = 0.0465$	p = ns	$p < 0.0001$	p = ns	$p = 0.0002$
B. Cumulative RP untreated patients (controls) (46 patients)										
	Week 0	72.9 ± 16.3	69.3 ± 16.4	62.2 ± 15.0	56.3 ± 12.8	130.9 ± 32.5	138.4 ± 39.1	3.83 ± 1.25	1.35 ± 0.71	6.39 ± 3.99
	Week 24	73.8 ± 19.0	70.0 ± 16.6	62.6 ± 15.3	58.1 ± 15.0	128.3 ± 28.2	138.1 ± 40.0	3.62 ± 1.03	1.33 ± 0.67	6.04 ± 3.44
	W0 vs. W24	p = ns	p = ns	p = ns	p = ns	p = ns	p = ns	p = ns	p = ns	p = ns

A: *Variation of blood perfusion (BP), Raynaud Condition Score (RCS), Raynaud's frequency (number of daily attacks) and duration (minutes) from baseline (W0) to week twenty-four (W24) during aminaphtone treatment, and after one (W25) and five (W29) weeks since treatment discontinuation, in patients with Raynaud's phenomenon (RP).* ***B:*** *Trend of blood perfusion and clinical symptoms in not-treated Raynaud's phenomenon patients. PU = perfusion units. Statistical significance between single times: Wilcoxon test.*

TABLE 3 | Variation of blood perfusion and clinical symptoms splitted by primary or secondary RP.

		Fingertip BP PU mean ± SD	Periungueal BP PU mean ± SD	Palm BP PU mean ± SD	Dorsum BP PU mean ± SD	Noise BP PU mean ± SD	Face BP PU mean ± SD	RCS score 0–10 mean ± SD	RP frequency No. of daily attacks mean ± SD	RP duration minutes mean ± SD
A. Primary RP patients treated with aminaphtone (11 patients)										
Timing	**Week 0**	57.1 ± 12.3	53.0 ± 12.1	53.1 ± 7.9	42.9 ± 10.4	104.4 ± 38.7	128.0 ± 45.0	7.09 ± 1.14	2.27 ± 0.47	19.09 ± 7.01
	Week 1	88.1 ± 16.8	79.3 ± 28.9	75.6 ± 20.2	57.9 ± 21.7	122.0 ± 36.2	131.2 ± 27.2	5.64 ± 0.81	1.64 ± 0.50	16.36 ± 5.05
	Week 4	103.4 ± 24.8	90.7 ± 24.1	96.8 ± 39.0	67.3 ± 17.5	127.8 ± 36.1	145.3 ± 32.7	3.67 ± 1.41	1.11 ± 0.33	8.00 ± 3.00
	Week 12	110.8 ± 20.4	94.2 ± 21.2	106.34 ± 37.7	73.1 ± 14.8	146.6 ± 41.2	156.6 ± 38.2	3.33 ± 1.12	1.12 ± 0.34	6.67 ± 3.16
	Week 24	108.2 ± 32.0	100.8 ± 34.3	70.9 ± 29.1	76.7 ± 23.8	145.0 ± 30.7	129.7 ± 31.0	3.22 ± 1.09	1.13 ± 0.33	6.00 ± 3.00
	Week 25	102.9 ± 48.7	92.7 ± 37.6	78.0 ± 23.27	66.8 ± 19.7	157.7 ± 53.6	137.6 ± 28.7	3.33 ± 1.32	1.12 ± 0.32	7.33 ± 3.16
	Week 29	93.7 ± 52.5	77.2 ± 34.5	61.6 ± 29.4	62.7 ± 18.3	157.6 ± 51.4	131.7 ± 29.3	4.11 ± 2.15	1.22 ± 0.44	8.36 ± 2.65
Statistical significance	W0 vs. W1	$p = 0.0005$	$p = 0.0217$	$p = 0.0012$	$p = 0.0269$	$p = 0.0186$	$p =$ ns	$p = 0.0039$	$p = 0.0019$	$p =$ ns
	W0 vs. W4	$p = 0.0007$	$p = 0.0020$	$p = 0.0057$	$p = 0.0038$	$p = 0.0558$	$p =$ ns	$p < 0.0003$	$p = 0.0006$	$p = 0.0016$
	W0 vs. W12	$p = 0.0002$	$p = 0.0007$	$p = 0.0016$	$p = 0.0008$	$p = 0.0057$	$p = 0.0558$	$p < 0.0001$	$p = 0.0006$	$p = 0.0017$
	W0 vs. W24	$p = 0.0016$	$p = 0.0039$	$p = 0.0155$	$p = 0.0012$	$p = 0.0352$	$p = 0.0569$	$p < 0.0001$	$p = 0.0006$	$p = 0.0006$
	W0 vs. W25	$p = 0.0238$	$p = 0.0333$	$p = 0.0196$	$p = 0.0069$	$p = 0.0465$	$p =$ ns	$p < 0.0001$	$p = 0.0006$	$p = 0.0023$
	W0 vs. W29	$p = 0.0728$	$p =$ ns	$p =$ ns	$p = 0.0098$	$p =$ ns	$p =$ ns	$p = 0.0009$	$p = 0.0006$	$p = 0.0008$
	W1 vs. W4	$p = 0.0124$	$p = 0.0165$	$p = 0.0382$	$p = 0.0020$	$p = 0.0563$	$p = 0.0506$	$p = 0.0175$	$p = 0.0006$	$p = 0.0080$
	W1 vs. W12	$p = 0.0008$	$p = 0.0063$	$p = 0.0326$	$p = 0.0118$	$p = 0.0084$	$p = 0.0138$	$p = 0.0027$	$p = 0.0133$	$p = 0.0071$
	W1 vs. W24	$p = 0.0563$	$p =$ ns	$p =$ ns	$p = 0.0552$	$p = 0.0563$	$p =$ ns	$p = 0.0016$	$p = 0.0133$	$p = 0.0030$
	W1 vs. W25	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p = 0.0382$	$p =$ ns	$p = 0.0046$	$p = 0.0509$	$p = 0.0080$
	W1 vs. W29	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p = 0.0542$	$p =$ ns	$p =$ ns	$p = 0.0353$	$p = 0.0071$
	W4 vs. W12	$p = 0.0040$	$p = 0.0109$	$p = 0.0062$	$p = 0.0558$	$p = 0.0077$	$p = 0.0382$	$p = 0.0533$	$p =$ ns	$p =$ ns
	W4 vs. W24	$p =$ ns	$p =$ ns	$p = 0.0521$	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns
	W4 vs. W25	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns
	W4 vs. W29	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p = 0.0158$	$p =$ ns	$p =$ ns	$p =$ ns
	W12 vs. W24	$p =$ ns	$p =$ ns	$p = 0.0124$	$p =$ ns	$p =$ ns	$p = 0.0548$	$p =$ ns	$p =$ ns	$p =$ ns
	W12 vs.W25	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p = 0.0145$	$p =$ ns	$p =$ ns	$p =$ ns
	W12 vs. W29	$p =$ ns	$p =$ ns	$p = 0.0268$	$p =$ ns	$p =$ ns	$p < 0.0037$	$p =$ ns	$p =$ ns	$p =$ ns
	W24 vs. W25	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns
	W24 vs.W29	$p =$ ns	$p = 0.031$	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p = 0.0353$
	W25 vs. W29	$p = 0.0223$	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns	$p =$ ns

(Continued)

TABLE 3 | Continued

		Fingertip BP PU mean ± SD	Periungueal BP PU mean ± SD	Palm BP PU mean ± SD	Dorsum BP PU mean ± SD	Noise BP PU mean ± SD	Face BP PU mean ± SD	RCS score 0–10 mean ± SD	RP frequency No. of daily attacks mean ± SD	RP duration minutes mean ± SD
B. Secondary RP patients (SSc) treated with aminaphtone (35 patients)										
Timing	**Week 0**	57.1 ± 15.7	52.2 ± 35.0	57.0 ± 22.5	42.1 ± 30.2	107.2 ± 48.1	125.6 ± 33.3	7.3 ± 1.7	2.54 ± 0.74	21.4 ± 8.5
	Week 1	94.8 ± 20.1	86.8 ± 41.3	80.9 ± 22.5	66.6 ± 27.1	120.2 ± 52.5	138.9 ± 40.9	5.8 ± 1.2	1.91 ± 0.92	14.9 ± 5.7
	Week 4	108.8 ± 25.1	92.2 ± 19.5	88.54 ± 20.7	69.8 ± 14.7	128.5 ± 48.9	145.2 ± 33.9	4.1 ± 1.4	1.29 ± 0.62	8.3 ± 4.3
	Week 12	102.6 ± 26.7	91.8 ± 16.8	90.1 ± 16.6	74.0 ± 13.7	138.4 ± 41.4	153.3 ± 34.3	3.2 ± 1.3	1.18 ± 0.46	5.6 ± 2.7
	Week 24	103.81 ± 27.6	87.9 ± 22.8	78.2 ± 20.5	71.0 ± 16.9	135.5 ± 43.2	138.3 ± 33.7	3.5 ± 0.9	1.24 ± 0.74	5.2 ± 2.5
	Week 25	87.5 ± 23.3	78.1 ± 25.1	70.0 ± 21.6	64.1 ± 18.1	139.1 ± 48.0	133.0 ± 29.9	3.2 ± 1.2	1.24 ± 0.74	5.8 ± 2.8
	Week 29	78.5 ± 18.5	78.5 ± 38.3	63.7 ± 18.8	61.8 ± 27.0	127.7 ± 39.7	130.6 ± 27.3	3.9 ± 1.2	1.32 ± 0.64	8.2 ± 4.1
Statistical significance	W0 vs. W1	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0516$	$p = 0.0176$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W4	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0244$	$p = 0.0005$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W12	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0026$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W24	$p < 0.0001$	$p < 0.0001$	$p = 0.0003$	$p < 0.0001$	$p = 0.0137$	$p = 0.0205$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W25	$p < 0.0001$	$p < 0.0001$	$p < 0.0078$	$p = 0.0001$	$p = 0.0096$	$p = 0.0544$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W0 vs. W29	$p < 0.0001$	$p = 0.0002$	p = ns	$p = 0.0003$	$p = 0.0524$	p = ns	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W4	$p < 0.0001$	$p = 0.0005$	$p = 0.0007$	$p = 0.0039$	$p = 0.0037$	$p = 0.0048$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W12	$p < 0.0001$	$p = 0.0024$	$p = 0.0016$	$p = 0.0003$	$p = 0.0005$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W24	$p = 0.0548$	p = ns	p = ns	p = ns	p = ns	p = ns	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W25	p = ns	p = ns	p = ns	p = ns	$p = 0.0526$	p = ns	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W1 vs. W29	$p = 0.0010$	p = ns	$p = 0.0014$	p = ns	p = ns	p = ns	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	W4 vs. W12	p = ns	p = ns	p = ns	$p = 0.0015$	$p = 0.0154$	$p = 0.0011$	$p < 0.0001$	$p = 0.0437$	$p = 0.0002$
	W4 vs. W24	p = ns	p = ns	p = ns	p = ns	p = ns	p = ns	$p = 0.0157$	p = ns	$p = 0.0001$
	W4 vs. W25	$p < 0.0001$	$p = 0.0065$	$p = 0.0003$	p = ns	p = ns	$p = 0.0381$	$p < 0.0001$	p = ns	$p = 0.0007$
	W4 vs. W29	$p < 0.0001$	p = ns	$p < 0.0001$	p = ns	p = ns	$p = 0.0253$	p = ns	p = ns	p = ns
	W12 vs. W24	$p = 0.0196$	p = ns	$p = 0.0010$	p = ns	p = ns	$p = 0.0338$	p = ns	p = ns	p = ns
	W12 vs.W25	$p < 0.0001$	$p = 0.0014$	$p < 0.0001$	$p = 0.0079$	p = ns	$p = 0.0018$	p = ns	p = ns	p = ns
	W12 vs. W29	$p < 0.0001$	$p = 0.0434$	$p < 0.0001$	$p = 0.0224$	p = ns	$p = 0.0014$	$p = 0.0003$	$p = 0.0230$	$p = 0.0003$
	W24 vs. W25	$p < 0.0001$	$p = 0.0002$	$p = 0.0066$	$p = 0.0069$	p = ns	p = ns	p = ns	p = ns	p = ns
	W24 vs.W29	$p < 0.0001$	p = ns	$p < 0.0001$	$p = 0.0395$	p = ns	p = ns	$p = 0.0472$	p = ns	$p = 0.0005$
	W25 vs. W29	$p < 0.0008$	p = ns	$p = 0.0140$	p = ns	$p = 0.0336$	p = ns	$p < 0.0001$	p = ns	$p = 0.0006$

Variation of blood perfusion (BP), Raynaud Condition Score (RCS), Raynaud's frequency (number of daily attacks) and duration (minutes) from baseline (W0) to week twenty-four (W24) during aminaphtone treatment, and after one (W25) and five (W29) weeks since treatment discontinuation, in patients with primary ***(A)*** *or secondary* ***(B)*** *Raynaud's phenomenon (RP). PU = perfusion units. SSc = systemic sclerosis. Statistical significance between single times: Wilcoxson test.*

The results concerning clinical efficacy were similar in both primary and secondary RP patients (see **Tables 3A,B** for further details), as well as in patients with limited or diffuse skin disease, and in patients with "Early," "Active," or "Late" NVC pattern of microangiopathy.

Any statistically significant change as in blood perfusion, as in clinical symptoms (RCS, frequency and duration of Raynaud's attacks), was not observed in the control group of RP patients (either primary or secondary RP) between W0 and W24 (see **Table 2B** for perfusion and clinical values).

One and five weeks after treatment discontinuation, a progressive reduction of skin blood perfusion was recorded (see **Table 2A** for statistical details). However, five weeks after aminaphtone discontinuation blood perfusion values were yet significantly higher than those at baseline in the majority of skin areas (**Table 2A**). Also clinical efficacy was still sustained 5 weeks after treatment discontinuation (**Table 2A**).

Serious adverse events were not observed during the study. Aminaphtone was stopped in two patients due to headache, which recovered one days after treatment discontinuation. One patient was lost during follow-up. Blood cell count, liver aminotransferase, and creatinine values were also routinary assessed every 3 months and no abnormal variation of these parameters was observed.

DISCUSSION

This is the first feasibility study that has evaluated the effects of aminaphtone treatment on both skin blood perfusion and clinical symptoms in patients affected by either primary or secondary RP.

The study demonstrates that aminaphtone treatment increases in short-time skin blood perfusion at the level of hands and face, as well as ameliorates RP clinical symptoms, with a sustained efficacy until 6 months. The results were similar for both primary and secondary RP patients. Of interest, any statistically significant difference was not observed concerning skin blood perfusion and RP clinical improvement between patients with different pattern of nailfold microangiopathy ("early," "active," or "late"), as well as between lcSSc and dcSSc patients, supporting the clinical efficacy of aminaphtone in different subgroups of RP patients (Sulli et al., 2017). Furthermore, skin blood perfusion increased after aminaphtone treatment also at the level of face, which usually shows similar blood perfusion values as in SSc patients as in healthy subjects (Sulli et al., 2014; Ruaro et al., 2016).

This study demonstrates also a progressive statistically significant improvement of the RCS, frequency and duration of RP attacks from baseline to 12 weeks of treatment. Similar results have been highlighted even by other studies where endothelin receptor antagonists or phosphodiesterase 5 inhibitors were administered to patients with secondary RP (Selenko-Gebauer et al., 2006; García de la Peña-Lefebvre et al., 2008; Roustit et al., 2013; Kamata and Minota, 2014; Lee et al., 2014).

Raynaud's phenomenon significantly impacts on quality of life in all subjects. It provokes the deterioration of patient quality of life, not only in terms of pain, but also due to the extreme difficulty in performing normal daily activities. An international survey involving 443 people with self-reported RP showed that 64% had poor ability to control their attacks and only 16% believed that one current medication was effective (Hughes et al., 2015). Treatments were generally considered tolerable but seldom fully effective, and the results confirmed an unmet need for new treatments, as the approach to the management of the disorder was based on published information, expert opinion, and current practices (Hughes et al., 2015).

Current treatments for RP include calcium channel blockers, i.v. prostanoids, and topical glyceryl trinitrate (applied locally to the digits), while key strategic treatment are the increased use of phosphodiesterase type V inhibitors in severe RP (Kowal-Bielecka et al., 2017). Other treatments being researched include botulinum toxin (for severe digital ischemia/ulceration), and several other drugs including oral prostanoids (Shah et al., 2013; Fardoun et al., 2016; Motegi et al., 2016; Żebryk and Puszczewicz, 2016). In view of costs and feasibility, the experts in EULAR propositions recommended that calcium antagonists were first-line therapy in the treatment of secondary RP in SSc, and intravenous prostanoids were recommended when calcium antagonists had failed. As both types of drugs may induce side effects of vascular origin, the experts recommend particular attention if prostanoids are combined with calcium antagonists (Kowal-Bielecka et al., 2009).

Recently, increased availability and interest in nailfold capillaroscopy and laser technologies, by assessing morphological and functional capillary/microcirculatory variations, paves the way for studies on early intervention and vascular protection in RP/SSc patients (Cutolo et al., 2010a,b, 2013, 2014; Rosato et al., 2010; Ruaro et al., 2014).

Our results highlight the effectiveness of aminaphtone in the treatment of RP. An interesting observation, not reported among the results of the study, was that some patients with oedematous/puffy fingers (8 patients complaining of primary RP, and 6 patients affected by secondary RP to SSc; among these 5 with the Early and 1 with the Active pattern of microangiopathy) reported an improvement, until complete resolution of symptoms, during treatment with aminaphtone. This was possibly related to the reduction of the oedematous phase that this molecule has been shown to induce in several studies (De Anna et al., 1989; Martinez-Zapata et al., 2016).

The mechanism of actions of aminaphtone is unclear. Recent studies have reported that aminaphtone reduces vessel permeability and tissue oedema (De Anna et al., 1989; Scorza

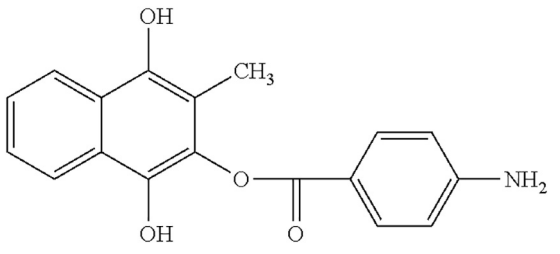

FIGURE 3 | Structure formula of aminaphtone (C18-H15-N-O4).

et al., 2008a,b). Furthermore, results from *in vitro* studies suggest that among different mechanisms of action, aminaphtone may down-regulate the E-selectin (ELAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intra Cellular Adhesion Molecule-1 (ICAM-1) expression, as well as cytokine/chemokine and endothelin-1 production on cultured human endothelial cells (Scorza et al., 2008a,b; Salazar et al., 2016). Of note, endothelin-1 generates vasoconstriction on microvessels and its serum level is increased in both patients with primary and secondary RP (Sulli et al., 2009).

Aminaphtone down-regulates both gene transcription and protein production of almost all the most important molecules responsible for the inflammatory state, including IL-6 (Salazar et al., 2016). It also decreases the levels of TGF-β which can lead to pulmonary fibrosis by activating the fibroblasts to produce an excessive deposition of collagen (Salazar et al., 2016).

Probably all these actions of aminaphtone support the first results of Parisi et al. (2015) who reported the possible effectiveness of aminaphtone in association with standard therapy in RP patients, resulting in a synergic effect on vasospastic phenomena.

As reported inside technical sheet, concerning pharmacokinetic properties, administered to humans aminaphtone is partially metabolized to phthiocol and eliminated through the urine within the 72nd hour. The maximum excretion level was observed to be 6 h after administration. Concerning preclinical safety data, the tests of acute toxicity (4 animal species for doses up to 3 g/kg), subacute toxicity (2 animal species up to 100 mg/kg, for 90 days) and chronic toxicity (50 mg/kg in the dog for 280 days) showed no symptoms of tissue lesions or changes in organ functions. Aminaphtone also had no teratogenic or mutagenic effects. The structure formula of aminaphtone is reported in **Figure 3**.

This study has some limitations. It's not randomized, not blind, and underpowered to detect small treatment effects. However, the high statistical significance of the results despite the small cohort of enrolled patients suggests the possibility to confirm these results by performing larger randomized clinical studies. The two patient groups cannot be completely compared: blood perfusion was assessed only at basal time and after 6 months in the control group, as the decision of including a control group into this pilot study came later, after enrolment completion. In order to assess the trend of blood perfusion during the same months of the year in both patient groups, a control group of patients who performed the first LASCA evaluation in the same month of the aminaphtone-treated group was enrolled. Also the possible effect of aminaphtone on digital ulcer healing/prevention was not assessed in this study, as it was not addressed to this endpoint. In both groups, aminaphtone and control group, only one patient developed a new digital ulcer; however, patients in the control group were showing a less aggressive disease at baseline. Despite this, aminaphtone treatment was found effective in increasing skin BP and ameliorating RP clinical symptoms, while any modification of skin BP was not detected in the untreated group of patients. Another points is the nature of this uncontrolled clinical study that does not provide the sureness that all patients have taken two tablets of aminaphtone per day for six months (the treatment cost is covered by patient in our country): at best of our knowledge, the patients declared adherence to the treatment, but this may not be proven and the eventuality might be the cause of a slight reduction of blood perfusion after week 12 of treatment. Finally, the seasonal variation of temperature should not have influenced the results of the study, as all patients were enrolled in November, and the study carried out during the six colder months of the year.

By considering recent data showing that 5–15% of patients diagnosed as affected by primary RP may shift to secondary RP during follow-up, the possible role of aminaphtone in the prevention of this transition should be longitudinally investigated (Cutolo et al., 2007a; Ingegnoli et al., 2010; Bernero et al., 2013; Trombetta et al., 2016; Gualtierotti et al., 2017).

CONCLUSION

Aminaphtone treatment was well tolerated and improved in short time skin blood perfusion and RP clinical symptoms, with a sustained efficacy until six months. A randomized, blind, controlled, clinical trial including a larger number of subjects is advisable to confirm these early results and to assess the possible role of aminaphtone also in the treatment/prevention of other SSc-related clinical manifestations.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are not publicly available for ethical and privacy reasons, but are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

BR and AS were involved in the conception and design of the study, acquisition of data, basic analysis and interpretation of data, drafting of the manuscript, and revising it critically for important intellectual content. CP, SP, and EA were involved in the acquisition of data, basic analysis and interpretation of data, drafting of the manuscript, and revising it critically for important intellectual content. AS performed the statistical analysis. All authors read and approved the final manuscript.

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BRIEF RESEARCH REPORT
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Innovations in the Assessment of Primary and Secondary Raynaud's Phenomenon

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Objectives: Raynaud's phenomenon (RP) is characterized by intense vasospasm of the digital arteries that causes characteristic color changes in fingers. There are two main types of RP: Primary RP (PRP) and Secondary RP (SRP). PRP is a benign condition. Whilst SRP is associated with several connective tissue diseases (CTD), in particular systemic sclerosis (SSc). The objectives of this report were: to present a short review on morphological (nailfold videocapillaroscopy, NVC) and functional techniques (laser tools and thermography) that allow for a correct diagnosis and treatment of RP and to investigate blood perfusion (BP) by laser speckle contrast analysis (LASCA) in different skin areas of hands and face in PRP, SRP to SSc, and healthy subjects (CNT).

Methods: 31 PRP patients (LeRoy criteria), 70 SRP to SSc (ACR/EULAR criteria) and 68 CNT were enrolled. BP was assessed by LASCA at the level different areas of hands and face. NVC was performed to distinguish between PRP and SRP, and to detect the proper pattern of nailfold microangiopathy in SSc patients.

Results: Both PRP and SRP showed a statistically significant lower BP than CNT at the level of fingertips ($p < 0.0001$), periungual ($p < 0.0001$), palmar aspect of 3rd finger ($p < 0.0001$), and palm areas ($p < 0.0001$). Moreover, BP was significantly lower in PRP than in SRP to SSc with the "Early" pattern of microangiopathy in the same areas as above ($p < 0.04$).

Conclusion: By considering a small cohort of patients, BP of hands was found lower in PRP than in SSc patients with the "Early" NVC pattern of microangiopathy.

Keywords: Raynaud's phenomenon, systemic sclerosis, microvascular damage, nailfold videocapillaroscopy, peripheral blood perfusion, laser techniques

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SHORT REVIEW AND INTRODUCTION

Raynaud's phenomenon (RP), first described in 1862 by Maurice Raynaud (Fardoun et al., 2016; Wigley and Flavahan, 2016), is present in 5–10% of the world's population. It is a clinical consequence of recurrent vasospasm of the small arteries and arterioles of the fingers and toes triggered by cold or even emotional stress (Wigley and Flavahan, 2016), at times also affecting the nose, ears, or lips (Block and Sequeira, 2001;

Herrick, 2005; McMahan and Wigley, 2010; Hughes and Herrick, 2016). The skin usually turns white (ischemia), blue (deoxygenation) and then red (reperfusion) (Block and Sequeira, 2001; Herrick, 2005; McMahan and Wigley, 2010; Hughes and Herrick, 2016).

There are two main categories, i.e., Primary (PRP) and Secondary RP (SRP) and most are PRP that have an isolated finding if there is no underlying pathology (idiopathic). SRP is present in various conditions, like connective tissue diseases (CTD), such as systemic sclerosis (SSc).

Ninety percent of SSc patients have RP which is the most common presenting feature and may precede diagnosis by many years (Block and Sequeira, 2001; Herrick, 2005; McMahan and Wigley, 2010; Hughes and Herrick, 2016). The suggested criteria for PRP include symmetric attacks, the absence of tissue necrosis, ulceration or gangrene, the absence of a secondary cause, negative tests for antinuclear antibodies and a normal erythrocyte sedimentation rate (LeRoy and Medsger, 1992, 2001).

When PRP diagnosis is made no underlying disease has yet been identified, making prediction of if and when it may turn into SRP difficult (Ingegnoli et al., 2010; Avouac et al., 2011; Bernero et al., 2013; Cutolo et al., 2017a). Nailfold video-capillaroscopy (NVC) is able to distinguish SRP from both PRP and healthy subjects by detecting morphological microcirculation abnormalities (Block and Sequeira, 2001; Cutolo et al., 2003; Herrick, 2005; Ingegnoli et al., 2017; Pizzorni et al., 2017b; Herrick and Murray, 2018). Follow-up nailfold capillaroscopic analysis should be performed every 6 months in PRP patients (Cutolo et al., 2003; Bernero et al., 2013).

Nailfold capillaries in PRP are usually normal in shape without any specific alterations (Ingegnoli et al., 2013; Smith et al., 2016a) or abnormal capillaroscopic findings, i.e., giant capillaries and microhemorrhages, whilst their presence is diagnostic for the "Early" NVC pattern of scleroderma microangiography (Cutolo et al., 2003, 2017a; Bernero et al., 2013; Ingegnoli et al., 2013; Smith et al., 2016a).

Indeed, abnormal nailfold capillaroscopic images (more specifically "scleroderma patterns") were included in the 2013 European League Against Rheumatism and American College of Rheumatology classification criteria for SSc to this aim (van den Hoogen et al., 2013).

Digital vasculopathy is structural and functional in SRP due to SSc. NVC cannot measure blood perfusion (BP) under standard conditions (Mugii et al., 2009) but other techniques, like laser and thermography as well as emerging technologies are able to evaluate and quantify skin blood flow and perfusion in SSc (Wigley et al., 1990; Clark et al., 2003; Murray et al., 2009; Rosato et al., 2009, 2011; Cutolo et al., 2010, 2014, 2018b; Pauling et al., 2012a,b, 2015; Della Rossa et al., 2013; Ruaro et al., 2014, 2016, 2017b, 2018c; Sulli et al., 2014; Lambrecht et al., 2016; Wilkinson et al., 2018). Laser Doppler flowmetry (LDF) evaluates blood flow at a single skin point, providing an index of skin perfusion (Cutolo et al., 2010, 2014; Ruaro et al., 2017b, 2018c).

Laser Doppler imaging (LDI) may also be used to evaluate the microcirculatory blood flow (Wigley et al., 1990; Clark et al., 2003; Murray et al., 2009; Rosato et al., 2009, 2011). LDI assesses more than one area and is more effective than a single probe Doppler (Wigley et al., 1990; Clark et al., 2003; Murray et al., 2009; Rosato et al., 2009, 2011). LDI can help to differentiate between PRP and patients with SRP to scleroderma (Wigley et al., 1990; Clark et al., 2003; Murray et al., 2009; Rosato et al., 2009, 2011). Although Murray et al. suggested that combining laser Doppler with other imaging modalities (e.g., nailfold capillaroscopy and thermal imaging) is more effective than laser Doppler alone, these functional imaging tools are not yet widely available (Murray et al., 2009).

Laser speckle contrast analysis (LASCA) can quantify the blood flow over a defined area and is based on the principle that when laser light illuminates a tissue it forms a speckle pattern (Della Rossa et al., 2013; Ruaro et al., 2014; Lambrecht et al., 2016; Cutolo et al., 2018b). Changes in this pattern are analyzed by software and the static areas show a stationary speckle pattern, in contrast with the moving objects like red blood cells that cause the speckle pattern to fluctuate and appear blurred. The level of blurring (contrast) is analyzed and interpreted as BP (Cutolo et al., 2017a; Ingegnoli et al., 2017). LASCA is a fast imaging technique, with a high resolution and reliability, as recently demonstrated in two studies (Lambrecht et al., 2016; Cutolo et al., 2018b).

LASCA has been applied in research studies on RP and SSc (Della Rossa et al., 2013; Ingegnoli et al., 2013, 2017) and one demonstrated that peripheral BP evaluated by both LDF and LASCA correlates to the extent of the microangiopathy (Ruaro et al., 2014).

Laser speckle contrast imaging (LCSI) is similar to LASCA and provides a five-fold increase in spatial resolution over LASCA. However, it is more time consuming (Pauling et al., 2015).

Thermal imaging (TI), an indirect method, makes use of a thermal camera to image the skin temperature to show the underlying blood flow (Clark et al., 2003; Murray et al., 2009; Pauling et al., 2012a,b; Wilkinson et al., 2018). TI evaluated RP in several studies and the response to lower temperatures (cold) was able to differentiate between PRP and SRP to SSc (Murray et al., 2009). However, it has a poor sensitivity in detecting BP variations and has a low spatial resolution (Murray et al., 2009).

Non-invasive assessment of the morphological and functional peripheral circulation may supplement the physical examination and provide a quick, accurately diagnosis, ultimately guiding the correct treatment for both PRP and SRP (Filaci et al., 1999, 2001; Faggioli et al., 2006; Pyrpasopoulou and Aslanidis, 2007; Aschwanden et al., 2008; Caramaschi et al., 2009; Miniati et al., 2009; Shah et al., 2011; Guiducci et al., 2012; Roustit et al., 2012; Cutolo et al., 2013, 2017b; Herrick, 2013, 2017; Cutolo and Sulli, 2015; Gladue et al., 2016; Smith et al., 2016b; Trombetta et al., 2016; Burmester et al., 2017; Kowal-Bielecka et al., 2017; Ruaro et al., 2017a; Rotondo et al., 2018).

Most PRP patients have no serious symptoms and respond well to conservative non-medical treatment like keeping warm and avoiding drugs with vasoconstrictive effects. Whilst other cases require pharmacological treatment like calcium channel blockers as first-line therapy (Herrick, 2013). Although various treatment options are available for the management of SSc-related SRP, these approaches at most reduce the severity of the symptoms but do not resolve the clinical situation (Herrick, 2013;

Cutolo and Sulli, 2015; Gladue et al., 2016; Herrick, 2017; Kowal-Bielecka et al., 2017).

The revised European League Against Rheumatism (EULAR) recommendations for RP in SSc patients (SSc-RP) treatment state that **"*calcium channel blockers should be used as first-line therapy and PDE-5 inhibitors in patients with SSc with severe RP and/or those who do not satisfactorily respond to calcium channel blockers*"** (Kowal-Bielecka et al., 2017). The experts recommended that ***"intravenous prostanoids are considered when oral therapies (including calcium channel blockers and PDE-5 inhibitors) have failed"*** and they also recognize that ***"fluoxetine is a useful alternative for treatment of SSc-RP, in particular in patients with SSc who cannot tolerate or do not respond to vasodilators"*** (Kowal-Bielecka et al., 2017).

As aforementioned, the current therapies for RP are often ineffective. Therefore, the biggest challenge is identifying a drug able to halt RP progression or better still, to prevent the microvascular anomalies which involve tissue hypoperfusion and hypoxia.

That is why an NVC-based assessment of microvascular structure and an evaluation of functional impairment by laser tools and thermography may be useful to assess the efficacy of pharmacological therapies during the treatment of RP patients.

Interestingly, some studies used NVC to detect the microvascular changes as possible markers of response to immunosuppressive/anti-fibrosing treatment and vasoactive drugs (Filaci et al., 1999, 2001; Faggioli et al., 2006; Pyrpasopoulou and Aslanidis, 2007; Caramaschi et al., 2009; Miniati et al., 2009; Shah et al., 2011; Guiducci et al., 2012; Cutolo et al., 2013; Smith et al., 2016b; Trombetta et al., 2016; Ruaro et al., 2017a). Early studies on the effect of Cyclosporin have shown a moderate improvement in clinical symptoms and SSc nailfold microangiopathy, after a 12 month treatment cycle (Filaci et al., 2001; Caramaschi et al., 2009).

Similarly, Cyclophosphamide administration was reported to be significantly associated with an improvement in microvascular damage and a regression of the capillaroscopic pattern severity (Caramaschi et al., 2009).

A recent study showed no progression (therefore a positive disease modifying effect) of the microvascular damage (mainly no further capillary loss) during the 12-month follow-up in patients with early SSc and diffuse skin involvement treated with Rituximab (Smith et al., 2016b).

Recent studies have reported that the use of autologous haemopoietic stem cell transplantation in patients with severe diffuse SSc improved microangiopathy and the NVC pattern changed from "Late" to "Active" (Miniati et al., 2009). Three studies reported an improvement in nailfold microvascularization after iloprost treatment (Faggioli et al., 2006; Pyrpasopoulou and Aslanidis, 2007; Shah et al., 2011; Rotondo et al., 2018). Various studies used NVC with laser techniques to access the drug response in SSc patients treated with a combination of intravenous prostanoids and endothelin-1 receptor blockers, reporting a significant capillary loss reduction (Guiducci et al., 2012; Cutolo et al., 2013, 2014, 2016; Trombetta et al., 2016; Ruaro et al., 2017a).

The objectives of this study were:

(i) to provide a short review in the introduction on morphological (NVC) and functional techniques (laser tools and thermography) that allow for a correct early diagnosis and treatment of primary and PRP;
(ii) to present a pilot study that compares BP measured by LASCA in different skin areas of the hands and face in patients with PRP, SRP to SSc and healthy subjects (CNT).

PATIENTS AND METHODS OF THE PILOT STUDY

Study Population

A total of 31 PRP patients were enrolled after having obtained their written informed consent for the use of imaging and the demographic data as educational material and for publications.

All the PRP patients fulfilled the LeRoy criteria (LeRoy and Medsger, 2001) as did 68 SSc patients, who met the ACR/EULAR 2013 criteria for SSc (van den Hoogen et al., 2013) during routine clinical assessment in our Rheumatology Department, from October, 2016 to Mach, 2017. The study was carried out according to the ethical standard of Good Clinical Practice. A complete medical history was collected and all participants had a clinical examination (**Table 1**).

The inclusion criteria were a diagnosis of PRP or SRP to SSc, and all patients had been on a stable drug regimen for at least 2 months prior study entry.

The exclusion criterion was being on a drug regimen that could potentially influence blood flow.

If the patients were being treated with prostanoids and endothelin-1 receptor antagonists, they were temporarily withdrawn 1 month before instrumental assessment.

All SSc patients were taking aspirin (average dosage 100 mg/day) at the time of the study. Other concomitant treatment included: proton pump inhibitors (used by #52 patients), antihypertensive drugs i.e., angiotensin-converting enzyme (ACE) inhibitors (#9 patients), cyclosporine (average dosage 150 mg/day: #12 patients), methotrexate (average dosage 7.5 mg/week: #12 patients). The PRP therapy treatment was: proton pump inhibitors (used by #8 patients), antihypertensive drugs i.e., ACE inhibitors (#3 patients).

Both LASCA and NVC were performed on the same day in all PRP and SSc patients.

Laser speckle contrast analysis was also performed in the 70 healthy subjects (CNT) matched with the RP patients for age and gender (see **Table 1** for demographic data).

Laser Speckle Contrast Analysis (LASCA)

Skin BP was analyzed by the LASCA technique (Pericam PSI, Perimed, Milan, Italy) at the level of dorsal and palmar aspect of hands and the whole face, in both SSc patients and healthy subjects as previously described (Ruaro et al., 2014, 2016; Sulli et al., 2014). Different regions of interest (ROIs) were created, as previously reported, i.e., at the level

TABLE 1 | Clinical findings in patients with primary Raynaud's phenomenon (PRP), systemic sclerosis (SSc) and healthy subjects (CNT).

	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
	CNT# 70	PRP # 31	SSc # 68	Early # 22	Active # 23	Late # 23	lcSSc # 54	dcSSc # 14
Age (years)	59 (22)	58 (24)	61 (18)	59 (20)	60 (14)	62 (13)	60 (17)	61 (14)
Gender (M/F)	4/66	1/30	3/65	1/21	1/22	1/22	3/51	1/13
Smokinghabit	3/67	2/29	3/65	2/20	1/22	0/23	2/52	1/13
RPduration (years)	NA	2 (1)	10 (8)	7 (6)	8 (7)	14 (12)	12 (8)	9 (8)
SScduration (years)	NA	NA	7 (6)	2 (2)	4 (4)	7 (7)	6 (6)	8 (6)

The SSc study group was also classified according to the microangiopathy patterns and skin involvement (RP = Raynaud's phenomenon; mRSS = modified Rodnan skin score; Early, Active, Late = patterns of microangiopathy at nailfold videocapillaroscopy; lcSSc = limited cutaneous SSc; dcSSc = diffuse cutaneous SSc).

of fingertips, periungual areas, dorsal and palmar aspect of the 3rd finger bilaterally, the dorsum and palm of both hands and face (forehead, tip of nose, zygomas and perioral region) (see **Figure 1** for ROI areas) (Sulli et al., 2014; Ruaro et al., 2016, 2018b).

The average BP from either fingertips or periungual areas was calculated by summing the perfusion values of eight fingers together and then dividing the final value by the number of fingers.

The average BP from the two palmar and dorsal areas of the fingers, palm and dorsum of the hands and zygoma was calculated by summing the perfusion values of the two sides (right and left) and then dividing the final value by two. The BP was quantified as perfusion units (PU; Sulli et al., 2014; Ruaro et al., 2016). The same operator (BR) performed the examination in all PRP, SRP-SSc patients and CNT.

Nailfold Videocapillaroscopy (NVC)

All patients were assessed by nailfold videocapillaroscopy (NVC), (equipped with a 200× contact lens, connected to image analysis software – Videocap, DS MediGroup, Milan, Italy) so as to distinguish PRP from SRP and to determine the correct nailfold microangiopathy pattern ("Early," "Active," or "Late" pattern, according to the Cutolo's criteria) in the SSc patients (Sulli et al., 2008; Smith et al., 2010, 2013; **Table 1**). The same operator (CP) performed the examination in all PRP and SRP-SSc patients and CNT.

Statistical Analysis

The statistical analysis was carried out by parametric procedures and confirmed by non-parametric tests. The Mann-Whitney *U* test was performed to compare unpaired groups of variables, along with the Kruskal-Wallis test to compare continuous variables with nominal variables that had more than two levels.

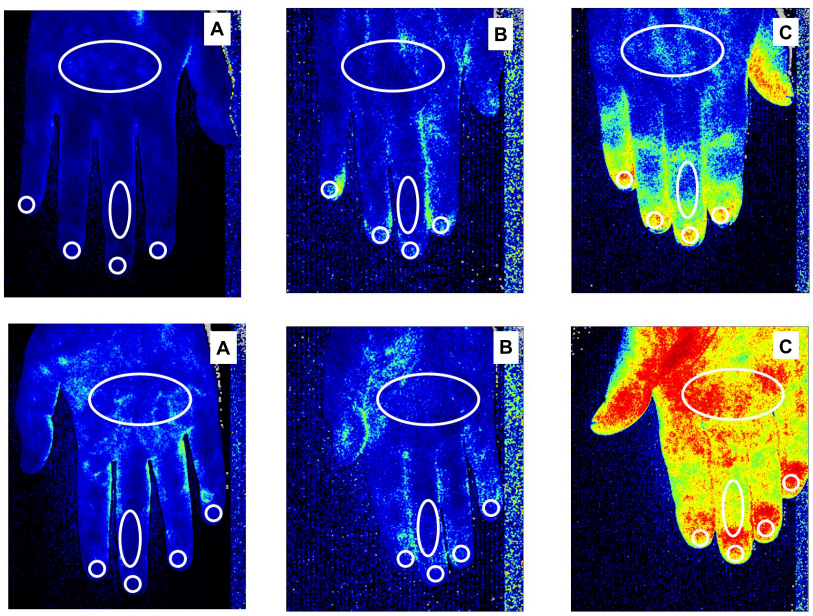

FIGURE 1 | Laser Speckle Contrast Analysis (LASCA) images of secondary Raynaud's phenomenon (RP) to systemic sclerosis, in a patient with a "Late" pattern of scleroderma microangiopathy **(A)**, primary RP **(B)** and a healthy subject **(C)**, showing the regions of interest (ROI - white circles) created at the level of dorsum and palm of the hand, dorsal and palmar aspect of the 3rd finger, periungual areas and fingertips to evaluate blood perfusion. Color code: blue corresponds to a low BP, yellow an intermediate BP and red a higher BP. Noteworthy is the fact that subjects with a late pattern have a prevalence of blue, indicating a low perfusion level.

Any *p*-values below 0.05 were considered statistically significant. The results are given as, median and interquartile range (IQR).

RESULTS

Both PRP and SSc patients had statistically significant lower BP than the healthy subjects at the fingertip ($p < 0.0001$), the periungual area ($p < 0.0001$), the palmar aspect of the 3rd finger ($p < 0.0001$) and the palm areas ($p < 0.0001$). Conversely, all three groups had similar BP values in the other areas of the hand (dorsal aspect of the 3rd finger and dorsum of hand) and face (forehead, tip of nose, zygomas and perioral region). Moreover, BP was statistically significantly lower in PRP than in SSc patients with the "Early" pattern of microangiopathy at fingertip ($p = 0.04$), periungual ($p < 0.05$), palmar aspect of the 3rd finger ($p = 0.0008$) and the palm areas ($p = 0.0009$). No statistically significant difference was observed between PRP and the "Early" pattern of microangiopathy in the other areas evaluated.

A statistically significant progressive decrease in BP was confirmed in SSc patients with a progressive pattern of nailfold microangiopathy ("Early," "Active," and "Late") at the fingertip, periungual, palmar aspect of the 3rd fingers and palm areas ($p < 0.05$). No statistically significant difference was observed between NVC patterns and BP at the level of the other areas (dorsum of hands, whole face and different areas of face) ($p > 0.05$) (**Table 2**).

If the three nailfold microangiopathy patterns ("Early," "Active," and "Late") are evaluated separately, there is a statistically significant difference only between the "Early" and "Late" group, at the level of the fingertip, periungual, palmar aspect of the 3rd fingers and palm areas ($p < 0.05$). No statistically significant difference was observed in the other areas.

There were very few smokers in our study and there was no statistically significant difference in the smoking habit between the groups.

DISCUSSION

Our pilot study shows that the hand BP, evaluated by LASCA, was lower in PRP than in SSc patients with an "Early" NVC microangiopathy pattern.

The results of this study also confirm that SSc patients had a significant lower median BP than healthy subjects and the progressive decrease of BP in SSc patients with different: "Early," "Active," or "Late" NVC pattern of microangiopathy at the level of hand.

Indeed, some authors have reported different perfusion values in PRP and SRP to SSc patients, but the perfusion was evaluated either after, or during, different forms of stress, such as the cold or occlusion test, in contrast with our study where the perfusion was evaluated at basal condition (Pauling et al., 2012a, 2015).

We would like to attest that all the PRP patients had a functional disorder/dysfunction in microvascular circulation and our data emphasize the importance of the perfusion reduction, even in a functional phenomenon such as in PRP patients.

Moreover, our data are in agreement with those of other studies that report NVC as being the best method to evaluate

TABLE 2 | Blood perfusion (BP) in systemic sclerosis (SSc), primary Raynaud's phenomenon (PRP) and healthy subjects (CNT).

	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Statistical significance					
	CNT # 70	PRP # 31	SSc # 68	Early # 22	Active # 23	Late # 22	CNT vs. PRP	CNT vs. SSc	PRP vs. SSc	E vs. A	E vs. L	A vs L
BP fingertips	187 (72)	90 (28)	88 (25)	92 (58)	88 (19)	82 (40)	$p < 0.0001$	$p < 0.0001$	$p = 0.6$	$p = 0.2$	$p = 0.006$	$p = 0.1$
BP palmar aspect of the 3rd phalanx	134 (74)	84 (19)	81 (27)	88 (25)	80 (20)	72 (35)	$p < 0.0001$	$p < 0.0001$	$p = 0.4$	$p = 0.06$	$p = 0.007$	$p = 0.2$
BP palm of hands	114 (27)	81 (22)	79 (31)	85 (22)	83 (31)	68 (39)	$p < 0.0001$	$p < 0.0001$	$p = 0.05$	$p = 0.4$	$p = 0.01$	$p = 0.04$
BP periungual areas	143 (51)	78 (28)	76 (38)	82 (34)	76 (47)	68 (42)	$p < 0.0001$	$p < 0.0001$	$p = 0.7$	$p = 0.1$	$p = 0.02$	$p = 0.3$
BP dorsal aspect of the 3rd phalanx	55 (28)	59 (16)	58 (24)	61 (19)	59 (25)	57 (24)	$p = 0.5$	$p = 0.09$	$p = 0.8$	$p = 0.2$	$p = 0.3$	$p = 0.6$
BP dorsum of hands	51 (27)	50 (13)	52 (18)	56 (22)	50 (19)	49 (16)	$p = 0.4$	$p = 0.07$	$p = 0.9$	$p = 0.1$	$p = 0.1$	$p = 0.8$
BP forehead	109 (44)	113 (32)	110 (33)	112 (21)	110 (29)	111 (31)	$p = 0.1$	$p = 0.09$	$p = 0.3$	$p = 0.2$	$p = 0.3$	$p = 0.8$
BP tip of nose	129 (45)	139 (42)	130 (42)	132 (42)	129 (36)	130 (56)	$p = 0.2$	$p = 0.09$	$p = 0.3$	$p = 0.5$	$p = 0.09$	$p = 0.3$
BP zygoma	127 (48)	155 (45)	145 (58)	150 (45)	145 (55)	143 (83)	$p = 0.4$	$p = 0.1$	$p = 0.2$	$p = 0.3$	$p = 0.2$	$p = 0.1$
BP perioral region	144 (48)	141 (39)	135 (46)	134 (45)	136 (46)	134 (56)	$p = 0.1$	$p = 0.1$	$p = 0.3$	$p = 0.2$	$p = 0.1$	$p = 0.3$
BP whole face	135 (34)	146 (28)	136 (42)	140 (32)	131 (36)	130 (68)	$p = 0.2$	$p = 0.3$	$p = 0.1$	$p = 0.2$	$p = 0.1$	$p = 0.2$

SSc patients with different capillaroscopic patterns of nailfold microangiopathy (Early, Active, Late), evaluated by LASCA in different areas of the hands (fingertips, periungual areas, dorsal and palmar aspect of the 3rd phalanx bilaterally, dorsum and palms of both hands) and face (forehead, tip of nose, zygomas and perioral region). Statistical significance: Mann-Witney U-test (# = numbers, E = Early, A = Active, L = Late). The statistical significance columns show the blood perfusion difference between the CNT (healthy subjects) and the PRP (primary Raynaud's phenomenon) and the systemic sclerosis (SSc) patients. The three capillaroscopic patterns of nailfold microangiopathy (Early, Active, Late), evaluated by LASCA, are also documented.

microcirculation morphological and permanent damage and to make a differential diagnosis between PRP and SRP (Murray et al., 2009; Ingegnoli et al., 2017; Herrick and Murray, 2018).

As previously reported our data confirm that patients with the "Late" SSc microangiopathy pattern had a lower blood flow than those with the "Active" or "Early" SSc patterns at NVC (Ruaro et al., 2014, 2018b). In our precedent article we also reported that when BP was assessed by the LASCA technique significantly lower values were observed in the SSc patients than in the healthy subjects at the level of the fingertips, periungual areas and palm of the hands, with a statistically significant negative correlation between the extent of the nailfold microangiopathy and the BP values at the level of the same skin areas in SSc patients (Ruaro et al., 2014, 2018b).

The increased interest in microcirculation has led to a rapid development of new assessment methods. However, these techniques lack the support of validation studies as to their application in clinical practice. Nevertheless, microvascular structure evaluation by NVC combined with functional investigation by laser techniques or TI, not only helps in the distinction between primary and SRP, but is also able to evaluate therapy response and disease progression (Filaci et al., 2001; Caramaschi et al., 2009; Guiducci et al., 2012; Cutolo et al., 2013, 2014, 2016; Smith et al., 2013, 2016b; Trombetta et al., 2016; Ruaro et al., 2017a, 2018a, Pizzorni et al., 2017a; Soulaidopoulos et al., 2017; Markusse et al., 2017).

In particular, the assessment of the number of capillary changes seems the best validated NVC parameter and is today evaluable with automated systems (Cutolo et al., 2018a).

In summary we are of the opinion that morphological evaluation by NVC is the best method for the early detection and quantification of microvascular abnormalities that characterize SRP. We also believe that clinicians should not underestimate RP which should have a scheduled follow-up as it might well be a precocious *cloaked clinical sign* of abnormal microcirculation and a risk factor for the development of a CTD, especially SSc.

Last but not least, the main message of this work is that while today there is no curative treatment all RP patients, because it is a very heterogeneous phenomenon, still there are many treatment options to improve quality of life of these patients. The early detection of disease and immediate intervention appears to make a difference, such as well-designed clinical trials and collaboration with networks, such as the European Reference Network on Rare and Complex Connective Tissue and Musculoskeletal Diseases Project and specialized centers carrying the research in this field with the aim of defining ideal diagnostic and therapeutic options (Smith et al., 2018).

ETHICS STATEMENT

This study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Ethics approval was obtained from the local Ethical Board and all patients gave written informed consent to enter the study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Association Between Changes in BLyS Levels and the Composition of B and T Cell Compartments in Patients With Refractory Systemic Lupus Erythematosus Treated With Belimumab

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Introduction: Belimumab is a monoclonal antibody against soluble BLyS used for treatment of refractory Systemic Lupus Erythematosus (SLE). Although B cells are the main target of this therapy, a BLyS-dependent T cell activation pathway has also been demonstrated. The aim of the study is to analyze B and T cells phenotype modifications in a cohort of SLE patients treated with belimumab in correlation with serum BLyS levels.

Materials and Methods: Fourteen SLE patients were enrolled in the study. Lymphocyte immunophenotyping by flow cytometry and determination of serum BLyS levels by high sensitivity ELISA were performed before the first infusion of belimumab, after 6 and 12 months of treatment. Sex and age-matched healthy controls were enrolled for the comparisons.

Results: Baseline number of total B cells, especially switched memory B cells, were lower in SLE patients compared to control subjects. After 6 months of treatment, the total number of B cells, particularly, naive and transitional B cells, was significantly reduced in correlation with the reduction of BLyS levels. No significant association was found between baseline counts of B cells and reduction of SLEDAI-2K over time. In terms of response prediction, a significant association between SLEDAI-2K improvement at 12 months and the decrease of total number of B cells within the first 6 months of therapy was observed. Concerning the T cell compartment, the baseline percentage number of CD8+ effector memory was associated with SLEDAI-2K at baseline and with its improvement after 12 months of therapy. Furthermore, T cell lymphopenia and low number of circulating recent thymic emigrants were also observed compared to control subjects measured at baseline.

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Discussion: The effects of belimumab on B cell subpopulations could be explained by the direct blockage of soluble BLyS, while the mild effects on T cells might be explained indirectly by the reduction of disease activity by means of therapy. B cell immunophenotyping during belimumab might be useful for monitoring the response to treatment.

Keywords: B lymphocyte, T lymphocyte, BLyS levels, belimumab, systemic lupus erythematosus

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the production of several autoantibodies. Both innate and adaptive immunity are involved in the pathogenesis of the disease, but recent evidences showed that adaptive immunity has a central role (Mak and Kow, 2014). An unbalance between effector and regulatory T cells (TREG), as well as the involvement of particular subsets of B cells, are described in the development of the disease (Tsokos et al., 2016; Piantoni et al., 2018). SLE patients have a severe defect in the B cell tolerance check, resulting in high numbers of autoreactive mature naïve B cells, which subsequently give rise to autoantibody producing plasma cells (Vossenkämper et al., 2011). The defect most likely occurs at the transitional stage between new bone marrow emigrants and mature naïve B cells in the periphery; hence, an expansion of transitional B cells may be detected in peripheral blood of SLE patients (Vossenkämper et al., 2011).

The mechanisms of selection of transitional B cells in humans are not yet fully understood (Sims et al., 2005); however, a crucial signal for the B cell development through this stage is thought to be delivered by B lymphocyte stimulator (BLyS, also known as BAFF), a member of the tumor necrosis factor superfamily (Mackay et al., 2010).

Belimumab is a human monoclonal antibody against soluble BLyS used in the treatment of SLE. In fact, BLyS overexpression leads to a lupus-like syndrome in mice, and its inhibition delays lupus onset in mouse models of spontaneous SLE (Boneparth and Davidson, 2012). Moreover, BLyS was found in high concentration in sera of SLE patients (Stohl et al., 2003).

A clinical trial study on 13 SLE patients treated with various dosages of belimumab demonstrated a selective depletion of naïve and transitional B cell subpopulations; in contrast, memory B cells and plasma cells were less susceptible to selective BLyS inhibition (Jacobi et al., 2010). Nevertheless, further studies are needed to confirm these findings.

Moreover, the presence of BLyS receptor 3 (also known as BAFF-R) on T cells and the role of a BLyS-dependent T cell activation pathway have been well demonstrated (Ng et al., 2004). Therefore, it may be hypothesized that belimumab could exert its action also through an effect on T cells. However, not much information is available on the effects of belimumab on circulating T cells (Jacobi et al., 2010).

The aim of this study was therefore to analyze the B and T cell phenotype of circulating cells in a cohort of SLE patients treated with belimumab and to correlate them with serum BLyS levels.

MATERIALS AND METHODS

Patients

Fourteen consecutive patients with SLE, classified according to the revised American College of Rheumatology (ACR) criteria (Hochberg, 1997), and treated with belimumab according to common clinical practice were enrolled in this study. A written informed consent was obtained from all the patients. Their main clinical, laboratory and demographic features, obtained from clinical charts, are presented in **Table 1**. All patients participated in a previous study on T cell subsets characterization (Piantoni et al., 2018). SLE Disease Activity Index 2000 (SLEDAI 2K)-score was used to determine disease activity: a score $\geq$6 indicates high disease activity (Romero-Diaz et al., 2011). SLE responder index (SRI) index was used to evaluate the response after 12 months of treatment, as a reduction of $\geq$4 points in SLEDAI-2K, no new BILAG A or >1 new BILAG B, and no deterioration in PGA by $\geq$30 mm (Furie et al., 2009). Fourteen sex and age-matched healthy controls were enrolled for the comparisons. The study was approved by the local institutional ethical committee (approval number 2793) and conducted in accordance with the Declaration of Helsinki.

Methods

Flow Cytometry

Peripheral blood samples from all patients and controls were obtained at the start of the study (baseline = T0) and every 6 months of treatment (6 months = T6 and 12 months = T12). To identify T cell surface markers, 100 μl of whole blood were stained for 30 min at 4°C using monoclonal antibodies conjugated with fluorochromes (Beckman Coulter) and read by flow cytometry (Cytomics NAVIOS, Beckman Coulter Inc., Fullerton, CA, United States), as previously described (Tsokos et al., 2016). Moreover, FITC-CD31, PE-CD25, ECD-CD45RA, PC5-CD4, and PC7-CD127 were used to identify T-cells recent thymic emigrants (RTE; CD31+CD45RA+) among Treg (CD4+CD25+) and other CD4+ T-cell subsets. B-cell subpopulations were evaluated with FITC-IgD, PE-CD38, ECD-CD45, PC5-CD19, PC7-CD27, and APC-CD24, as shown in **Figure 1**. Absolute cells counts was determined by single platform analysis using Flow-Count beads (Beckman Coulter).

ELISA

Serum samples of 10 patients collected every 6 months (T0, T6, and T12) were measured by R&D BAFF ELISA human Duo Set for the determination of BLyS levels, according to manufacturer's guidelines.

Statistical Analysis

Comparisons between healthy controls and patients were made with Mann–Whitney test. The variations between baseline and different time points were evaluated using Wilcoxon signed-rank test or Friedmann ANOVA on ranks. Pearson correlation/linear regression or logarithmic regression were used to evaluate the association between quantitative variables. All relevant associations were confirmed using linear modeling and controlling for lymphocyte numbers throughout. Adjustment for inter-individual patient effects was done by including a random intercept in the linear regression model. Statistical analysis was performed by using the software package StatView (SAS Institute Inc., Cary, NC, United States), SPSS (version 25.0, SPSS, Chicago, IL, United States) and R software package version 3.5.2. [R Core Team (2018), R Foundation for Statistical Computing, Vienna, Austria]. A p-value (p) $\leq$ 0.050 was considered as statistically significant.

TABLE 1 | Demographic, clinical and laboratory features of SLE patients at baseline.

Demographic features	
Women	11 (79%)
Age, years	38 (31–49)
Disease duration, years	13 (8–23)
Caucasian ethnicity	14 (100%)
SLE manifestations *n* (%)	
Cutaneous manifestations (malar rash and/or discoid rash, oral ulcers)	7 (50%)
Articular involvement (arthritis/Jaccoud's arthropathy)	10 (71%)
Renal involvement	9 (64%)
Hematological involvement	7 (50%)
NPSLE	0
Serositis (pulmonary/pericardic effusion)	3 (21%)
Antiphospholipid Syndrome	3 (21%)
SLEDAI 2K-score	6 (3–11)
SDI	1 (0–2)
PGA	2 (1–2)
Laboratory parameters	
Reduced serum levels of C3 and/or C4	11 (79%)
Anti-dsDNA positivity	13 (93%)
aPL positivity *	5 (37%)
Treatment	
Steroids (prednisone)	13 (93%)
Dosage of prednisone (mg/week)	38 (25–135)
Use of hydroxychloroquine at 5 mg/Kg/day	11 (79%)
Use of immunosuppressive drugs **	11 (79%)

*Data are expressed as median (10th–90th percentile), if not otherwise specified. NPSLE, neuropsychiatric systemic lupus erythematosus; SLEDAI-2K score, Systemic Lupus Erythematosus Disease Activity Index 2000; SDI, Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index; PGA, Physician Global Assessment; Anti-dsDNA, anti-double-stranded DNA autoantibody; aCL, anticardiolipin antibodies; Ig, immunoglobulin; Anti-b2GPI, anti-beta2-glycoprotein I antibodies; LA, lupus anticoagulant; nv, normal values. *1 patient with triple, 2 patients with double, and 3 patients with single positivity for aPL. **3 patients were on treatment with mycophenolate mofetil, 2 patients with methotrexate, 2 with azathioprine, 2 with cyclosporine, 1 with leflunomide, and 1 one with both methotrexate and cyclosporine. The immunosuppressive therapy remained the same at T6 and at T12.*

RESULTS

Characterization of Differences in Baseline Composition of B and T Cell Compartments Between SLE Patients and Healthy Controls

A lower absolute count of total circulating CD19+ B cells (p = 0.050; **Table 2**), and in particular, a lower number of CD19+ switched memory cells was observed in patients with SLE at baseline, as compared to healthy controls (p = 0.01, **Table 2**). As previously reported (Piantoni et al., 2018), T cell lymphopenia was observed in patients with SLE, with increased percentages of effector cells (data not shown). Moreover, patients with SLE had lower absolute number of circulating RTE (p = 0.03).

Clinical and Laboratory Features of SLE Patients Treated With Belimumab

One patient moved to another center and was lost during follow-up after 7 months of treatment. During belimumab treatment there was a significant reduction in SLEDAI 2K activity index (**Table 3**). At baseline, eight patients had high disease activity. At T6, 11 patients reached low disease activity, which was maintained at T12 in 10 cases. On the other hand, 2 out of 3 patients with SLEDAI 2K-score $\geq$6 at T6 reached low disease activity at T12. Prednisone dosage was progressively reduced (**Table 3**). Other therapies were unchanged. Serum levels of BLyS significantly decreased over time (**Table 3**). T0-T12 SRI index showed a response to the treatment in 69% of our patients.

Analysis of Changes in the B Cell Compartment in SLE Patients During Follow-Up While on Belimumab Treatment

As shown in **Table 2**, after treatment with belimumab CD19+ B lymphocytes decreased in patients with SLE, both in percentages (T0 vs. T6: p = 0.02; T6 vs. T12: p = 0.02; T0 vs. T12: p = 0.002) and absolute numbers (T0 vs. T6: p = 0.009; T0 vs. T12: p = 0.005).

In particular, there was a decrease of naïve B cells, in percentages (T0 vs. T6: p = 0.002; T0 vs. T12: p = 0.002) and absolute numbers (T0 vs. T6: p = 0.002; T0 vs. T12: p = 0.003), and of transitional B cells absolute number (T0 vs. T6: p = 0.03; T0 vs. T12: p = 0.03). The percentage of switched memory B

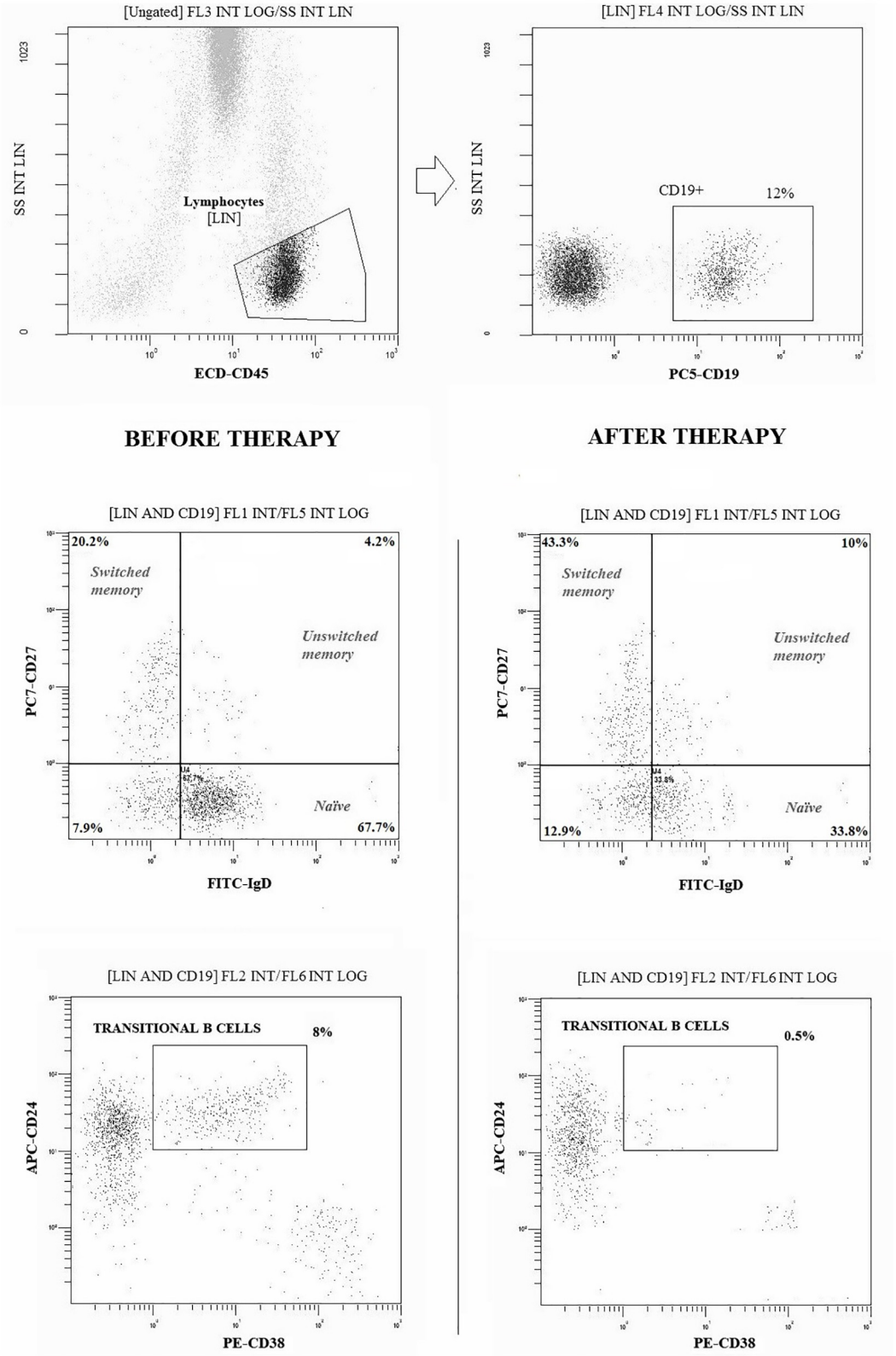

FIGURE 1 | Gating strategies and dot-plot analysis of B (CD19+) cell subpopulations in a representative patient before and after 12 months of therapy with belimumab. Naïve: CD27-IgD+; Switched Memory: CD27+IgD-; Unswitched Memory: CD27+IgD +; Transitional: CD24 high CD38 high. At least 20.000 events were analyzed for each sample.

cells increased (T0 vs. T6: p = 0.004; T0 vs. T12: p = 0.005), but their absolute number did not change. A linear model controlling for lymphocyte numbers confirmed these results (data not shown). A reduction of unswitched memory B cells after 12 month of therapy was shown in initial analysis (T0 vs. T12: p = 0.05), but the effect was not robust after controlling for lymphocytes.

Analysis of Changes in the T Cell Compartment in SLE Patients During Follow-Up While on Belimumab Treatment

Comparing distributions of memory, effector and regulatory T cell subsets before and after therapy with belimumab, we did not observe any differences, except for a reduction of the absolute number of CD8+ cells at T6 only (p = 0.050), especially CD8+ EM cells (T0 vs. T6: p = 0.04; T6 vs. T12: p = 0.050). Furthermore, a reduced percentage of total RTE cells in the CD4+ T cell compartment was noticed following treatment for 12 months (T0 vs. T12: p = 0.01; **Table 2**). After controlling for lymphocytes using linear mixed modeling, we confirmed a reduced percentage of CD4+ RTE and found an additional reduction of absolute number of CD4+ RTE cells after 12 months of therapy (R^2 = 0.478, estimate: −50.98, CI: −90.92 − −11.05, p = 0.02). Furthermore, we find also a reduction of the absolute number of naïve CD4+ T cells after 12 months of therapy (R^2 = 0.640, estimate: −36.84, CI: −71.40 − −2.28, p = 0.048).

Correlation Between Changes in B and T Cell Compartments and BLyS Levels

The relative change of BLyS levels at 6 and 12 months from baseline showed linear correlations with the percentage of naïve B cells (Pearson correlation = 0.645, p = 0.044 and 0.639, p = 0.002, respectively) and transitional B cells (Pearson correlation = 0.768, p = 0.009 and 0.623, p = 0.055, respectively). No correlations were found within the T cell compartment. After the correction for lymphocyte counts, relative change of BLyS was associated as an independent factor with a reduction in the number of naïve B cells (R^2 = 0.605, estimate: 0.02, CI = 0–0.03, p = 0.023).

Correlation Between Changes in the B and T Cell Compartment and Clinical Parameters During Follow-Up

No significant association was found between the baseline values of B cell numbers and the reduction of SLEDAI-2K over time (data not shown). However, at T12 the only 2 patients with high disease activity had higher percentages of transitional B cells and unswitched memory cells than 7 patients in which an initially high SLEDAI-2K decreased below 6 [9% (7–11) vs. 1% (0–4) and 29% (17–41) vs. 6% (4–8), respectively].

TABLE 2 | Comparisons among the number of B- and T-cell subsets (expressed as percentage and absolute number) between patients and healthy controls, and between patients before and after 6 and 12 months of treatment with belimumab.

	HC (n = 14)	T0 (n = 14)	T6 (n = 14)	T12 (n = 13)	p HC vs. patients at T0 (n = 14)	PT0 vs. T6 (n = 14)	p T0 vs. T12 (n = 13)	p T6 vs. T12 (n = 13)
B Cells								
CD19+ (% lymphocytes)	8 (6-9)	8 (3-22)	5 (2-7)	3 (1-5)	0.70	**0.02**	**0.0022**	**0.02**
CD19+ (cell/μl)	153 (120-229)	82 (15-361)	23 (11-57)	19 (6-55)	**0.050**	0.0088	0.0047	0.72
CD19+ SW (% CD19+)	17 (12-24)	16 (5-39)	35 (15-56)	44 (16-61)	0.80	0.0037	0.0046	0.15
CD19+ SW (cell/μl)	28 (19-48)	11 (5-29)	6 (2-20)	4 (2-25)	**0.01**	0.24	0.53	0.79
CD19+ UNSW (% CD19+)	2 (0-8)	5 (0-13)	6 (2-20)	7 (3-12)	0.20	0.27	0.86	0.19
CD19+ UNSW (cell/μl)	3 (2-12)	2 (1-9)	2 (1-6)	1 (0-4)	0.50	0.17	**0.050**	0.24
CD19+ naïve (% CD19+)	62 (40-71)	45 (15-82)	21 (8-43)	19 (8-35)	0.20	**0.0019**	**0.0019**	0.15
CD19+ naïve (cell/μl)	97 (40-155)	36 (4-235)	4 (2-13)	5 (1-10)	0.20	**0.0024**	**0.0029**	0.37
TRANSITIONAL (% CD19+)	1 (0-3)	0.6 (0-8)	0.5 (0-4)	0.5 (0-6)	0.60	0.38	0.63	0.28
TRANSITIONAL (cell/μl)	1 (0-4)	1 (0-9)	0 (0-1)	0.2 (0-1)	0.80	**0.03**	**0.03**	0.33
T cells								
CD4+ (% lymphocytes)	49 (40-65)	43 (30-64)	47 (30-55)	46 (34-58)	0.20	0.50	0.10	0.50
CD4+ (cell/μl)	1131 (716-1370)	365 (97-1002)	326 (170-693)	317 (99-592)	**0.0004**	0.07	0.90	0.50
CD4+ RTE (% CD4+)	18 (9-25)	20 (14-38)	17 (3-35)	10 (3-27)	0.20	0.30	**0.01**	0.07
CD4+ RTE (cell/μl)	240 (70-286)	95 (31-177)	68 (6-106)	30 (18-74)	**0.03**	0.09	0.10	0.30
CD8+ (% lymphocytes)	25 (20-30)	27 (19-43)	32 (18-40)	31 (20-41)	0.40	0.90	0.10	0.70
CD8+ (cell/μl)	516 (241-733)	340 (85-501)	258 (67-414)	254 (86-410)	**0.01**	**0.050**	0.80	0.70
CD8+ EM (% CD8+)	32 (13-45)	26 (15-37)	22 (14-37)	26 (9-51)	0.30	0.60	0.60	0.10
CD8+ EM (cell/μl)	133 (14-286)	61 (24-118)	53 (10-110)	54 (16-174)	0.09	**0.04**	0.50	**0.050**

Data are expressed as median (10th–90th percentile). HC, Healthy Controls; SW, Switched B-cells; UNSW, Unswitched B-cells; EM, Effector memory T-cells; RTE, Recent Thymic Emigrants T-cells. Mann–Whitney test or Wilcoxon signed-rank test was used for the comparisons, when appropriate. In bold p ≤ 0.050.

TABLE 3 | Comparisons of the clinical and laboratory features of SLE patients at different time points.

	T0 (n = 14)	T6 (n = 14)	T12 (n = 13)	Friedman's ANOVA on ranks (p-value)
Disease activity				
SLEDAI 2K-score	7.0(3.7)	3.7(2.2)	3.5(1.2)	**<0.001**
Laboratory parameters				
Serum levels of C3 (mg/dl) (nv = 80–160)	74.4(20.6)	73.1(22.2)	70.4(22.2)	0.417
Serum levels of C4 (mg/dl) (nv = 10–40)	12.1(6.2)	13.3(5.9)	12.3(6.3)	0.804
Serum levels of anti-dsDNA (UI/ml) (nv < 7)	93.4(186.7)	90.6(127.7)	62.0(73.9)	0.629
BLyS levels (pg/ml)*	572.7(571.0)	396.7(344.9)	387.0(335.5)	**0.045**
Treatment				
Dosage of prednisone (mg/week)	56.9(50)	32.2(19.2)	25.2(15.6)	**0.011**

*Data are expressed as mean (standard deviation). SLEDAI-2K score, Systemic Lupus Erythematosus Disease Activity Index 2000; Anti-dsDNA, anti-double-stranded DNA autoantibody; BLyS, B lymphocyte stimulator; nv, normal values; ns, not significant. Friedman's ANOVA on ranks was used for the comparisons. In bold $p \leq 0.050$. *BLyS levels were tested in only 10 patients.*

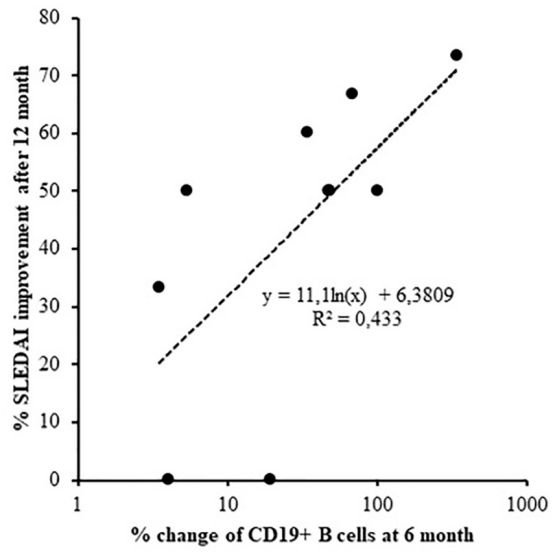

FIGURE 2 | Logarithmic regression between SLEDAI-2K change after 12 months of treatment and the reduction of CD19 cells after 6 months (Log regression, r = 0.658, p = 0.039).

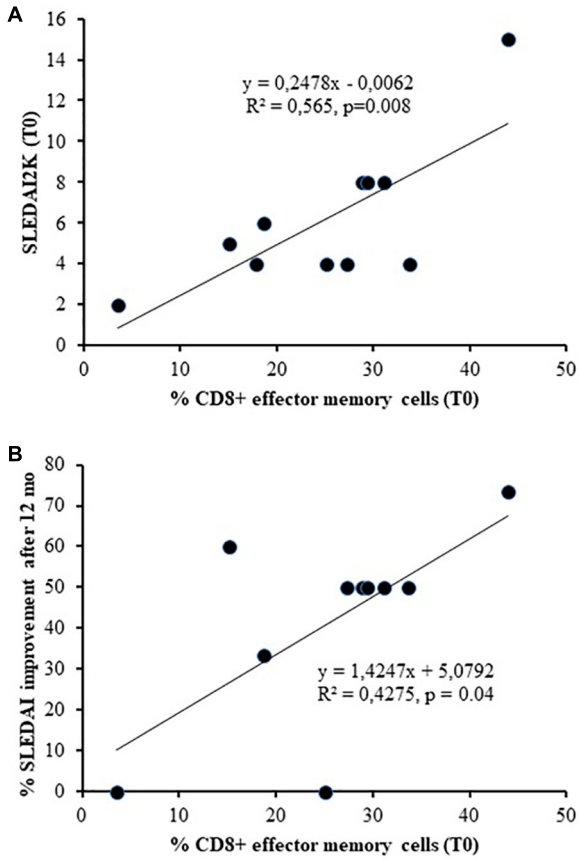

FIGURE 3 | Association between the baseline percentage number of CD8+ Effector Memory and SLEDAI-2K at T0 **(A)** (linear regression, r = 0.752, p = 0.008) and its improvement after 12 months of therapy **(B)** (linear regression, r = 0.654, p = 0.04).

In terms of response prediction, percent of SLEDAI-2K improvement at 12 months was associated with a decrease in total number of B cells within the first 6 months of therapy (Log regression, r = 0.658, p = 0.039; **Figure 2**).

Concerning T cell compartment, the baseline percentage of CD8+ EM was associated with SLEDAI-2K at T0 (linear regression, r = 0.752, p = 0.008) and with its improvement after 12 months of therapy (linear regression, r = 0.654, p = 0.04, **Figure 3**).

Performing logistic regression on SRI response showed no significant associations (data not showed).

DISCUSSION

Since lymphopenia is a common clinical manifestation and one of the classification criteria for SLE (Hochberg, 1997), the low absolute number of B and T cells observed in our patients at baseline were expected. The reduced number of RTE confirms data suggesting a reduced thymic output in patients with active SLE (Vieira et al., 2008), one of the possible causes of T cell lymphopenia.

After treatment with belimumab, we observed a reduction of B cells and of their naïve and transitional subpopulations. Our results confirm, in a real-life scenario, a previous study on 13 patients participating in a clinical trial and treated with various dosages of belimumab (in most cases, lower than in common clinical practice) (Jacobi et al., 2010). In this study the depletion of naïve and transitional B cells was significant after 3 months of therapy, whereas, the reduction of unswitched memory B cells became evident only after 12 months, similarly to our observation. Moreover, we confirm that the inhibition of BLyS did not influence the switched memory cell compartment (Jacobi et al., 2010). A short time initial expansion of memory B cells, which later returned to baseline levels, was demonstrated by more recent studies, without significant variations in anti vaccine antibody titers during time (Stohl et al., 2003; Ramsköld et al., 2018). It may be secondary to their release from germinal centers or to inhibition of their return to lymphoid tissues, or it may be consequent to promotion of differentiation of naïve to memory B cells (Stohl et al., 2003).

B cell survival is regulated by the crosstalk between B cell receptor (BCR) and BLyS-R pathways: BCR cross-link on immature B cells may induce apoptosis, which can be prevented by BLyS, whereas in mature B cells it can stimulate cell activation and growth (Mackay et al., 2010). The selective effects of belimumab on circulating B cells may therefore be explained by the differential expression of various BLyS receptors in different phases of B cell maturation: BLyS-R has a strong selectivity for BLyS and is preferentially expressed on naïve and transitional B cells, whereas BLyS inhibition by belimumab cannot block signaling mediated by APRIL through other receptors such as TACI and BCMA, which are expressed in other phases of B cell ontogeny (Mackay et al., 2010). The correlation between the relative change of BLyS levels and the modifications of the naïve and transitional B cell number in our cohort confirms this evidence. This correlation was shown to be weak as demonstrated by the application of the linear model corrected for lymphocyte counts. This might indicate that the main effect of belimumab on subpopulations could be not via serum BLyS (which we measured) but may be via blocking membrane BLyS. However, the effect on unswitched memory cells is delayed and might be explained by the reduced production of marginal-zone B cells that are expected to be BLyS-dependent (Jacobi et al., 2010).

Since survival of autoreactive B cells depends more on the interaction between BLyS and its receptors than on the stimulation of BCR, which is downregulated in these cells, differently from their non-reactive counterparts, it may be hypothesized that belimumab can promote the deletion of autoreactive B cells, especially in their first stages of maturation (Liu and Davidson, 2011). However, this has not yet been demonstrated in SLE patients, and the effects on autoreactive plasma cells and autoantibodies are modest (Jacobi et al., 2010). The clinical efficacy of belimumab might be, at least in part, independent of autoantibody depletion.

Similarly, no direct correlation of B cell number variations with clinical features or anti-dsDNA was found in our cohort. Only an association between SLEDAI-2K percentage improvement after 12 months of therapy and the decrease of total number of B cells within the first 6 months was found, showing their possible role as predictor of response.

Moreover, patients with high disease activity after 12 months of treatment had higher percentages of transitional and unswitched B cells at T12, suggesting that the number of circulating B cells belonging to these subsets might also be used as a marker of response to belimumab. This was confirmed by a recent study which suggested that evaluation of B cell counts might be useful at the beginning of belimumab treatment and that high baseline B cell counts predicted no response to the treatment (Ramsköld et al., 2018). Future studies are needed to evaluate the possible mechanisms underlining the failure of this therapeutic approach: among other possible explanations, involvement of BLyS-independent mechanisms of B cell survival, the presence of natural BLyS-neutralizing autoantibodies, or the rise of anti-drug antibodies would be interesting to look for.

The possible effect of belimumab on T cells was hypothesized considering that *in vitro* studies of human T cells showed that BLyS can provide a complete costimulatory signal together with anti-T cell receptor (TCR) stimulation (Huard et al., 2004). In the above mentioned study by Jacobi et al. (2010), no variation of T cell counts was observed, but effector memory T cells were not evaluated. Indeed, a reduction of CD8+ EM was the only variation among T cell subpopulations observed in our cohort. Their baseline percentage number was associated with SLEDAI-2K at T0 and with its improvement after 12 months of therapy, suggesting the possible role of CD8+ EM as a useful marker of response to treatment. However, it cannot be excluded that the variation of their number can indirectly be explained by the reduction of disease activity obtained through the therapy. In fact, effector T cells are particularly expanded in patients with high disease activity (Piantoni et al., 2018). *Vice versa*, the lack of RTE defect correction observed in SLE patients treated with belimumab suggests that the reduced thymic output cannot be corrected by reducing disease activity through an action on BLyS.

In conclusion, our results confirm the effects of belimumab on B cell subpopulations. These can be directly explained by the blockage of soluble BLyS interaction with BLyS-R. On the other hand, the effects on the composition of the T cell compartment are mild, which is not totally unexpected, since the intensity of BLyS-R expression by peripheral T cells is low (Ng et al., 2004). The principal limits of this study include the small sample size, presence of concomitant immunosuppressive therapies and low absolute cell number at baseline. Nevertheless, future studies on larger numbers of patients are needed to evaluate whether changes in B cell subsets after therapy initiation could be considered as marker of response to treatment with belimumab in SLE patients.

ETHICS STATEMENT

The study was approved by the institutional ethical committee of Brescia (approval number 2793) and conducted in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

FR, SA, SP, and TL designed, set up, and carried out the experiments. GP, FR, RK, and SP analyzed the data. LA, RR, FF, PA, and AT performed the clinical evaluation of the patients and the collection of the samples. LA, SP, AT, GP, FF, and PA designed and supported the study. All authors checked the final version of the manuscript.

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P2X7 Receptor Expression in Patients With Serositis Related to Systemic Lupus Erythematosus

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Introduction: P2X7R is an extracellular ATP-gated receptor involved in inflammatory and autoimmune processes mainly acting through NLPR3-inflammasome activation and IL-1β release, also implicated in lymphocyte proliferation and cellular apoptosis. Several observations from animal models and patients' studies highlight a possible link between P2X7R-NLRP3 axis and Systemic Lupus Erythematosus (SLE) pathogenesis. The P2X7R-inflammasome axis in addition to the direct production of IL-1β and IL-18, indirectly mediates the release of other cytokines implicated in the pathogenesis of SLE, such as IL-6. The aim of this study was to investigate the role of P2X7R and NLRP3-inflammasome in SLE.

Methods: Forty-eight SLE patients, 16 with (SLE-S) and 32 without (SLE-NS) history of serositis, and 20 healthy control (HC) subjects were enrolled. Demographic, clinical, and therapeutic data were collected. IL-1β and IL-6 plasma levels were evaluated by ELISA. Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by Ficoll gradient sedimentation and employed as follows: (1) evaluation of P2X7R and NLRP3 expression by RT-PCR; (2) determination of P2X7R activity as Benzoyl ATP (BzATP)-induced $[Ca^{2+}]_i$ increments using Fura-2-AM fluorescent probe; (3) isolation of monocytes/macrophages and assessment of *in vitro* IL-1β and IL-6 release following stimulation with lipopolysaccharide (LPS) and BzATP, either separately or in combination.

Results: Plasma IL-1β levels were unmodified in SLE respect to HC whereas IL-6 levels were higher in SLE than in HC, resulting significantly increased in SLE-S. Macrophages isolated from SLE patients released lower quantities of IL-1β after stimulation with BzATP, whereas IL-6 release was significantly augmented in SLE-NS respect to both HC and SLE-S after all types of stimulation. The $[Ca^{2+}]$i increase following BzATP stimulation was significantly lower in PBMCs from SLE patients than in PBMCs from HC. RT-PCR showed significantly reduced P2X7R and significantly augmented NLRP3 expression in PBMCs from SLE patients.

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Conclusion: Our data indicate reduced P2X7R expression and function in SLE patients compared with HC and, conversely, increased IL-6 signaling. The possible consequences of reduced P2X7R, mainly on cytokines network deregulation and lymphocyte proliferation, will be further investigated as well as the role of IL-6 as a possible therapeutic target especially in lupus serositis.

Keywords: P2X7R, Systemic Lupus Erythematosus, NLRP3 inflammasome, IL-1β, IL-6, serositis

INTRODUCTION

With the first reports of Burnstock in 1970s, adenosine triphosphate (ATP) has passed from a simple molecule devoted to energy reserve, to a relevant extracellular signaling molecule able to mediate numerous physiological and pathological processes (Burnstock, 2006).

Under physiological conditions ATP is poorly present in the extracellular space reaching concentrations in the order of nanomolar (nM). On the contrary, in pathological conditions ATP can behave as a danger associated molecular pattern (DAMP) being released from damaged or dying cells or from intact cells following either stimulation or mechanical or oxidative stress (Pellegatti et al., 2005; Burnstock, 2006; Giuliani et al., 2018).

P2X7 receptor (P2X7R) is the most investigated and well defined receptor for extracellular ATP.

P2X7R forms a homo-trimer ion channel allowing the efflux of K^+ and influx of Na^+ and Ca^{2+} after low ATP activation. After prolonged stimulation by high ATP, the receptor forms a pore that allows the passage of hydrophilic solutes of molecular weight up to 900 Da (Virginio et al., 1999). P2X7R expressed in all immune cells including monocytes/macrophages, T and B lymphocytes, dendritic cells (DCs), mast cells and natural killer cells. Its role in NLRP3 inflammasome activation and IL-1β release, is crucial for inflammatory responses (Di Virgilio et al., 2017). P2X7R activation by extracellular ATP provokes the assembly of NLRP3 inflammasome which determines the activation of procaspase-1 to caspase-1, which in turn mediates the cleavage of pro-IL-1β and pro-IL-18 to their active forms (IL-1β and IL-18) subsequently released into the extracellular space (Piccini et al., 2008). Given the powerful IL-1β-mediated inflammatory response involved in many pathological processes (Dinarello, 2009), production and release of this cytokine is strictly controlled first of all by regulation of the inflammasome. The NLRP3 inflammasome is able to directly determine the production of IL-1β and IL-18. With the activation of P2X7R, however, there is also the release of other inflammatory cytokines such as IL-1α, TNF-α, and IL-6, independently of the inflammasome (Gicquel et al., 2015).

In addition, P2X7R is involved in regulation of cell growth and proliferation. In particular, through Ca^{2+} influx P2X7R is able to activate NFAT (nuclear factor of activated T cell) that promotes transcription of the IL-2 gene and consequent T lymphocytes proliferation (Yip et al., 2009). NFAT mediates activation of two relevant transcription factors: the nuclear factor kappa B (NF-κB), involved in both pro-inflammatory cytokines (IL-1β, IL-18, IL-6, IL-8, and TNF-α) production (Liu et al., 2011) and apoptosis, and the hypoxia inducible factor 1α (HIF-1α) released during hypoxia, also activated during neoplasms allowing transcription of multiple genes involved in apoptosis resistance, inflammation, angiogenesis, tumor invasiveness and metastasis (Tafani et al., 2014).

P2X7R therefore, displays a dual role depending on both the degree of activation by ATP (in terms of concentration and duration of stimulation), and the expression of the receptor itself that can vary between different cells. The level of P2X7R expression and the cell type where it is expressed may induce a prevalent pro-proliferative or pro-apoptotic activity (Adinolfi et al., 2012). Thanks to all these characteristics, P2X7R might become an important therapeutic target in inflammatory and neoplastic diseases.

Systemic Lupus Erythematosus (SLE) is traditionally considered the prototype of autoimmune diseases in which adaptive immunity is the main driver characterized by an aberrant activation of auto-reactive B-lymphocytes and consequent production of autoantibodies, and immuno-complexes formation. Recent evidence emphasize a possible role of innate immunity, and in particular of the purinergic system, in the pathogenesis of SLE (Bortoluzzi et al., 2016; Di Virgilio and Giuliani, 2016). Several mouse models (NZB/WF1, MLR/lpr, BXSB/Yaa, and pristane-induced) and some data in humans provided demonstration of the role of innate immunity and especially of P2X7R-NLRP3 inflammasome, in SLE pathogenesis (Turner et al., 2006; Zhao et al., 2013). Innate immunity can use processes of programmed cell death as a form of host defense by limiting the possibility of the infectious agents to replicate. In the case of SLE, there is a loss of control of these processes with an inadequate clearance of cellular and nuclear debris, which can act as DAMPs, perpetuating the activation of the innate immune system with continuous positive feedback. In addition to apoptosis, other forms of dysfunctional cell death are implicated in SLE pathogenesis, such as pyroptosis mediated by inflammasome activation after recognition by sensor proteins (including NOD-like receptor (NLR)), of pathogen associated molecular patterns (PAMPs) and DAMPs. Once activated, caspase-1 promotes cell death through DNA cleavage, nuclear condensation, plasma membrane pore formation and lysosome exocytosis allowing release into the extracellular space of lysosomal enzymes, partially processed pathogens and autoantigens that being processed by the antigen presenting cells (APCs), stimulate the autoimmune response (Bergsbaken et al., 2011; Magna and Pisetsky, 2015). Netosis, another type of cell death firstly associated with neutrophils, causes the extrusion of nuclear DNA, histones and granular entrapped antimicrobial proteins,

called neutrophil extracellular traps (NETs). In SLE, impaired clearance of NETs (like inadequate clearance of apoptotic bodies) is responsible for accumulation of several autoantigens, including self-dsDNA and activation of inflammasome (Villanueva et al., 2011). NETs can also bind another family of pattern recognition receptors (PRRs), the Toll like receptors (TLRs), increasing pro-IL-1β/pro-IL-18 levels and NLRP3 expression (Villanueva et al., 2011). TLR-9 by binding NETs, induces production of type I IFN essential for differentiation of monocytes to DCs that stimulate autoreactive B and T lymphocytes (Lande et al., 2011) and enhance IL-6 and TNF-α release (Torigoe et al., 2018).

SLE can affect any organ system leading to a broad spectrum of clinical manifestation. Serositis is one of lupus related manifestation typically characterized by an increase in acute phase reactants which is a not common occurrence in SLE, unless the concomitant presence of an infectious event. Serositis characterizes other "inflammasome driven" pathologies such as Familial Mediterranean Fever (FMF) and responds promptly to colchicine, a therapy proposed also for the treatment of lupus related serositis.

Colchicine affects several different P2X7R dependent activities through β-tubulin, its primary molecular target, it prevents microtubule polymerization and at high concentrations causes microtubule depolymerization. Colchicine diminishes ATP-induced cationic dye uptake in mouse macrophages, witnessing the requirement of microtubules for P2X7R-dependent pore formation. Moreover, the colchicine interaction with microtubule rearrangement, necessary for IL-1β release, inhibits the ATP-induced P2X7R-dependent release of this cytokine in mouse macrophages (Marques-da-Silva et al., 2011).

The aim of the present study was to explore more deeply the role of the innate immune system in SLE evaluating the P2X7R and NLRP3 expression and activity in SLE patients. Since, serositis is a clinical manifestation associated with inflammatory behavior and one of the most characteristic manifestations of inflammasome-mediated diseases as FMF, the secondary aim was to analyze P2X7R in lupus-related serositis.

Finally, to more extensively investigate the activity of P2X7R, we tested both IL-1β and IL-6, as representative of two different pathways by which this purinergic receptor can mediate inflammatory responses.

MATERIALS AND METHODS

Patients and Study Design

Patients with SLE satisfying the 1997 revised American College of Rheumatology criteria (Hochberg, 1997), were recruited consecutively from the Rheumatology Unit, S. Anna Hospital, University of Ferrara. Clinical (past or ongoing manifestation), serological, demographic characteristics and concomitant therapy were retrospectively recorded. We did a secondary analysis in lupus related serositis. Additional analysis was carried out for the other main clinical manifestations (renal, articular, neurological, and cutaneous) described in the supplementary material. Disease activity and cumulative damage were assessed by SLE disease activity index-2000 (SLEDAI-2K) (Gladman et al., 2002) and the Systemic Lupus International Collaborating Clinics (SLICC) (Gladman et al., 2000), respectively, and recorded in clinical records and dedicated database.

Sero-immunologic tests included: (1) anti-nuclear antibodies (ANA) assessed by indirect immunofluorescence on Hep2 cells (positivity was defined at a titer $\geq$ 1:160); (2) C3 and C4 dosage measured by nephelometry and hypo-complementaemia was defined by local lab reference values (e.g., C3 $<$ 0.8 and C4 $<$ 0.11 g/l detected in at least two separate occasions); (3) anti-DNA antibodies detected by immunofluorescence on *Crithidia luciliae* (positivity was certified if checked in at least two separate measurements, with a cut-off titer of 1:40); (4) anti-SSA, anti-SSB, anti-Sm, and anti-RNP antibodies detected by immunoblot technique (Line blot, Euroimmun); (5) Lupus anticoagulant (LA) measured accordingly with the recommendation of the Scientific and Standardization Committee of the International Society of Thrombosis and Hemostasis, and anti-CL and anti-beta2GPI measured by enzyme linked immunosorbent assay (ELISA) (Harris et al., 1987). If the result was confirmed in two separate measurements performed 12 weeks apart, positivity for anti-PL and LA was assigned (Horbach et al., 1996). Erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) were measured according to procedures of local laboratory.

Healthy subjects (HC; $n = 20$), volunteers from Ferrara Blood Bank, were considered as control group.

We also analyzed an additional small control-group composed of five patients with an history of serositis affected by diseases other than SLE and excluding FMF (one rheumatoid arthritis and serositis ongoing at the time of sample collection, one systemic sclerosis, one dermatomyositis, one inflammatory bowel disease, and one Sjogren's syndrome).

All control subjects and patients underwent venous blood collection in EDTA tubes after giving written informed consent. The study was approved by the local ethics committee and conducted in accordance with the amended Helsinki Declaration.

Separation of Plasma and Peripheral Blood Mononuclear Cells (PBMCs)

Plasma was obtained by centrifugation of blood samples at 1,000 $\times$ *g* for 15 min at 4°C, divided into aliquots and frozen at −80°C until use. The remaining cellular component was used to separate peripheral blood mononuclear cells (PBMCs) by stratification on a Ficoll (Health Care, Uppsala, Sweden) gradient as previously described (Falzoni et al., 1995). PBMCs were divided into three aliquots of 2×10^6 cells each, and used for: (1) setting up short-term cell cultures, (2) measurement of calcium fluxes in fluorimetry, and (3) RNA extraction.

Preparation and Stimulation of PBMCs Short-Term Cultures

Peripheral blood mononuclear cells were suspended at the concentration of 5×10^5 cells/ml of 10% FBS supplemented RPMI (Gibco, Thermo Scientific, Waltham, MA, United States) without antibiotics and placed into 12-well plates for cell culture, using

four wells for each patient. After an overnight incubation at 37°, 5% CO_2, non-adherent cells were removed and the adherent mononuclear cells (90% macrophages, as verified detecting shape modification at optical microscopy) were incubated in 10% FBS-supplemented RPMI under the following conditions: (1) no additions (control) for 5 h; (2) 1 μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, United States) for 4 h; (3) 1 μg/ml LPS for 4 h, followed by 300 μM BzATP (Sigma-Aldrich) for 1 h; (4) none for 4 h, followed by 300 μM BzATP for 1 h. At the end, the supernatants were collected, centrifuged and frozen at −80°C until use.

IL-1β and IL-6 ELISA

IL-1β and IL-6 concentrations in both plasma and PBMCs supernatants were measured by ELISA using the human IL-1β/IL-1F2 and the human IL-6 Quantikine ELISA kits (R&D System, Bio-Techne, Minneapolis, MN, United States), respectively, following manufacturer's instructions. Standard and samples were tested in duplicate. Optical density was detected using a Thermo Scientific Multiskan FC plate reader. Cytokines concentrations were expressed as pg/ml.

Measurement of Intracellular Ca^{2+} Concentrations $[Ca^{2+}]_i$

Variations of intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were measured using the Fura-2/AM (Sigma-Aldrich) fluorescent probe. For this 2×10^6 PBMCs were loaded with 2 μM Fura-2/AM for 20 min at 37°C. After washes, PBMCs were employed for the assay at 37°C in a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Cernuso SN, Milan, Italy) using 500 μM BzATP as stimulus. In some experiments, PBMCs were pre-incubated for 1 h at 37°C in 5% CO_2 with 20–200 μM chloroquine (Sigma-Aldrich), washed and tested for variation of $[Ca^{2+}]_I$ as above.

RNA Extraction

Peripheral blood mononuclear cells were suspended in Trizol (Thermo Fisher Scientific, Waltham, MA, United States) and RNA extracted using the PureLink RNA Mini Kit (Thermo Fisher Scientific) following manufacturer's instructions. RNA was suspended in RNAse free water and its concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

RT-PCR

RNA retro-transcription was performed using the High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific) following manufacturer's instructions. RT-PCR for P2X7R, NLRP3, and GAPDH as endogenous control was performed using an RT-PCR kit (Thermo Fisher Scientific) in a PCR Biometra UNO- Thermoblock (DASIT, Cornaredo, Milan, Italy) using the following primers: Human p2x7 assay ID: Hs00175721_m1, Human NLRP3 assay ID: Hs00366465_m1. Pre-developed taqman endogenous control human GAPDH code 4326317E was used as housekeeping gene.

TABLE 1 | Demographic, clinical features and pharmacologic treatments of the SLE patients.

	SLE patients (*n* = 48)
Demographic parameters	
No. female/male	45 (93.7%)/3 (6.3%)
Age (mean ± SD) (years)	41.9 ± 10.2
Disease duration (months)	130.6 ± 96.7
Past clinical pattern, n° (%)	
Cutaneous (acute/subacute/chronic)	37 (77.1%)
Articular	31 (64.6%)
Serositis	16 (33.3%; three ongoing) type of serositis: • 4 pericardial • 7 pleural • 5 both pleural + pericardial
Renal	12 (25%) → glomerulonephritis histological class: • 1 both II and III class • 2 III class • 1 both III and V class • 5 IV class • 1 both IV and V class • 2 V class
Neurological	9 (18.8%) → type of neurological involvement: • 6 ischemic lesions • 1 headache • 1 depression • 1 myasthenia
Anemia	6 (12.5%)
Leukopenia	17 (35.4%)
Thrombocytopenia	3 (6.25%)
Serological parameters n°(%)	
ANA positivity	48 (100%)
ENA positivity	31 (64.6%)
Anti-DNA positivity	40 (83.3%)
Hypocomplementemia	40 (83.3%)
aPL (aCL, β2GPI, and/or LA) positivity	15 (31.2%)
Ongoing treatment	
Hydroxychloroquine (200 mg/day)	38 (79.2%)
Corticosteroids (2.5 up to 12.5 mg/day)	40 (83.4%)
Mycophenolate mofetil	8 (16.7%)
Cyclosporine A	2 (4.2%)
Azathioprine	4 (8.3%)
Methotrexate (10–15 mg/week)	3 (6.3%)
PEX	1 (2.1%)
Rituximab	1 (2.1%)
Belimumab	1 (2.1%)
Cumulative dosage of steroids gr (mean ± SD)	19.8 ± 18.0
Ongoing clinical manifestation n° (%)	
Serositis	3 (6.25%)
Arthralgia	6 (12.5%)
Cutaneous manifestation	6 (12.5%)
Renal (proteinuria > 500 mg/24 h)	5 (10.4%)
Neurological	0
Leukopenia	4 (8.34%)

(Continued)

TABLE 1 | Continued

	SLE patients (n = 48)
Current serological parameters	
Anti-DNA	40 (83.3%)
Hypocomplementemia	40 (83.3%)
ESR (mm/h) (mean ± SD) (years)	16.4 ± 9.3
CRP (mean ± SD) (years)	0.7 ± 1.6
Current clinimetric parameters	
SLEDAI-2K (mean ± SD)	4.2 ± 4.4
SLICC (mean ± SD)	0.6 ± 0.8

SLEDAI-2K, SLE disease activity index-2000; SLICC, SLE International Collaborating Clinics Damage Index; aPL, antiphospholipid antibodies including anti-cardiolipin antibodies (aCL) and lupus anticoagulant (LA); β2GPI, beta-2 glycoprotein 1 antibodies; ENA, extractable nuclear antigens antibodies; PEX, plasma exchange; ESR, erythrocyte sedimentation rate; CRP, C reactive protein.

Statistical Analysis

For descriptive analysis, discrete variables were expressed as absolute and relative frequencies while continuous variables as mean ± standard error (SE). The comparison between the groups was performed with the chi-squared test, *t*-test or Mann–Whitney and Wilcoxon test for non-parametric variables. The correlation index was used to evaluate the relationship between continuous variables, the correlation Spearman's ranks index was used, where indicated. Analyses were performed using Graphpad version 6.

RESULTS

Clinical Characteristics

A total of 48 SLE patients (45 women and 3 men) were enrolled, 16 (33.3%) of which had a history of serositis (previous or ongoing at the time of blood collection). The mean age (mean ± SD) of SLE population was of 41.9 ± 10.2 years, disease duration of 130.6 ± 96.7 months, the mean SLEDAI-2K 4.2 ± 4.4 and the mean SLICC equal to 0.6 ± 0.8. Demographic, clinical and pharmacologic treatments of all patients were collected (**Table 1**). No significant difference in clinical characteristics between patients with (SLE-S) or without (SLE-NS) a history of serositis was found, except for CRP, which was significantly higher in SLE-S (**Table 2**).

TABLE 2 | Comparison between patients with a positive history of serositis (SLE-S) vs. patients without history of serositis (SLE-NS).

	SLE-NS n° (%)	SLE-S (n°; %)	p (SLE-NS vs. SLE-S)
No. of patients	**32 (66.7%)**	**16 (33.3%)**	
Demographic parameters			
No. female/male	31/1	14/2	0.25
Age (mean ± SD) (years)	40.8 ± 8.4	44.2 ± 13.1	0.28
Disease duration (months)	134.7 ± 98.3	122.5 ± 96.2	0.68
Ongoing treatment			
Hydroxychloroquine (200 mg/day)	25 (78.1%)	13(81.2%)	1
Corticosteroids (2.5 up to 12.5 mg/day)	26 (81.3%)	6 (18.8%)	0.70
Mycophenolate mofetil	7 (21.9%)	1 (6.25%)	0.24
Cyclosporine A	2 (6.3%)	0	0.55
Azathioprine	2 (6.25%)	2(12.5%)	0.59
Methotrexate (10–15 mg/week)	1 (3.3%)	2 (15.38%)	0.21
PEX	0	1 (6.25%)	0.33
Rituximab	1 (3.1%)	0	1
Belimumab	0	1 (6.25%)	0.33
Ongoing dosage of steroids mg (mean ± SD)	4.3 ± 3.6	5.3 ± 4.5	0.42
Cumulative dosage of steroids gr (mean ± SD)	21.6 ± 20.6	18.9 ± 16.8	0.64
Current serological parameters			
Anti-DNA	20 (62.5%)	11 (68.8%)	1.00
Hypocomplementemia	13 (40.63%)	11 (68.8%)	0.125
ESR (mm/h) mean ± SD years	15.4 ± 1.5	18 ± 2.7	0.29
CRP mean ± SD years	0.4 ± 0.02	1.4 ± 0.67	0.04
Current clinimetric parameters			
SLEDAI-2K (mean ± SD)	3.8 ± 4.2	4.9 ± 4.6	0.42
SLICC (mean ± SD)	0.45 ± 0.8	0.8 ± 0.9	0.17

SLEDAI-2K, LE disease activity index-2000; SLICC, SLE International Collaborating Clinics Damage Index; aPL, antiphospholipid antibodies including anti-cardiolipin antibodies (aCL) and lupus anticoagulant (LA); β2GPI, beta-2 glycoprotein 1 antibodies; ENA, extractable nuclear antigens antibodies; PEX, plasma exchange; ESR, erythrocyte sedimentation rate; CRP, C reactive protein. Chi-square, t-test or Mann–Whitney for non-parametric data.

Evaluation of Plasma IL-1β and IL-6

Plasma IL-1β levels did not differ significantly between patients (SLE) and HC (**Figure 1A**) and a sub-analysis performed in SLE-S and SLE-NS gave similar results (**Figure 2A**). In a small population (n = 5) of patients with serositis without SLE, plasma IL-1β levels were significantly higher than in HC subjects (**Supplementary Figure 1A**), and even higher than SLE-S (**Supplementary Figure 1B**). Plasma IL-6 levels resulted higher, although not significantly, in SLE patients (3.09 ± 0.57 pg/ml) compared with (1.58 ± 0.67 pg/ml) (p = 0.191) (**Figure 1B**). Subanalysis of SLE patients group showed that plasma IL-6 was significantly higher in SLE-S (4.8 ± 0.97 pg/ml) vs. both SLE-NS (2.02 ± 0.37 pg/ml, p = 0.009) and HC (1.58 ± 0.67 pg/ml, p = 0.037) (**Figure 2B**). Subjects with serositis without SLE, showed plasma IL-6 levels higher than SLE subjects (**Supplementary Figures 1C,D**).

Evaluation of IL-1β and IL-6 in Cultured Macrophages Supernatants

Release of IL-1β from macrophages maintained for 5 h in RPMI or stimulated for 4 h with 1 μg/ml LPS was not significantly different between SLE and HC (pg/ml; mean ± SE) (**Figures 3A,B**). Stimulation of macrophages with 300 μM BzATP for 1 h following LPS treatment provoked IL-1β release significantly lower in SLE-S compared to SLE-NS (988.7 ± 103.6 vs. 1237 ± 70.39; p = 0.048) whereas no significant difference

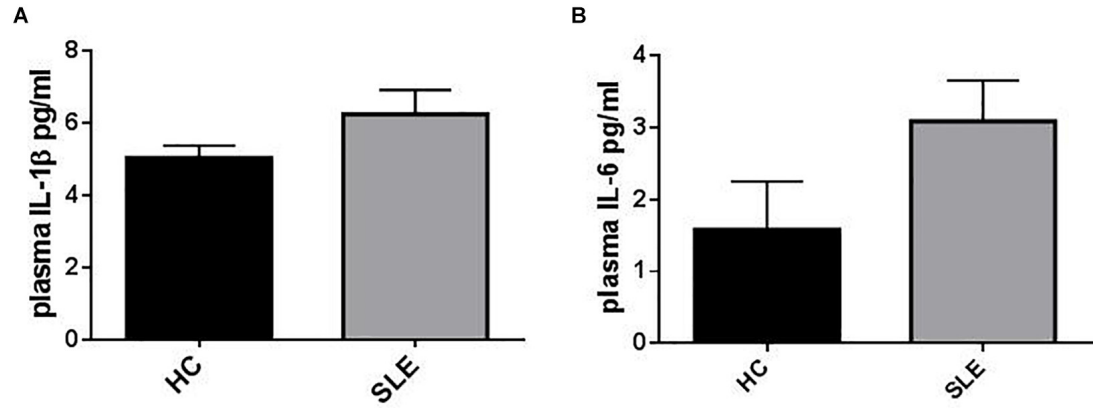

FIGURE 1 | Representation of IL-1β and IL-6 plasma levels in patients (SLE) vs. healthy control (HC). **(A)** Plasma IL-1β levels were not statistically different between HC and SLE (SLE vs. HC = 6.24 ± 0.61 vs. 5.42 ± 0.384, $p = 0.26$). Unpaired *t*-test. **(B)** Plasma IL-6 levels resulted higher in SLE vs. HC (SLE vs. HC = 3.09 ± 0.57 vs. 1.58 ± 0.67, $p = 0.191$). Unpaired *t*-test. Data are means ± SE.

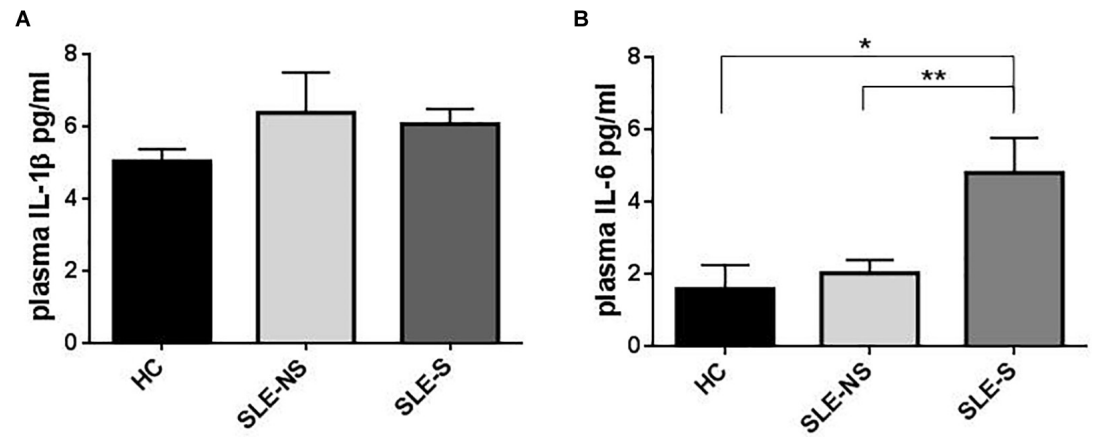

FIGURE 2 | Representation of IL-1β and IL-6 plasma levels in patients with serositis (SLE-S), without serositis (SLE-NS) compared to healthy control (HC). **(A)** Plasma IL-1β levels were not statistically significant between HC, SLE-S, and SLE-NS (SLE-NS vs. HC = 6.39 ± 1.13 vs. 5.42 ± 0.384, $p = 0.45$), (SLE-S vs. HC = 6.07 ± 0.38 vs. 5.42 ± 0.38, $p = 0.246$), (SLE-S vs. SLE-NS = 6.07 ± 0.38 vs. 6.39 ± 1.13, $p = 0.80$). Unpaired *t*-test. **(B)** Plasma IL-6 levels resulted significantly higher in SLE-S compared to both HC and SLE-NS: (SLE-NS vs. HC = 2.02 ± 0.37 vs. 1.58 ± 0.67, $p = 0.54$), (SLE-S vs. HC = 4.8 ± 0.97 vs. 1.58 ± 0.67, $p = 0.037$), (SLE-S vs. SLE-NS 4.8 ± 0.97 vs. 2.02 ± 0.37, $p = 0.009$). Unpaired *t*-test. Data are means ± SE. Only significant differences are shown. $^{*}p < 0.05$, $^{**}p < 0.01$.

was found with HC (**Figure 3C**). The most relevant difference between SLE and HC was visible when macrophages were stimulated with 300 μM BzATP alone (**Figure 3D**). In this case IL-1β release was significantly lower in SLE patients respect to HC (SLE-NS vs. HC = 11.84 ± 4.68 vs. 66.19 ± 12.31, $p < 0.0001$; SLE-S vs. HC = 11.95 ± 4.99 vs. 66.19 ± 12.31, $p = 0.0002$) without difference between patients with serositis vs. patients without serositis (SLE-S vs. SLE-NS, $p = 0.987$). Patients with serositis without SLE, showed IL-1β release after stimulation with BzATP significantly higher respect to both SLE-S and SLE-NS (**Supplementary Figure 2D**). IL-1β released from unstimulated macrophages and after stimulation with LPS alone was not significantly different between HC, SLE patients and serositis patients (**Supplementary Figures 2A,B**). IL-6 was found significantly higher in supernatants of macrophages from SLE compared to HC in all different experimental conditions (pg/ml; mean ± SE) (**Figure 4**). IL-6 released from unstimulated macrophages maintained for 5 h in RPMI was significantly higher in SLE patients vs. HC (SLE-NS vs. HC: 329.0 ± 83.74 vs. 11.83 ± 0.51, $p = 0.0102$; SLE-S vs. HC = 29.18 ± 4.85 vs. 11.83 ± 0.51, $p = 0.0021$) and in patients without serositis vs. patients with serositis (SLE-NS vs. SLE-S, $p = 0.014$) (**Figure 4A**). The release of IL-6 from macrophages stimulated for 4 h with 1 μg/ml LPS was significantly higher in SLE patients vs. HC (SLE-NS vs. HC: 1172 ± 74.25 vs. 339.9 ± 41.37, $p < 0.0001$; SLE-S vs. HC = 728.8 ± 101.2 vs. 339.9 ± 41.37, $p = 0.002$) and in patients without serositis vs. patients with serositis (SLE-NS vs. SLE-S, $p = 0.001$) (**Figure 4B**). Stimulation for 4 h with 1 μg/ml LPS, and for 1 h with 300 μM BzATP produced a significantly higher IL-6 release in SLE patients vs. HC (SLE-NS vs. HC: 1159 ± 83.87

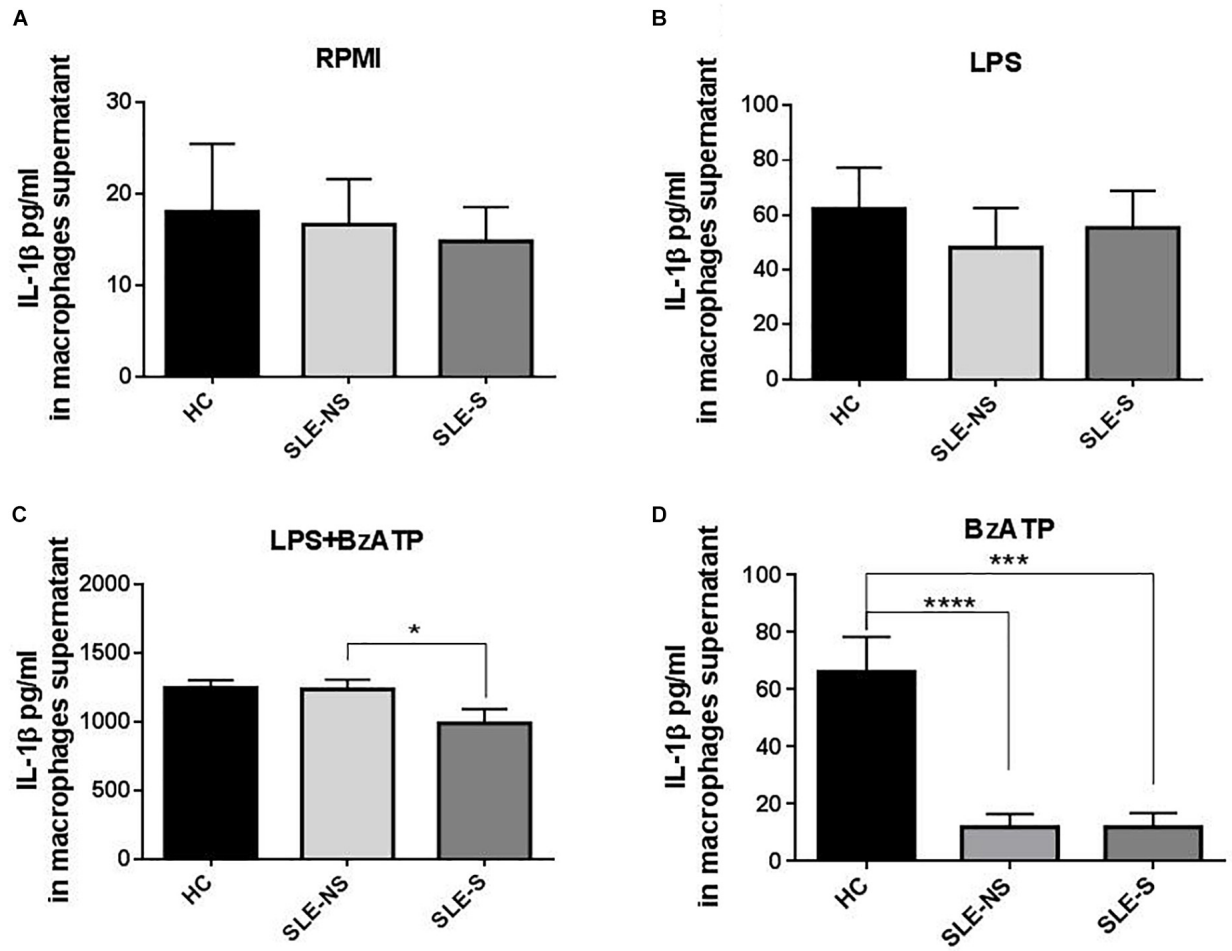

FIGURE 3 | Representation of IL-1β levels in macrophages supernatants from patients with serositis (SLE-S), without serositis (SLE-NS) and healthy controls (HC). **(A)** IL-1β released from unstimulated macrophages maintained for 5 h in 10% FBS supplemented RPMI was not significantly different between HC and SLE patients (SLE-NS vs. HC = 16.63 ± 5 vs. 18.05 ± 7.41, $p = 0.88$; SLE-S vs. HC = 14.82 ± 3.76 vs. 18.05 ± 7.41, $p = 0.67$) and between SLE-S vs. SLE-NS ($p = 0.80$). Unpaired *t*-test. **(B)** IL-1β released from macrophages stimulated for 4 h with 1 μg/ml LPS in 10% FBS-supplemented RPMI was not significantly different between HC and SLE patients (SLE-NS vs. HC = 48.11 ± 14.53 vs. 62.28 ± 15.09, $p = 0.556$; SLE-S vs. HC = 55.38 ± 13.47 vs. 62.28 ± 15.09, $p = 0.736$), and between SLE-S vs. SLE-NS ($p = 0.745$). Unpaired *t*-test. **(C)** IL-1β released from macrophages stimulated for 4 h with 1 μg/ml LPS and for 1 h with 500 μM BzATP in 10% FBS-supplemented RPMI was not different between HC and SLE patients (SLE-NS vs. HC = 1237 ± 70.39 vs. 1246 ± 58.61, $p = 0.936$; SLE-S vs. HC = 988.7 ± 103.6 vs. 1246 ± 58.61, $p = 0.072$). IL-1β was significantly lower in SLE-S compared to SLE-NS ($p = 0.0476$). Unpaired *t*-test. **(D)** IL-1β released from macrophages stimulated for 1 h with 300 μM BzATP in 10% FBS-supplemented RPMI was significantly lower in SLE patients respect to HC (SLE-NS vs. HC = 63.91 ± 18.94 vs. 208.4 ± 34.87, $p < 0.0006$; SLE-S vs. HC = 59.29 ± 24.02 vs. 208.4 ± 34.87, $p = 0.0015$). IL-1β was not different between SLE-S vs. SLE-NS ($p = 0.881$). Unpaired *t*-test. Data are means ± SE. Only significant differences are shown. *$p < 0.05$, ***$p < 0.005$, ****$p < 0.001$.

vs. 330.7 ± 43.46, $p < 0.0001$; SLE-S vs. HC = 719.7 ± 112.6 vs. 330.7 ± 43.46, $p = 0.004$) and in patients without serositis vs. patients with serositis (SLE-NS vs. SLE-S, $p = 0.003$) (**Figure 4C**). Similarly, IL-6 released following macrophage stimulation for 1 h with BzATP was significantly higher in SLE patients vs. HC (SLE-NS vs. HC: 328 ± 92.1 vs. 13.38 ± 0.76, $p = 0.019$; SLE-S vs. HC = 43.95 ± 9.49 vs. 13.38 ± 0.76, $p = 0.005$) and in patients without serositis vs. patients with serositis (SLE-NS vs. SLE-SS, $p = 0.028$) (**Figure 4D**).

IL-6 released from macrophages of patients with serositis without SLE was significantly lower than that secreted by macrophages of SLE-NS when stimulated by LPS (**Supplementary Figure 3B**) while was significantly higher than that secreted by macrophages of HC following LPS+BzATP stimulation (**Supplementary Figure 3C**). No significant difference of IL-6 levels were found in patients with serositis after stimulation by RPMI and BzATP alone (**Supplementary Figures 3A–D**).

Evaluation of Variations of Intracellular Ca^{2+} Concentration ($\Delta[Ca^{2+}]_i$) With Fura-2/AM in PBMCs After BzATP Stimulation

Variation of intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$ (Fura-2/AM) nM ± SE) after stimulation of PBMCs with 500 μM

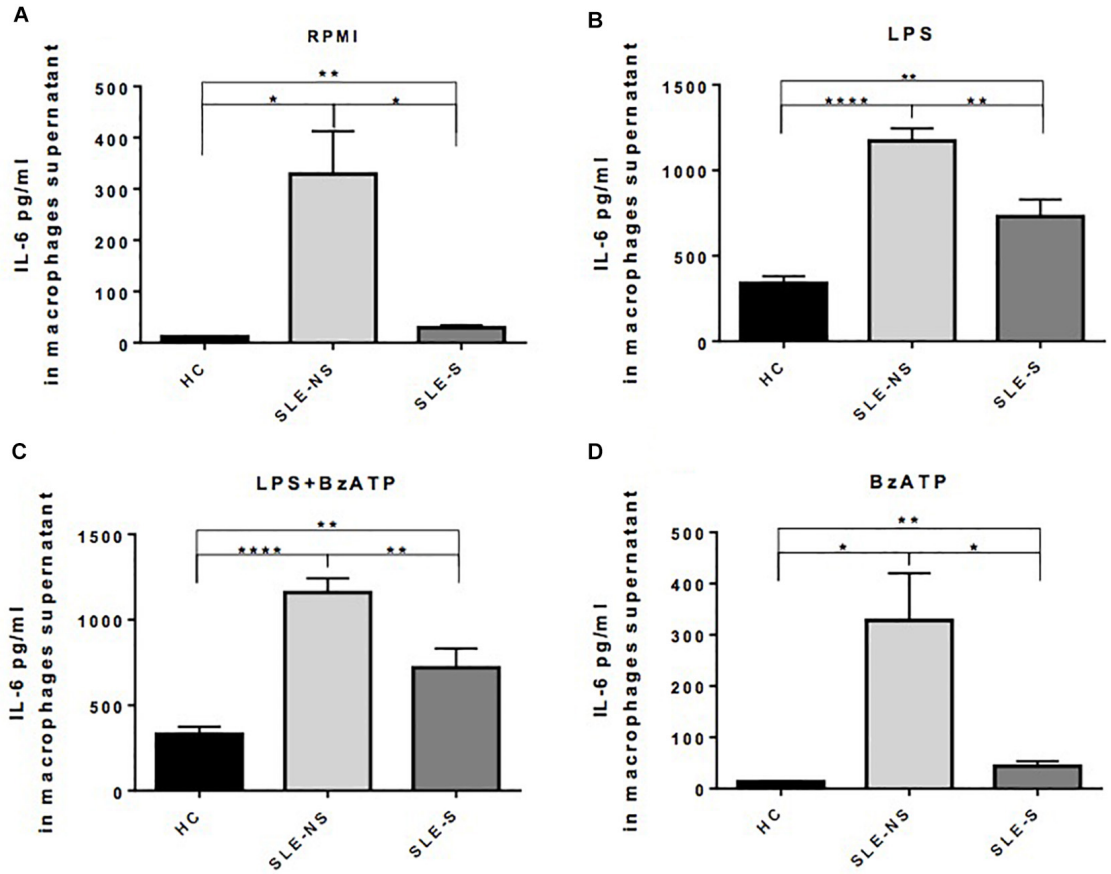

FIGURE 4 | Representation of IL-6 levels in macrophages supernatants from patients with serositis (SLE-S), without serositis (SLE-NS) and healthy controls (HC). **(A)** IL-6 released from unstimulated macrophages maintained for 5 h in 10% FBS supplemented RPMI was significantly higher in SLE patients vs. HC (SLE-NS vs. HC: 329.0 ± 83.74 vs. 11.83 ± 0.51, $p = 0.0102$; SLE-S vs. HC = 29.18 ± 4.85 vs. 11.83 ± 0.51, $p = 0.0021$) and in patients without serositis vs. patients with serositis (SLE-S vs. SLE-NS, $p = 0.014$). **(B)** IL-6 released from macrophages stimulated for 4 h with 1 μg/ml LPS in 10% FBS-supplemented RPMI was significantly higher in SLE patients vs. HC (SLE-NS vs. HC: 1172 ± 74.25 vs. 339.9 ± 41.37, $p < 0.0001$; SLE-S vs. HC = 728.8 ± 101.2 vs. 339.9 ± 41.37, $p = 0.002$) and in patients without serositis vs. patients with serositis (SLE-S vs. SLE-NS, $p = 0.001$). **(C)** IL-6 levels from macrophages stimulated for 4 h with 1 μg/ml LPS, and for 1 h with 300 μM BzATP in 10% FBS-supplemented RPMI were significantly higher in SLE patients vs. HC (SLE-NS vs. HC: 1159 ± 83.87 vs. 330.7 ± 43.46, $p < 0.0001$; SLE-S vs. HC = 719.7 ± 112.6 vs. 330.7 ± 43.46, $p = 0.004$) and in patients without serositis vs. patients with serositis (SLE-S vs. SLE-NS, $p = 0.003$). **(D)** IL-6 released from macrophages stimulated for 1 h with 300 μM BzATP in 10% FBS-supplemented RPMI was significantly higher in SLE patients vs. HC (SLE-NS vs. HC: 328 ± 92.1 vs. 13.38 ± 0.76, $p = 0.019$; SLE-S vs. HC = 43.95 ± 9.49 vs. 13.38 ± 0.76, $p = 0.005$) and in patients without serositis vs. patients with serositis (SLE-S vs. SLE-NS, $p = 0.028$). Data are means ± SE. Only significant differences are shown. *$p < 0.05$, **$p < 0.01$, ****$p < 0.001$.

BzATP, a direct measurement of P2X7R activity, was significantly lower in SLE than in HC (SLE vs. HC = 64.1 ± 6.84 vs. 105.5 ± 12.75, $p = 0.008$; SLE-NS vs. HC = 67.69 ± 9.23 vs. 105.5 ± 12.75; $p = 0.0267$; SLE-S vs. HC = 58.25 ± 10.28 vs. 105.5 ± 12.75, $p = 0.0215$), with no significant difference between patients without serositis vs. patients with serositis (SLE-NS vs. SLE-S, $p = 0.674$) (**Figure 5A**). PBMCs from five patients with serositis without any evidence of SLE, showed a BzATP-stimulated increase of $[Ca^{2+}]_i$ corresponding to 191.8 ± 50.92, significantly higher than that measured in all the other groups of subjects (serositis vs. HC $p = 0.008$; serositis vs. SLE-NS $p = 0.0005$; serositis vs. SLE-S $p = 0.0016$) (**Supplementary Figure 4**).

To evaluate the effect of chloroquine on P2X7R activity, PBMCs from four controls and three patients underwent an evaluation of Ca^{2+} influx in normal conditions and after treatment for 1 h with 200 μM chloroquine. The $\Delta[Ca^{2+}]_i$ was higher after chloroquine treatment respect to basal conditions (Wilcoxon matched-pairs signed rank test $p = 0.016$) both in controls and patients (pre vs. post treatment: 92.0 ± 11.07 vs. 132.3 ± 22.18; $p = 0.13$) and patients (48.67 ± 15.68 vs. 81 ± 20.65; $p = 0.25$) (**Figure 5B**).

Evaluation of P2X7R and NLRP3 Expression With RT-PCR

At RT-PCR, P2X7R resulted significantly less expressed in PBMCs of patients compared to controls (mean ± SE) (SLE vs. HC = 0.87 ± 0.1 vs. 1.29 ± 0.13; $p = 0.02$), particularly in patients with serositis (SLE-S vs. HC = 0.724 ± 0.11 vs. 1.29 ± 0.13; $p = 0.019$) (**Figure 6A**). On the contrary, NLRP3

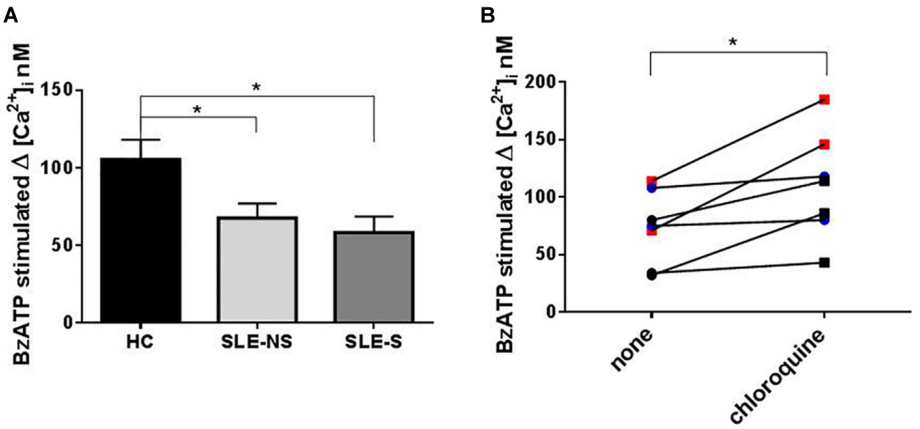

FIGURE 5 | Evaluation of Calcium influx with Fura-2 in PBMCs after BzATP stimulation and chloroquine pre-treatment. **(A)** The increase of intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) following stimulation with 500 μM BzATP was significantly lower in PBMCs from patients (SLE) vs. controls (HC) (SLE-NS vs. HC = 67.69 ± 9.23 vs. 105.5 ± 12.75, $p = 0.0228$; SLE-S vs. HC = 58.25 ± 10.28 vs. 105.5 ± 12.75, $p = 0.0147$). Unpaired *t*-test. Only significant differences are shown. **(B)** The effect of 200 mM chloroquine on $\Delta[Ca^{2+}]_i$ was evaluated on PBMCs from four controls and three patients. Each line represents a patients or a control subject. The $\Delta[Ca^{2+}]_i$ was higher after chloroquine treatment respect to basal conditions (Wilcoxon matched-pairs signed rank test, $p = 0.016$) both in controls and patients (pre vs. post treatment: 92.0 ± 11.07 vs. 132.3 ± 22.18, $p = 0.13$) and patients (48.67 ± 15.68 vs. 81 ± 20.65, $p = 0.25$). Data are means ± SE. $^*p < 0.05$.

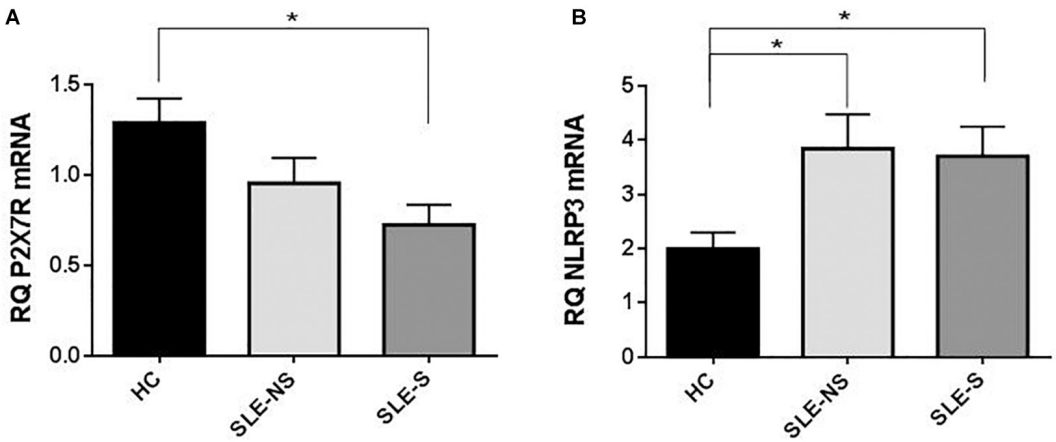

FIGURE 6 | Expression of P2X7R and NLRP3 mRNA in PMBCs from patients (SLE) vs. healthy control (HC). **(A)** P2X7R was significantly less expressed in SLE vs. HC (SLE vs. HC = 0.87 ± 0.10 vs. 1.29 ± 0.13, $p = 0.02$), particularly in patients with serositis (SLE-S vs. HC = 0.724 ± 0.11 vs. 1.29 ± 0.13, $p = 0.019$). **(B)** NLRP3 expression resulted significantly higher in SLE vs. HC (SLE vs. HC = 3.80 ± 0.46 vs. 1.99 ± 0.30, $p = 0.018$). Data are means ± SE. Only significant differences are shown. $^*p < 0.05$.

expression resulted significantly higher in PBMCs of patients than in controls (SLE vs. HC = 3.80 ± 0.46 vs. 1.99 ± 0.30; $p = 0.018$) also considering the two sub-categories separately (SLE-NS vs. HC = 3.846 ± 0.633 vs. 1.995 ± 0.302, $p = 0.013$; SLE-S vs. HC = 3.702 ± 0.552 vs. 1.995 ± 0.302, $p = 0.036$) (**Figure 6B**).

Evaluation of the Role of Clinical Factors on P2X7R Activity Evaluated as $\Delta[Ca^{2+}]_i$ (Fura-2)

No significant correlation between disease activity/disease duration and P2X7R activity assessed by Ca^{2+} influx measurement was detected (**Table 3**). For the evaluation of the effect of therapy, we have collected all patients taking immunosuppressive therapy in a single group called "ongoing major immunosuppressive therapy." We also assessed separately the influence of ongoing hydroxychloroquine and of ongoing steroids therapy (**Table 3**). Finally, the effect of steroid dosage (current or cumulative) was assessed (**Table 3**). No significant influence of therapy on $\Delta[Ca^{2+}]_i$ was detected.

DISCUSSION

The aim of our study was to evaluate the expression and the activity of P2X7R and NLRP3 inflammasome in a cohort

TABLE 3 | Correlation between disease activity, disease duration, and corticosteroids dosage with P2X7R activity.

	SLEDAI	Disease duration	Cumulative dosage of steroids	Current dosage of steroids	Ongoing hydroxychloroquine (38 patients)	Ongoing steroids (40 patients)	Ongoing major immunosuppressive therapy (18 patients)
	Spearman rho; *p*	rho; *p*	Spearman rho; *p*	Spearman rho; *p*	Yes vs. No; *p*	Yes vs. No; *p*	Yes vs. No; *p*
ΔCa^{2+} (Fura-2) nM (±SD)	−0.4; $p = 0.1$	0.08; $p = 0.73$	0.18; $p = 0.47$	−0.13; $p = 0.61$	70.8 ± 30.8 vs. 53.6 ± 34.2; $p = 0.32$	66.3 ± 33.9 vs. 61.5 ± 4.9; $p = 0.84$	53.8 ± 37.7 vs. 76.2 ± 22.6; $p = 0.15$

Spearman correlation.

of patients with SLE, analyzing more deeply the cases that presented a history of serositis, a clinical manifestation that more than others recalls auto-inflammatory diseases such as FMF. A sub-analysis performed on SLE patients showed no differences between SLE-NS and SLE-S as regard clinical parameters, disease activity, demographic characteristics, and treatment, except the CRP levels (**Table 2**).

Evidence in both mouse models and humans had revealed a possible role for P2X7R and NLRP3 inflammasome in SLE pathogenesis. MRL/lpr mice showed increased renal expression of P2X7R, NLRP3, ASC and caspase 1, resulting in increased production of IL-1β and IL-18 and treatment with the P2X7R antagonist Brilliant Blue G reduced proteinuria, serum anti-DNA antibodies and, at renal histology, glomerular cellularity, signs of vasculitis and IgG and C3 deposition (Zhao et al., 2013). Also in lupus-like nephritis induced by intraperitoneal injection of pristane, caspase-1-/- mice presented reduction of autoantibodies (anti-DNA and anti-RNP) and hypergammaglobulinemia compared to pristane-treated wild type mice (Peairs et al., 2009). In humans, a study on renal biopsies showed increased P2X7R expression in lupus patients compared to controls (Turner et al., 2006) and increased IL-1β serum levels in SLE patients has been demonstrated (Cigni et al., 2015).

Contrary to these previous observations, our study does not reveal a direct role of P2X7R as an inducer of the inflammatory response in patients with SLE. P2X7R expression (as mRNA) (**Figure 6**) and activity (as BzATP $\Delta[Ca^{2+}]_i$) (**Figure 5**) were indeed reduced in PBMCs from SLE patients respect to those from HC. In addition, BzATP-stimulated IL-1β release, from macrophages of SLE patients was reduced respect to controls (**Figure 3**). In our patients, plasma IL-1β levels were only slightly, not significantly increased compared to healthy subjects and cultured macrophages from these patients released substantially similar levels of IL-1β after stimulation with LPS alone or followed by BzATP. These results suggest that P2X7R reduced expression and activity in SLE patients might be partially compensated by increased expression of NLRP3, as revealed by RT-PCR (**Figure 6**). The P2X7R defect in SLE patients was underlined by the highest difference between controls and patients in IL-1β release when macrophages were stimulated by BzATP alone which can be considered a powerful P2X7R agonist (**Figure 3D**).

Since P2X7R activation is also implicated in the production of other inflammatory cytokines such as IL-1α, TNF-α, and IL-6, independently of the NLRP3 inflammasome (Gicquel et al., 2015), to more extensively investigate the P2X7R activity we also tested IL-6 levels as representative of a different pathway by which P2X7R can support inflammatory responses. Plasma IL-6 levels were higher in SLE subjects, especially in SLE-S subgroup (**Figures 1B**, **2B**). Macrophages from SLE patients released significantly higher levels of IL-6 *in vitro*, even in basal conditions and after stimulation with LPS and/or BzATP (**Figure 4**). IL-6 release was particularly increased in the SLE-NS sub-group suggesting that macrophages from SLE-S patients might be de-sensitized to IL-6 production *in vitro*. In SLE, IL-6 levels seem not to be correlated to an inflammatory state as suggested by the lack of correlation between CRP and IL-6 levels (both in plasma and in supernatants) (**Supplementary Table 6**). These results lead us to hypothesize that in our SLE patients a pathogenetic pathway resulting in increased production of IL-6, would prevail on the IL-1β pathway mediated by P2X7R that indeed appears downregulated. IL-6 release could be independent of the P2X7R-NLRP3 axis. The inflammasome may indeed be activated by different pathways stimulating other PRRs, such as TLRs able not only to promote IL-1β and IL-18 gene transcription, but also to increase NLRP3 activity and IL-6 production (Villanueva et al., 2011; Klonowska-Szymczyk et al., 2017).

To ascertain that the observed data were associated not only to serositis but more generally to SLE, a sub-analysis performed in other major lupus-related clinical manifestations (nephritis, arthritis, skin, and neuropsychiatric involvement) excluded the association with the activity and expression of P2X7R, NLRP3 and production of IL-1 β and IL-6 (see **Supplementary Tables 1–4**). The analysis carried out on the group of patients with serositis, suffering from pathologies other than SLE and FMF, showed that P2X7R was more active in these subjects respect to SLE patients, as evidenced by increased IL-1β in both plasma and supernatants of macrophages after stimulation with pure agonist BzATP (**Supplementary Figures 1A, 2D**) and augmented BzATP $\Delta[Ca^{2+}]_I$ increase (**Supplementary Figure 4**). These results, despite the limitations of the small sample size (five patients with serositis), reinforce our hypothesis that P2X7R is downregulated in patients with SLE, regardless of the type of clinical manifestation.

Cytokine production is not the only activity mediated by P2X7R. In case of pore formation following prolonged stimulation with ATP, P2X7R could mediate a cytotoxic effect promoting cell death. A reduced expression of P2X7R would be implicated in defective apoptosis of different cellular types

like T follicular helper (Tfh) cells in germinative centers with consequent enhanced activation of B lymphocytes (Perruzza et al., 2017). A study conducted on 42 SLE patients, showed that enhanced expansion of Tfh cells was correlated with diminished cell death mediated by P2X7R (Gualtierotti et al., 2017; Faliti et al., 2019). In accordance with this evidence, reduced expression of P2X7R detected in our study might be implicated in SLE pathogenesis through a different mechanism mediated by its role in cellular growth control. Further studies will be needed to clarify the role of P2X7R in SLE, and especially if the reduced expression of the receptor has a major role in SLE pathogenesis or if this finding could be considered a consequence of the disease itself. In presence of a persistent inflammatory state as in SLE (consistent with elevated IL-6 levels found in the patients of this study), there is an increase in ATP levels in the extracellular microenvironment able to activate P2X7R. An adaptation mechanism, secondary to the chronic inflammatory state, could, therefore, induce a negative feedback effect on P2X7R expression. On the other side, the increase in IL-6 could represent an activated pathway in response to a primary P2X7R downregulation. The role of IL-6 in SLE has been evaluated in the pathogenesis of different clinical manifestations. For example, it has been detected in serum and urine of SLE patients with lupus nephritis (Peterson et al., 1996) and in cerebrospinal fluid in patients with neuropsychiatric manifestations (Yoshio et al., 2016), especially in presence of cognitive impairment (Wiseman et al., 2017). These evidences would make reasonable an attempt of targeting IL-6 in SLE. A phase I study evaluated the effect of high-affinity human anti-IL-6 monoclonal antibody sirukumab in SLE patients with LN of class III and IV that have already received induction therapy with mycophenolate mophetil (MMF) or cyclophosphamide and presented significant proteinuria despite maintenance treatment with MMF or azathioprine. After 6 months of treatment (21 patients treated with sirukumab and 4 with placebo) no significant improvement in proteinuria and disease activity was found after adding the anti-IL-6 treatment to immunosuppressive therapy (Rovin et al., 2016). Another phase II study in SLE patients with the humanized monoclonal antibody tocilizumab against the chain of the IL-6 receptor, showed a reduction of auto-antibodies production and plasma-cells and significant clinical improvement especially in case of arthritis and fatigue (Illei et al., 2010). Finally a case report demonstrated the efficacy of tocilizumab in lupus serositis (Kamata and Minota, 2012; Ocampo et al., 2016).

Moreover, also IL-1β and P2X7R-inflammasoma axis, were considered possible therapeutic targets in other reports. Anakinra, an IL-1β receptor antagonist, was for example shown to be effective and safe in a small study conducted on SLE patients with refractory arthritis (Ostendorf et al., 2005). Inflammation parameters (ESR and CRP) are generally altered in the SLE during few conditions like infection or serositis (Ryu et al., 2017). In addition, this manifestation responds promptly to colchicine, a treatment generally used in diseases considered "inflammasome guided" as the FMF (Morel et al., 2015; Padeh and Berkun, 2016). The primary molecular target of colchicine is β-tubulin inducing microtubule de-polymerization. In mouse macrophages the lack of microtubule rearrangement provoked by colchicine, affected several different P2X7R activities such as pore formation and IL-1β release (Marques-da-Silva et al., 2011). These aspects made us to hypothesize that P2X7R-inflammasome axis could be a fundamental actor in the pathogenesis of lupus serositis which is usually successfully treated with colchicine.

Our study has many limitations: first, the patients enrolled had a prolonged disease duration (over 10 years) so they have been subjected to a long period of treatment. The majority of patients did not present active disease expressed in term of SLEDAI-2K (4.2 ± 4.4). Considering the serological domains of SLEDAI-2K, 83.3% of patients had both anti-DNA positivity and hypocomplementemia and consequently, a limited sample size presented an active clinical manifestation at the enrollment. To assess the influence of the presence of an active manifestation on P2X7R activity and expression, we compared patients in clinical remission (clinical SLEDAI-2K without serology = 0) with patients whit at least one active clinical manifestation at the time of enrollment (pooling different clinical manifestations together for the low number of subjects). This analysis showed that the presence of clinical activity does not influence the previous results (**Supplementary Table 5**).

Another limitation consists in the fact that the included patients presented heterogeneity in disease activity and treatment so being a potential bias of this study.

Taking these aspects into consideration, we have evaluated the correlation between the main clinical variables and the activity of P2X7R through a direct measure of its mechanism of action (represented by BzATP stimulated $\Delta[Ca^{2+}]_i$-Fura-2/AM) and no significant correlations with disease duration, SLEDAI-2K and ongoing therapy were detected. In particular, in our series, 79% of patients were treated with hydroxychloroquine, a cornerstone in the SLE therapy, with an inhibitory action on TLR (Torigoe et al., 2018). Compared to the steroid, hydroxychloroquine accumulates in the body over a long period exerting a prolonged effect. To evaluate if this drug could have played a role in the reduction of the activity of P2X7R, we carried out a test on few samples in which PMBCs were pre-treated with 200 μM chloroquine and subsequently stimulated with BzATP to evaluate the $\Delta[Ca^{2+}]_i$. The results obtained, showed that in the same subject, the chloroquine pre-treatment determined an increased $\Delta[Ca^{2+}]_i$ suggesting that the reduced activity of P2X7R in SLE patients would not be due to the pharmacological action of this drug. Furthermore, despite the ongoing treatment and the absence of high disease activity, an increase of inflammatory cytokine IL-6 was found in patients both in supernatants of stimulated monocytes and plasma. The limited number of patients (especially those with serositis), and the wide variability among patients in cytokine expression, did not allow in some cases to reach statistical significance.

CONCLUSION

Compared to auto-inflammatory diseases, in which inflammasome has the main pathogenetic role, SLE is a complex condition where alterations of adaptive and innate immunity coexist. During different phases of the disease, multiple pathways

can influence each other and change their activity. Further studies should enroll patients at disease onset and naïve from therapy, to evaluate if P2X7R represents a bridging role between environmental risk factors and autoimmunity development in the early stages of the disease, especially in those patients where a trigger, for example infectious, is recognizable. In this case, P2X7R might be overexpressed during the initial phase of illness and downregulated once the autoimmune process becomes established. In this phase of the disease, other pathways and other cytokines, such as IL-6, could be prevalent and should be considered as important therapeutic targets in the treatment of SLE, especially in presence of clinical manifestations with a more "inflammatory" track as serositis.

ETHICS STATEMENT

All subjects provided written informed consent. The study was approved by the local ethics committee and conducted in accordance with the amended Helsinki Declaration.

AUTHOR CONTRIBUTIONS

FF and AB formulated the concept and designed the manuscript. FF, ALG, and MP conducted the experiments. FF and ALG contributed to statistical analysis and wrote the manuscript. FF, ALG, MEP, MG, FD, and AB revised the manuscript critically, approved the final manuscript, and agreed to be accountable for all aspects of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00435/full#supplementary-material

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A Model for Apoptotic-Cell-Mediated Adaptive Immune Evasion *via* CD80–CTLA-4 Signaling

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Apoptotic cells carry a plethora of self-antigens but they suppress eliciting of innate and adaptive immune responses to them. How apoptotic cells evade and subvert adaptive immune responses has been elusive. Here, we propose a novel model to understand how apoptotic cells regulate T cell activation in different contexts, leading mostly to tolerogenic responses, mainly *via* taking control of the CD80–CTLA-4 coinhibitory signal delivered to T cells. This model may facilitate understanding of the molecular mechanisms of autoimmune diseases associated with dysregulation of apoptosis or apoptotic cell clearance, and it highlights potential therapeutic targets or strategies for treatment of multiple immunological disorders.

Keywords: apoptosis, immunotolerance, autoimmunity, costimulatory pathway, CD80, CTLA-4, coinhibitory pathway, immune evasion

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APOPTOSIS

Apoptosis, or programmed cell death (PCD), is the physiological form of cell death that plays an important role in tissue homeostasis and regeneration, as well as maintenance of robust organ functions. While cell death by necrosis may have immunostimulatory and inflammatory effects (Sauter et al., 2000), apoptosis shows no immunostimulatory capacities, and may serve beneficial functions to the host (Sauter et al., 2000; Mahajan et al., 2016).

Our understanding of many aspects of apoptosis has constantly increased over the past ~30 years, due to tremendous effort and a myriad of studies using various model systems. For example, mouse models, due to the power of mouse genetics and with the availability of gene-targeting approaches, have been a mainstay tool to understand both the various functions of apoptosis-related genes in development and the association between gene functions or apoptosis states and disease phenotypes in mammals (see, for instance, Reyes et al., 2010; Gómez-Sintes et al., 2011; Yamaguchi et al., 2011; Wu et al., 2015). Given that the apoptosis machinery is evolutionarily conserved, from worms to mammals, other eukaryotic models have also been used such as drosophila, especially considering the ease of genetic screens and availability of a large number of fly lines (see, for instance, Richardson and Kumar, 2002; Gullaud et al., 2003; Denton et al., 2008; Xu et al., 2009). Even yeast, which, while it lacks main regulators of mammalian apoptosis such as caspases and the B cell lymphoma 2 (Bcl-2) family members, was used to study apoptosis *via* heterologous expression of many such genes, given the advantages of the yeast model (e.g., easy manipulation *via* molecular biology or genetics, low cost, and the availability of powerful tools such as the yeast two-hybrid system) (Fleury et al., 2002; Kazemzadeh et al., 2012). In addition to the *in vivo* models,

in vitro models have also been frequently used to understand the signaling pathways or molecular interactions that regulate apoptosis at the cellular level, in physiological or disease conditions (Calissano et al., 2009; Spencer and Sorger, 2011). Importantly, with the advent of stem cell technologies and *in vitro* differentiation methods, many human (stem cell-derived) cell types, including neurons, were used to understand apoptosis-related molecular disease mechanisms in the human genetic background (Csöbönyeiová et al., 2016; Fang et al., 2018).

Induction of cell death by apoptosis in mammals is initiated by two major signaling cascades: the "extrinsic" and "intrinsic" pathways of apoptosis (Nagata and Tanaka, 2017). In the intrinsic pathway, activation of apoptosis is triggered by either developmental signals or genotoxic substances resulting in the release of many proteins including cytochrome C from the mitochondria by pro-apoptotic members of the Bcl-2 family (Nagata and Tanaka, 2017). The released cytochrome C subsequently mediates the formation of apoptosomes in the respective cell's cytosol, which are multiprotein complexes consisting of cytochrome C, pro-caspase 9, and apoptotic protease-activating factor 1 (APAF1) that process pro-caspase 9 to its mature form (Liu et al., 1996; Zou et al., 1997, 1999). Mature caspase 9 finally mediates the maturation of inactive pro-caspase 3 to its active form caspase 3 (Nagata and Tanaka, 2017). In the extrinsic pathway of apoptosis, binding of FasL (Fas Ligand, expressed on the surface of the apoptosis-inducing cell) to Fas (CD95, tumor necrosis factor receptor superfamily member 6) on the cell destined to undergo apoptosis results in a conformational change in the Fas trimer allowing for the formation of the death-inducing signaling complex (DISC) (Nagata and Tanaka, 2017). DISC is a multiprotein complex containing the Fas-associated death domain protein (FADD) and pro-caspase 8 (Chinnaiyan et al., 1995; Kischkel et al., 1995; Muzio et al., 1996). DISC activation results in the production of mature caspase 3 by DISC-matured caspase 8 (Nagata and Tanaka, 2017). Finally, caspase 3 activated by both apoptosis pathways triggers the apoptosis program *via* the cleavage of >500 cellular substrates (Nagata and Tanaka, 2017). While FasL expression is restricted to cytotoxic T lymphocytes, T helper type-2 (Th2) cells, and Natural Killer (NK) cells (Kägi et al., 1994; Lowin et al., 1994), Fas is expressed by most cell types (Nagata and Tanaka, 2017). Therefore, FasL-Fas interaction-induced apoptosis is very important for tissue homeostasis. Besides FasL, other ligands such as tumor necrosis factor-alpha (TNF-α), lymphotoxin-alpha (LT-α), TNF-like protein-1A (TL1A), and Apo2L/TNF-related apoptosis-inducing ligand (TRAIL) can also trigger Fas-dependent apoptosis *via* the extrinsic pathway (Yamada et al., 2017).

APOPTOTIC CELLS AND INNATE IMMUNITY

It was initially thought that apoptotic cells (ACs) might be immunologically null, however a plethora of evidence has since then indicated that ACs are immunologically active, exerting, in most cases, anti-inflammatory and immunosuppressive effects. Early, in 1997, a pioneering study (Voll et al., 1997a) showed that peripheral blood-derived macrophages exposed to ACs exhibited enhanced production of the immunosuppressive cytokine interleukin (IL)-10, which is an important immune regulatory molecule that prevents inflammatory immune responses, tissue damage, and the development of autoimmunity. Recently, ACs were shown to induce upregulation of the transcription factor aryl hydrocarbon receptor (AhR) in a Toll-like receptor (TLR) 9-dependent manner, which enhanced production of IL-10 to mediate AC-dependent immunosuppression (Shinde et al., 2018). Consequently, AhR knockout induced autoimmune responses and systemic lupus erythematosus (SLE) disease in a mouse model (Shinde et al., 2018). However, it is important to note that, while IL-10 is mainly considered to have anti-inflammatory effects on a wide range of target cells, recent findings suggest a more complex modulatory function of this important cytokine. Because of its role as an important B cell growth and differentiation factor (that promotes B cell proliferation and IgG production), IL-10 was suggested to contribute to the pathology of SLE *via* activation of autoreactive B cells (reviewed in Geginat et al., 2016). IL-10 levels were shown to increase in SLE patients and polymorphisms in the IL-10 promoter were strongly associated with SLE development (Peng et al., 2013). In line with these findings, neutralization of IL-10 blocked autoantibody production in SLE patients (Llorente et al., 1995). However, both the source of the pathogenic IL-10 production in SLE patients and its possible contribution to other autoimmune diseases remain to be further characterized (Geginat et al., 2016).

Besides IL-10, ACs were shown to induce the production of many anti-inflammatory cytokines such as transforming growth factor beta (TGF-β), platelet activating factor (PAF), and prostaglandin E2 (PGE2) (Voll et al., 1997b; Cvetanovic and Ucker, 2004). In addition, macrophage exposure to ACs caused a reduction in the macrophages' expression of the pro-inflammatory and immunostimulatory cytokines tumor necrosis factor (TNF)-α, IL-12, IL-1β, IL-18, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fadok et al., 1998; Kim et al., 2005).

Therefore, ACs are able to modulate the activation state of, and cytokine secretion from, antigen-presenting cells (APCs) which influences both innate and subsequent adaptive immune responses to the ACs. This immune modulation also has consequences for T cell activation upon encountering ACs. For example, suppression of macrophage-derived IL-12 production may prevent the differentiation of self-reactive T helper type-1 (Th1) $CD4^+$ cells and autoimmunity (Trembleau et al., 1995), while AC-induced IL-10 represses the expression of MHC-II and costimulatory molecules required for antigen presentation and subsequent T cell activation (Couper et al., 2008).

APOPTOTIC CELLS AND ADAPTIVE IMMUNITY

The Route to T Cell Activation

Upon T cell receptor (TCR) activation by ligand binding, such as by specific-antigen-bound major histocompatibility complex

(MHC) on the surface of APCs, the TCR-associated CD3 chains become tyrosine-phosphorylated leading to recruitment of kinases and scaffold proteins and formation of a supramolecular complex that triggers signaling pathways and transcriptional cascades responsible for T cell differentiation and clonal expansion, as well as effector cell generation (Rathmell et al., 2003; Smith-Garvin et al., 2009; Marko et al., 2010; Schultze et al., 2012). Among those signaling and transcriptional events are the upregulation of the glucose receptor Glut 1 (Frauwirth et al., 2002) and the glutamine receptors Snat1 and Snat2 (Carr et al., 2010), and the activation of the MAPK and PI3K/Akt pathways (Kannan et al., 2012). Collectively, these events are required to fulfill the metabolic needs of the proliferating T cells and to support cell cycle progression and cytokine production (Appleman et al., 2002).

However, TCR ligation alone is insufficient to trigger or maintain robust T cell activation, as this process is tightly regulated by a complex array of costimulatory and coinhibitory ligands and receptors (Esensten et al., 2016). The prototypical costimulatory receptor is CD28, while the prototypical coinhibitory receptors are cytotoxic T lymphocyte antigen-4 (CTLA-4) and PD-1 (Buchbinder and Desai, 2016). Shared ligands between both types of receptors include CD80 (B7-1) and CD86 (B7-2). Stimulation of CD28 potentiates and sustains IL-2 production from T cells and prevents peripheral immunotolerance development (Bour-Jordan et al., 2011; Kow and Mak, 2013). These T cells activated by CD28-B7 signaling then mature and differentiate, subsequently inducing B cell proliferation and differentiation into plasma cells producing antigen-specific antibodies (Kow and Mak, 2013).

Although CD80 can bind to and activate CD28, significant evidence suggests that it also contributes a strong coinhibitory function. In fact, the binding of CD80 to the coinhibitory receptor CTLA-4 occurs with higher affinity than its binding to the costimulatory receptor CD28 (K_D = 0.2 and 4 μM, respectively) (van der Merwe and Davis, 2003; Collins et al., 2005; Butte et al., 2008). Furthermore, the crystal structure of the CD80-CTLA-4 complex showed that CD80 homodimers bind bivalent CTLA-4 homodimers in an unusually stable, high-avidity complex (Ikemizu et al., 2000; Stamper et al., 2001). CTLA-4-mediated coinhibitory signaling is critical for negative regulation of T cell activation and proliferation, as evidenced by the severe lymphoproliferation and multi-organ inflammatory lymphocytic infiltrates observed in mice lacking CTLA-4 signaling (Tivol et al., 1995) or in cancer patients receiving anti-CTLA-4 antibodies (Pardoll, 2012).

Even when the TCR and CD28 are ligand-activated, CTLA-4 activation can inhibit cell cycle progression and cause proliferative arrest of T cells by suppressing IL-2 production (Krummel and Allison, 1996; Walunas et al., 1996). It thus seems that CTLA-4 has a superdominant, overarching role in the regulation of T cell activation. CTLA-4 selectively reverses CD28-mediated costimulation (Walunas et al., 1996; Tai et al., 2007). Moreover, since CTLA-4 is a higher affinity CD80/86 binding partner, CTLA-4 can compete out CD28 for CD80/CD86 binding, also leading to suppression of T cell activation. Besides CD28 and CTLA-4, other costimulatory and coinhibitory receptors and ligands were later discovered and reported to modulate APC-T cell interaction (for review, see Kow and Mak, 2013; Attanasio and Wherry, 2016; Schildberg et al., 2016).

An Integrative Model of Apoptotic-Cell-Mediated Immune Evasion and Tolerance

Strong evidence indicates an essential role for the coinhibitory pathway in suppressing adaptive immune responses. First, the role of the coinhibitory signaling in regulating self-tolerance or autoimmunity is supported by the finding that various coinhibitory ligands are expressed, besides APCs, on non-hematopoietic cells which was suggested to play a role in maintaining tissue tolerance by suppressing self-reactive T cells in the periphery (Anderson et al., 2016; Schildberg et al., 2016; Ward-Kavanagh et al., 2016; Janakiram et al., 2017). Notably, some tumors and infectious pathogens evade immune recognition by exploiting such natural tolerance mechanisms (Odorizzi and Wherry, 2012; Pardoll, 2012; Wang et al., 2013; Attanasio and Wherry, 2016; Baumeister et al., 2016).

Moreover, CTLA-4-mediated coinhibition was shown to be essential for terminating T cell activation as CTLA-$4^{-/-}$ mice develop massive lymphoproliferation and early death (Tivol et al., 1995; Waterhouse et al., 1995). CTLA-4 was also suggested as a master switch for peripheral tolerance (Bluestone, 1997). Importantly, the essential role of CTLA-4 in autoimmune regulation was further highlighted by the fact that blockade of CTLA-4 signaling in multiple animal models resulted in aggravation of autoimmune diseases (Karandikar et al., 1996; Luhder et al., 1998; Chitnis et al., 2001). Moreover, CTLA-4 gene single-nucleotide polymorphisms (SNPs) in humans were associated with autoimmune disorders. For example, a SNP in the 6.1-kb (kilobase) 3′ region of the CTLA-4 gene was associated with higher risk of Grave's disease, autoimmune hypothyroidism, and type 1 diabetes mellitus (Ueda et al., 2003). Blockade of the CTLA-4- or PD-1-mediated coinhibitory signaling accelerated cardiac allograft rejection in C57BL/6 mice receiving BALB/c hearts (Ito et al., 2005). It was suggested that CTLA-4 and PD-1 may play redundant or complementary functions that differentially target different stages of tolerance (priming, activation, or reactivation of T cells, respectively) (Linsley and Ledbetter, 1993; June et al., 1994; Dahl et al., 2000).

The role of the costimulatory/coinhibitory molecules in immune responses to ACs *in vivo* (in mice) has also been suggested by the finding that antigen-coupled ACs (derived from splenocytes) induced T cell tolerance *via* enhanced IL-10 and PD-L1 expression on AC-ingesting macrophages. These ACs also enhanced Treg activation that maintained immunotolerance in that model (Kushwah et al., 2010). PD-L1 upregulation was dependent on IL-10, as IL-10 neutralization with antibodies reduced the PD-L1 response to ACs.

Since ACs were reported to induce IL-10, and given that IL-10 is mostly an anti-inflammatory cytokine as discussed above, which is important for the induction of tolerance and suppression of dendritic cell maturation (Faulkner et al., 2000; Corinti et al., 2001), it seemed plausible to suggest that adaptive immune responses to ACs could be mediated by IL-10 effects. However, that hypothesis has been difficult to

fully establish due to different conclusions from various studies. For example, ACs were shown to induce IL-10 production by monocytes (Voll et al., 1997a), but not macrophages (Fadok et al., 1998). But antigen-coupled ACs enhanced IL-10 and PD-L1 expression in AC-ingesting macrophages and induced T cell tolerance in mice (Getts et al., 2011). Importantly, however, while statistically significant, the IL-10-induced PD-L1 upregulation in that study was subtle when compared to the IL-10-neutralized control (only ~20% increase in PD-L1 mean fluorescence intensity (MFI) over the control). Conversely, using macrophage cell lines and mouse primary macrophages, we found that the upregulation of another costimulatory/coinhibitory molecule, CD80, was more pronounced (on average > 4-fold upregulation, relative to the unstimulated control) (Yakoub et al., 2018).

In another study, dendritic cells exposed to ACs exhibited reduced T cell proliferation and activation and reduced lipopolysaccharide (LPS)-triggered upregulation of the costimulatory molecule CD86 (Stuart et al., 2002). In that study, ACs did not significantly affect IL-10 levels secreted by dendritic cells, and even IL-10 neutralization by soluble IL-10R did not affect expression of the costimulatory molecules on the dendritic cells (Stuart et al., 2002). Even further, ACs could inhibit LPS-induced activation of bone marrow-derived dendritic cells derived from IL-10-deficient mice; and neutralizing another anti-inflammatory cytokine, TGF-β1, could not suppress the inhibition of dendritic cell maturation by ACs (Stuart et al., 2002), contrary to what was suggested (Chen et al., 2001). In total, these reports suggest that the effect of ACs on adaptive immune responses cannot, or cannot completely, be attributed to secondary effects of the cytokine secretion modulated by ACs, and that ACs may have direct effects on the machinery regulating adaptive immune responses.

Similar to the results in macrophages showing upregulation of CD80 by ACs (Yakoub et al., 2018), ACs were reported to induce CD80 and CD86 expression on *in vitro* differentiated human dendritic cells, which involved both soluble factors secreted and cell-cell contact to achieve the full effects of the ACs (Johansson et al., 2007; Pathak et al., 2012). Notably, however, these effects of ACs on CD80/86 expression on dendritic cells required "pre-activation" of the ACs with anti-CD3 and anti-CD28 antibodies, whereas non-pre-activated ACs showed no effect on CD80/86 expression on dendritic cells (Johansson et al., 2007; Pathak et al., 2012). These results suggest a differential AC response between dendritic cells and macrophages, as such AC activation was not required to modulate CD80 levels on macrophages (Yakoub et al., 2018). In a similar concept, "heat-stressed," but not unstressed, ACs induced the costimulatory molecules CD40, CD80, and CD86 on dendritic cells and secretion of the immunostimulatory IL-12, resulting in enhanced T cell responses (Feng et al., 2002).

While the immunosuppressive or tolerogenic effects of ACs are established by a plethora of evidence (Kabelitz and Janssen, 1997; Steinman et al., 2000; Fadok et al., 2001; Ferguson et al., 2002; Liu et al., 2002; Stuart et al., 2002; Morelli et al., 2003; Rovere-Querini et al., 2004; Gray et al., 2007), there were also instances where ACs were reported to have immunostimulatory effects (Hoffmann et al., 2000; Ignatius et al., 2000; Feng et al., 2002; Buttiglieri et al., 2003; Goldszmid et al., 2003; Ishii et al., 2003; Casares et al., 2005). It is thus possible to propose a model (**Figure 1**) whereby exposure to ACs in a non-inflammatory/non-immunostimulatory context (that does not involve immunogenic stimuli that trigger T cell activation) mounts a tolerogenic response *via* T cell inhibition through the coinhibitory pathway, whereas exposure to activated ACs in an immunostimulatory context mounts an immunogenic response *via* T cell activation through the costimulatory pathway. In support of this model is our finding that under non-immunostimulatory conditions, the AC-induced CD80 upregulation on macrophages was coupled with CD28 downregulation on T cells (Yakoub et al., 2018), which may possibly enhance the coinhibitory functions of CD80 as it binds the coinhibitory receptor CTLA-4 with much higher affinity and stability than it does the costimulatory receptor CD28 as described above.

CD80 binding to CTLA-4 conveys an inhibitory signal to T cell activation that overrides costimulatory signals, counteracting the initiation of T cell activation and proliferation and indeed inducing their apoptosis (Walunas et al., 1994). Moreover, other mechanisms could also contribute to AC-mediated suppression of adaptive immune responses. For example, ACs (derived from dendritic cells) engulfed by dendritic cells induced TGF-β1 secretion and differentiation of naïve T cells into $Foxp3^+$ Tregs (Kushwah et al., 2010). Naïve T cells can differentiate upon antigen recognition into effector T cell subsets such as Th1, Th2, and Th17, or into immunosuppressive Tregs. AC-ingesting dendritic cells suppressed the development of the effector Th17 cells, but enhanced the development of Tregs where dendritic cell-T cell interaction and the costimulatory/coinhibitory signaling were suggested to play a role in Treg induction (Yamazaki et al., 2003; Torchinsky et al., 2009).

CD80 expressed on T cells was also shown to bind PD-L1 on APCs with an affinity greater than that of CD80-CD28 binding (Butte et al., 2007; Rollins and Gibbons Johnson, 2017), which may negatively regulate T cell activation. Similarly, CD80 on APCs, which ACs upregulate (Yakoub et al., 2018), was suggested to bind PD-L1 on T cells (Schildberg et al., 2016), which may downregulate T cell activation, if not directly, indirectly by competing out CD28 and thus reducing the costimulatory signal that is essential for T cell activation and sustenance of the adaptive immune response. Notably, PD-L1 expression on parenchymal tissues including pancreatic islets mediated tolerance and inhibited self-reactive CD4 T cells (Keir et al., 2006); and interference with CD80-PD-L1 binding enhanced activation of CD4 and CD8 T cells *in vivo* and accelerated development of autoimmune diabetes in NOD mice (Paterson et al., 2011). Interestingly, it was also proposed that CD80/86 binding to CTLA-4 and PD-L1 on T cells enhances T cell motility, reducing T cell-APC contacts and the strength of the immune synapse, while enhancing contacts with, and activation of, Tregs (Dilek et al., 2013). Moreover, binding of PD-L1 on ACs to PD-1 on T cells is also possible (Kushwah et al., 2010), which may also strengthen the coinhibitory signal

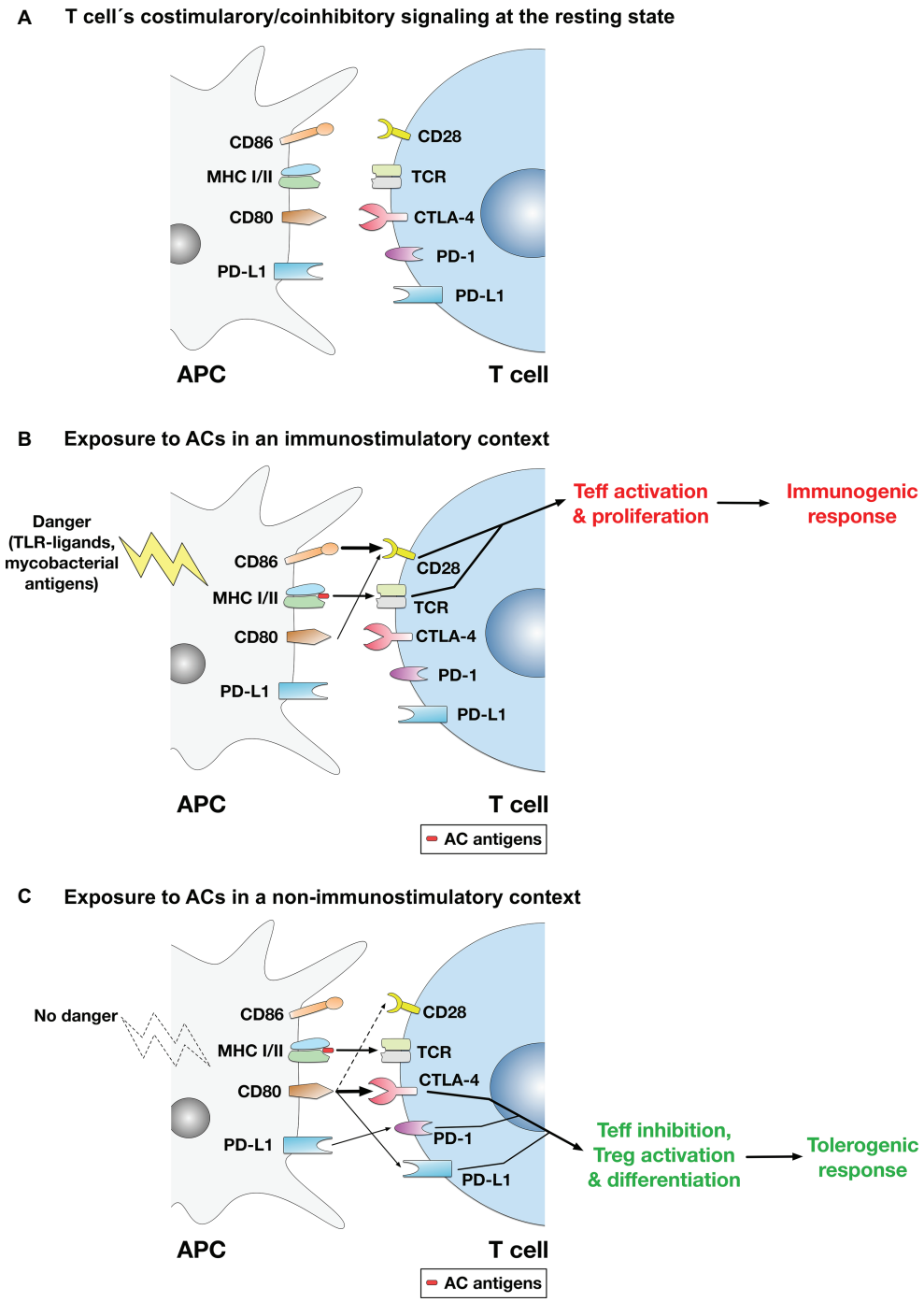

FIGURE 1 | A model for the regulation of T cell-mediated adaptive immune responses to apoptotic cells. **(A)** At the resting state, no activation of the TCR or the costimulatory pathway takes place. **(B)** In immunostimulatory contexts (such as AC ingestion by APCs in the presence of LPS, TLR ligands, or mycobacterial antigens), TCR binding to AC antigens presented in the context of MHC-I/II and the costimulatory signaling mediated by binding of CD28 by mainly CD86 (or CD86 and CD80) take place. Thus, activation of the costimulatory pathway ensures effector T cell (Teff) activation and proliferation, leading to mounting of an immune response to AC antigens. **(C)** In non-immunostimulatory contexts, such as AC ingestion by APCs in the absence of additional immunopotentiating stimuli (e.g., TLR ligands or mycobacterial antigens), the costimulatory signaling mediated by CD28 is downregulated. Rather, CD80 is upregulated, which binds mainly to CTLA-4 (CD80 binds CTLA-4 with much higher affinity than CD28), initiating the coinhibitory signaling that leads to Teff suppression and apoptosis. Even if some costimulatory signal is conveyed (by binding of some CD80 molecules to CD28), the CTLA-4-mediated coinhibitory signal predominates and usually overrides costimulation. Other signals are possible (e.g., binding of CD80 to PD-L1 on T cells or binding of PD-L1 on APCs to PD-1 on T cells) and might have some contribution to the overall coinhibitory signaling delivered to T cells, although their significance and relative contribution have yet to be established. Overall, the coinhibitory signaling to T cells triggers the inhibition of Teff functions; and in this context, differentiation of Tregs may also be enhanced.

to T cells, although its significance needs to be established as previously discussed.

Whether ACs would induce a tolerogenic or immunogenic response may depend on presence of immunostimulatory conditions or cues, including: (1) the type of cell death induced in the ACs, e.g., caspase-dependent or independent (Larmonier et al., 2002); (2) type of the apoptosis-inducing agent or drug used in the experimental model (Casares et al., 2005; Obeid et al., 2007); (3) the stage of apoptosis (early vs. late) in the ACs (Weyd et al., 2013); (4) type of the apoptotic corpse (ACs or apoptotic blebs) ingested by the APC (Fransen et al., 2009); (5) cell-type of the ACs ingested by the APCs (Kushwah et al., 2010); (6) secretion of T cell immuostimulatory cytokines, such as TNF-α, or TLR ligands (Clayton et al., 2003); (7) type and ratio of the APCs (dendritic cells or macrophages) present in a particular tissue site (Denning et al., 2007); or (8) presence of potentially immunogenic or immunogenizing infectious pathogen (e.g., mycobacterial) antigens in the tissue microenvironment (Espinosa-Cueto et al., 2017).

While the distinction between APC types (dendritic cells or macrophages) in terms of the type of immune response (immunogenic vs. tolerogenic) to ACs cannot be completely settled given the varying reports thus far in this regard, there is still some preliminary evidence to propose that macrophages might mainly mediate tolerogenic responses to ACs while dendritic cells might mainly mediate the immunogenic responses. Dendritic cells and macrophages exhibit distinct locales and may thus mediate differential, locale-specific, AC responses (T cell activation or inhibition). For example, in the intestinal lamina propria, both APC types present commensal microbe and dietary antigens to T cells, with dendritic cells inducing effector Th17 T cells, and macrophages inducing Tregs (Denning et al., 2007). Tregs induced by AC-presenting macrophages showed induced anti-inflammatory cytokine production and reduced immunostimulatory cytokines, and displayed an anergic phenotype after restimulation with the antigen (Denning et al., 2007). Because macrophages are more abundant than dendritic cells in the lamina propria, T cell tolerance thus becomes the predominant response in that context. Importantly, macrophages were shown to be essential for clearing tumor ACs introduced into mice and eliciting tolerogenic responses to the ACs (Asano et al., 2011); as ablation of the spleen marginal zone macrophages in these mice diminished the immunosuppressive potential of the ACs and enabled triggering of an immune response to ACs (McGaha et al., 2011). It thus seems possible that the immunosuppressive or tolerogenic response to ACs is mainly mediated by macrophages as the APCs.

ROLE OF APOPTOTIC-CELL-MEDIATED IMMUNOSUPPRESSION IN DISEASES

An abundance of ACs (~70 billion) is produced daily in humans and defective clearance of these ACs has been associated with diseases, primarily autoimmune conditions. Therefore, we will briefly discuss some disease conditions, where we propose that the immunosuppressive properties of ACs may be exploited for therapeutic purposes.

Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic systemic autoimmune disorder characterized by progressive inflammatory bone and joint destruction. Immunologically, the autoimmune responses are mediated by either circulating autoantibodies directed against citrullinated peptides and rheumatoid factor or complement protein C3 (Abdolmaleki et al., 2018). The destructive autoimmune responses in RA are maintained by IL-6 and TNF-α secreted by tissue macrophages triggering the MMP and RANK-ligand-supported activation of osteoclasts (An et al., 2016). In RA patients, the sustained joint inflammation is maintained by an abnormal state of aberrant cell survival caused by perpetual T cell activation resulting in stimulation and proliferation of fibroblasts which was termed "apoptosis resistance" (Malemud, 2018). In line with these observations, anti-apoptotic proteins were shown to be upregulated in RA synovial fluids (reviewed in Williams et al., 2018). Therefore, induction of apoptosis has the potential to reduce joint damage and to further modulate autoimmune responses in RA by modulating the coinhibitory signaling to T cells as previously described. Experimental apoptosis induction has therefore been considered as potential therapeutic avenue in RA, e.g., by targeting intracellular apoptotic inhibitory molecules, but thus far has not reached clinical trials (Williams et al., 2018).

Systemic Lupus Erythematosus

SLE is a chronic systemic autoimmune disorder characterized by the presence of circulating nuclear antigens, including DNA and nucleosomes, and of autoantibodies against these nuclear antigens (Poon et al., 2014). SLE affects the skin, lungs, kidneys, and central nervous system. Impaired engulfment of ACs by phagocytes leads to accumulation of ACs in the lymph nodes and blood and in the skin after UV exposure. Impaired AC clearance eventually leads to secondary necrosis, which allows intracellular (self-) antigens, normally hidden within the AC to be exposed extracellularly, and thus recognizing these self-antigens by the immune system, causing the production of autoantibodies and autoimmunity. While mostly suggested to be caused by defective AC engulfment, super-stimulatory APCs that lead to hyperactive T cells were also suggested to recapitulate SLE in a mouse model (Zhu et al., 2005). Thus, targeting APC activity which is normally modulated by ACs in normal physiological conditions might be a successful strategy for therapy development for SLE.

Sjögren's Syndrome

Sjögren's Syndrome (SS) is an autoimmune disease targeting the salivary and lacrimal glands resulting in chronic dryness of mouth and eyes (Ainola et al., 2018). In SS, apoptotic particles blebbing from apoptotic epithelial cells, possibly caused by defects in the production of sex hormones, that contain typical SS autoantigens such as hY1RNA were shown to contribute to disease pathology (Ainola et al., 2018). In line with this increase in apoptosis rates, enhanced levels of both Fas and FasL were reported in salivary gland tissues, but not in lacrimal gland tissues or peripheral blood

lymphocytes of patients with SS, implicating Fas-mediated apoptosis in the destruction of salivary gland tissue (Ishimaru et al., 2001; Bolstad et al., 2003). The presence of both autoantigens and adjuvanting nucleic acids in these apoptotic particles was shown to stimulate plasmacytoid dendritic cells in salivary glands *via* TLR7 and TLR9, resulting in the activation of autoreactive T and B cells (Ainola et al., 2018). However, Ishimaru et al. reported that mice treated with an anti-murine FasL antibody (to suppress Fas-mediated apoptosis) unexpectedly showed exacerbations of the autoimmune lesions in both salivary and lacrimal glands (Ishimaru et al., 2001). Therefore, both the role of apoptosis in the pathology of SS and the therapeutic potential of ACs in the treatment of SS are poorly understood, warranting further investigations.

Autoimmune Lymphoproliferative Syndrome

Autoimmune Lymphoproliferative Syndrome (ALPS) is an inherited autoimmune disorder characterized by spleno- and hepatomegaly, lymphadenopathy, autoimmune lesions in multiple organs, as well as autoimmune hemolytic anemia, thrombocytopenia, or leukocytopenia, caused by cell-type specific autoantibody production (Turbyville and Rao, 2010; Price et al., 2014; Yamada et al., 2017). In ALPS patients, these symptoms are caused by spontaneous mutations in the Fas, FasL, or caspase 10 genes, resulting in a defective apoptosis of antigen-activated T- and B cells in the periphery, and the impaired limitation of immune responses (Yamada et al., 2017). Since the pathology of ALPS is caused by a disruption of lymphocyte apoptosis, it is plausible to speculate that a decrease in production of ACs (which exert immunoinhibitory/anti-inflammatory effects as discussed) may contribute to the autoimmune pathology in ALPS patients. The role of ACs in ALPS pathogenesis and its possible exploitation for therapy is thus an interesting area for future research.

Diabetes Mellitus

Type 1 diabetes mellitus can be caused by autoimmune responses to pancreatic beta-cell antigens resulting in insulin deficiency and hyperglycemia. While it was suggested that inefficient clearing of apoptotic pancreatic cells resulting in the release of damage-associated molecular patterns (DAMPs) in combination with autoantigens may contribute to the pathology of type 1 diabetes (Heimberg et al., 2001; O'Brien et al., 2006), ACs were also suggested as a tool to induce tolerance to beta-cell self-antigens. Indeed, ACs (apoptotic beta-cell infusion) could suppress beta-cell antigen-specific $CD4^+$ T cell proliferation and delay the onset of diabetes in the diabetes-susceptible, autoimmune (NOD) mice (Xia et al., 2007; Marin-Gallen et al., 2010). Therefore, ACs show a promising potential for the treatment of type 1 diabetes.

Transplantation

After transplantation, immunosuppressive drugs are given to the patients, to induce tolerance and prevent graft rejection. However, these drugs show many undesirable and potentially dangerous side effects. Thus, ACs were suggested to be used as possibly side effect-free tolerogenic tools (Morelli and Larregina, 2010). Donor's ACs given to transplant patients may tolerize or repress the recipient's immune responses to the transplant, prevent graft-versus-host reactions, and enhance graft survival (Kleinclauss et al., 2006; Wang et al., 2006, 2009; Bittencourt et al., 2011).

Cancer

Anti-tumor chemotherapeutic agents lead to production of massive cytotoxicity and generation of ACs. Given the adaptive immunosuppressive and tolerogenic effects of ACs, it is plausible to hypothesize that the apoptosis induced, and the ACs produced, by cancer treatments contribute to tumor survival indirectly by dampening immune responses to cancer cell antigens carried on these ACs. Thus, functionally blocking ACs in cancer may help reduce these undesirable effects of antineoplastic agents on anti-tumor immunity. In fact, some therapeutic strategies to target the coinhibitory molecules that mediate the adaptive immune responses to ACs have been investigated for their possible beneficial effects on anti-tumor immunity (Chen, 2004; Hirano et al., 2005; Curran et al., 2010).

CONCLUDING REMARKS

While ACs have been investigated as tolerogenic or immunosuppressive "vaccines," understanding the molecular mechanisms of the ACs' immunomodulating effects, especially their interaction with the costimulatory/coinhibitory pathway, may encourage attempts at targeting specific molecules that ACs exploit in mediating their effects. In general, the costimulatory/coinhibitory pathway has been explored as target for therapeutic purposes. For example, targeting of either CD28, PD-1, PD-L1, or CTLA-4 has been investigated and some therapies targeting this machinery are already in clinical use (e.g., the anti-CTLA-4 antibody Ipilimumab for the treatment of advanced metastatic melanoma, and the anti-PD-1 antibodies Pembrolizumab and Nivolumab for the treatment of advanced melanoma, advanced non-small cell lung cancer, and metastatic renal cell carcinoma) (Dilek et al., 2013).

Although the costimulatory/coinhibitory pathway has been a tempting target for disease therapy, some challenges remain and are yet to be overcome in the future to enable full harnessing of the therapeutic potential of this pathway. For example, some therapies targeting the costimulatory/coinhibitory pathway have even been discontinued in medical practice due to limited effectiveness (Smith et al., 2013), or have failed clinical trials at early stages (Khoury and Sayegh, 2004). Some proposed therapies can also pose significant risks (Frebel and Oxenius, 2013). This all reflects the fact that we have not yet reached complete and thorough understanding of the costimulation/coinhibition pathways and their intricate interactions with diseases. In conclusion, this perspective proposes a model to understand how ACs regulate the costimulatory/coinhibitory signaling pathways of regulating T cell activation in order to suppress adaptive immune responses, which may facilitate harnessing these molecular mechanisms in therapy development for various immunopathological conditions.

AUTHOR CONTRIBUTIONS

AY conceived and wrote the first draft of this manuscript. SS reviewed, critiqued, and revised the first draft. AY and SS rewrote and finally revised the final manuscript.

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The Influence of Overweight and Obesity on Treatment Response in Juvenile Idiopathic Arthritis

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Background: There is evidence that obesity could be a risk factor for the severity and response to treatment in adult patients with rheumatoid arthritis (RA) due both to the mechanical effect of overweight and to the potential pro-inflammatory effects of cytokines produced by adipose tissue.

Objectives: To evaluate the role of overweight and obesity in a cohort of young patients with juvenile idiopathic arthritis (JIA) in terms of incidence, disease activity, outcome, and response to treatments.

Methods: This single-center retrospective cohort study evaluated 110 children affected by JIA under treatment with conventional disease-modifying antirheumatic drugs (DMARDs) and biologic agents. Body mass index (BMI) categories of 5–84th (normal weight), 85–94th (overweight), and ≥95th (obese) percentile were used. Patients with systemic JIA, uveitis, chronic comorbidities, or under other potentially confounding systemic treatments were excluded. Uni- and multivariate analyses were performed.

Results: One hundred and ten JIA patients (polyarticular $n = 50$, oligoarticular $n = 38$, psoriatic $n = 12$, enthesitis-related arthritis $n = 8$, undifferentiated $n = 2$) were enrolled in the study, 75% girls and 25% boys. The mean age at treatment onset was 6.09 years. Baseline BMI was ≥5th and ≤84th percentile in 80 patients, 85–94th in 27, and ≥95th in 3.

We did not observe a significant association between BMI and erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), or number of active joints at baseline, while involvement of the joints of lower limbs was significantly greater ($p = 0.025$) in overweight/obese patients. However, a trend toward lower remission rates and higher number of relapses, both after DMARDs and biologics, in patients with higher BMI was observed.

Conclusion: This study focuses on the relationship between overweight/obesity and JIA. A significant correlation between obesity and a greater involvement of the joints of the lower limbs at baseline was demonstrated. Furthermore, our data suggest that obesity could negatively influence the course of the disease as well as treatment response.

Keywords: obesity, overweight, body mass index, juvenile idiopathic arthritis, disease-modifying antirheumatic drugs, biologics

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INTRODUCTION

Obesity is a medical condition characterized by an excess in body fat accumulation associated with a potentially negative impact on health (Fact Sheet on Obesity and Overweight, 2006). Body mass index (BMI, weight/height^2, kg/m^2) is the most widely used method to define obesity and overweight, although it does not take into account body composition and the share of adiposity (Cole et al., 2000). Referring to the US Centers for Disease Control and Prevention (CDC) BMI-for-age growth chart, obesity is defined among subjects aged 2 to 19 years in the presence of a BMI at or above the sex-specific 95th percentile, while overweight is defined as a BMI at or above the 85th percentile and below the 95th (Krebs et al., 2007; Katzmarzyk et al., 2015).

The prevalence of overweight and obesity in European countries accounts for 53.1%, and in the pediatric age, it is about 20%, ranging from 40% in Southern Europe to less than 10% in Northern European countries (Ahrens et al., 2014; Marques et al., 2018).

An increased accumulation of body fat represents a significant risk factor for metabolic complications and a modifiable variable for a number of chronic diseases (Finucane et al., 2011). A wealth of studies both in mice and in humans have shown increased levels of pro-inflammatory cytokines in adipose tissues, including TNF-α, interleukin 6 (IL-6), IL-1β, and several other inflammatory proteins (Hotamisligil et al., 1995; Berg and Scherer, 2005).

Recently, evidence has emerged in favor of the potential negative influence of obesity on rheumatoid arthritis (RA) disease progression, disease severity, and treatment response (Klaasen et al., 2011; Gremese et al., 2013; Heimans et al., 2013; Ellerby et al., 2014; Sandberg et al., 2014; Liu et al., 2017). Liu et al. (2017) in their meta-analysis documented the unfavorable effect of obesity on achieving remission in RA and its impact on disease activity and patient-reported outcomes during therapy. Conversely, only few data have been published about the effect of obesity on juvenile idiopathic arthritis (JIA).

Thus, the goal of our study was to investigate the relationship between BMI and JIA in terms of baseline disease activity and response to classic disease-modifying antirheumatic drugs (DMARDs) and biological agents.

MATERIALS AND METHODS

A retrospective study was conducted with an analysis of the medical records of children with JIA classified according to International League of Associations for Rheumatology (ILAR) criteria that were seen in the Pediatric Rheumatology Unit of Anna Meyer Children Hospital between January 2009 and January 2017 (Petty et al., 2004). Only patients who needed systemic drugs were selected. Arthritis related with uveitis and systemic JIA subtypes were considered exclusion criteria.

Demographic data, age at disease onset, anthropometric measurements, disease duration, laboratory data, joint involvement, and medications including DMARDs and biologic drugs were extracted from charts and inserted into a dedicated database. Patients were usually evaluated every 3 months with a thorough physical examination inclusive of assessment of the number and the type of active joints (swelling or both tenderness and limited range of motion) and laboratory investigations.

Height was measured with a wall-mounted stadiometer to the nearest 0.1 cm, while for weight, a calibrated beam balance platform scale to the nearest 0.1 kg was used. BMI was calculated as weight in kilograms divided by height in square meters. BMI-for-age percentiles were computed using the CDC reference values. Normal BMI was defined as the 5th percentile to less than the 85th percentile, overweight as the 85th to less than the 95th percentile, and obese as equal to or greater than the 95th percentile (Kuczmarski et al., 2002).

Relevant laboratory variables included erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). Clinically inactive disease status was defined as no joints with active disease, an ESR of ≤20 mm/h, and CRP ≤0.5 mg/dl.

The data were analyzed using the person time method. Patients on treatment with first-line drugs (DMARDs) were followed from the start of treatment up to the date of remission or last follow-up. The remission rates for person-months were therefore calculated. The rates of relapse were calculated as the number of relapse (first occurrence) after remission on person-months at risk. The amount of person-months was the sum of months at risk from the date of remission to the date of relapse or of the last follow-up. For subjects eligible for treatment with biological drugs, remission and relapse post-remission rates were calculated using the same method. We performed univariate and multivariate analysis and included in the models clinically and statistically relevant variables as covariate. All analyses were performed with the STATA 11 software.

Since we used deidentified data, according to national regulation, no ethics committee approval or informed consent was needed.

RESULTS

Demographic and clinical data are reported in **Table 1**. One hundred and ten JIA patients (polyarticular $n = 50$, oligoarticular $n = 38$, psoriatic $n = 12$, enthesitis-related arthritis $n = 8$, undifferentiated $n = 2$) were enrolled in the study, 75% girls and 25% boys. The mean age at conventional DMARDs treatment onset was 6.09 years. Baseline BMI was included between the 5th and 84th percentile in 80 patients, 85–94th in 27, and ≥95th in 3.

At baseline, ESR and CRP values were increased, although not significantly, in overweight/obese compared with healthy weighted individuals ($p = 0.373$ and 0.176 for ESR and CRP, respectively). The number of active lower limb joints was significantly lower ($p = 0.028$) in healthy weighted than in overweight/obese individuals (**Table 1**).

Sixty-nine patients out of 110 achieved remission on conventional DMARDs. The remission rate was 93.88/1000 person-months, with higher remission rates in healthy weighted children (98.87) than in obese children (83.58). At univariate analysis, however, the difference did not reach statistical significance. The hazard ratio (HR) in obese children compared with healthy weighted children was 0.8, CI 95% 0.46–1.40. An abnormal CRP value was the only covariate influencing the remission rate, close to statistical significance (HR 1.79, CI 95% 0.98–3.26) (**Table 2**).

TABLE 1 | Baseline characteristics of the population.

Variables		BMI 5–84th percentile	BMI ≥85th percentile	Total	*p*
Age (years) at enrollment	Mean (SD)	6.0 (4.04)	6.2 (3.4)	6.0 (3.8)	0.7782
Age at puberty (years)	Mean (SD)	11.4 (1.7)	10.5 (1.4)	11.1 (1.7)	0.1197
Gender	Females	57 (71.2%)	26 (86.6%)	83 (75.4%)	0.094
	Males	23 (28.7%)	4 (13.3%)	27 (24.5%)	
ESR (mm/h)	0–19	23 (30.2%)	6 (21.4%)	29 (27.8%)	0.373
	≥ 20	53 (69.7%)	22 (78.5%)	75 (72.1%)	
CRP (mg/dl)	0.0–0.49	32 (43.2%)	8 (28.5%)	40 (39.2%)	0.176
	≥ 0.5	42 (56.7%)	20 (71.4%)	62 (60.7%)	
Number of active joints	0–4	49 (61.2%)	13 (43.3%)	62 (56.3%)	0.092
	≥ 5	31 (38.7%)	17 (56.6%)	48 (43.6%)	
Number of active joints in lower limbs	0–4	66 (88%)	18 (69.2%)	84 (83.1%)	0.028*
	≥ 5	9 (12%)	8 (30.7%)	17 (16.8%)	
JIA category	ERA	6 (7.5)	2 (6.6)	8 (7.2)	0.645
	Undifferentiated	1 (1.2)	1 (3.3)	2 (1.8)	
	Oligoarticular	31 (38.7)	7 (23.3)	38 (34.5)	
	Polyarticular RF neg	33 (41.2)	16 (53.3)	49 (44.5)	
	Polyarticular RF pos	1 (1.2)	0 (0.0)	1 (0.9)	
	Psoriatic	8 (10.0)	4 (13.3)	12 (10.9)	

**Statistical significance.*

TABLE 2 | Remission with classical DMARDs.

Variables	*n* events	Person-months	Incidence rate × 1000 person-months	Hazard ratio (CI 95%)
All patients	69	734.9	93.8	
BMI				
5–84th percentile	48	485.4	98.8	1
≥85th percentile	17	203.4	83.5	0.8 (0.46–1.40)
Age at puberty (years)				
0–10	9	103.2	87.2	1
≥11	13	171.7	75.7	0.74 (0.3–1.84)
ESR (mm/h)				
0–19	15	154.2	97.2	1
≥20	50	498.8	100.2	1.23 (0.67–2.27)
CRP (mg/dL)				
0.0–0.49	20	246.98	80.98	1
≥0.50	43	391.64	109.80	1.79 (0.98–3.26)
Number of active joints				
0–4	39	371.2	105.0	1
≥5	30	363.6	82.4	0.80 (0.48–1.31)
Number of active joints in lower limbs				
0–4	52	576.3	90.2	1
≥5	11	109.5	100.4	1.28 (0.65–2.5)

Incidence rate × 1,000 person-months and hazard ratio. Univariate analysis.

The multivariate analysis showed a significant effect of elevated CRP value on the remission rate (HR 2.21, CI 95% 1.12–4.35). Lower remission rates were observed in children with an overweight/obese BMI and a higher number of active joints (more than five) at baseline as well as in patients with increased values of ESR and CRP compared to normal values (HR 1.77, CI 95% 0.62–5.06 and HR 1.72, CI 95% 0.75–3.98, for ESR and CRP, respectively), although without a statistical significance (**Tables 3** and **4**). Thirty-five patients out of 69 relapsed after remission, with a relapse rate of 13.40/1000 person-months. The relapse rate was slightly higher in obese

TABLE 3 | Remission with classic (DMARDs).

Variables		Hazard ratio	(CI 95%)
BMI (percentile)	0–84th	1	
	≥85th	0.77	0.42–1.43
CRP (mg/dL)	0.0–0.49	1	
	≥0.50	2.21	1.12–4.35
Number of active joints	0–4	1	
	≥5	0.67	0.37–1.20

Multivariate analysis. Cox regression.

TABLE 4 | Relapse post-remission with classic DMARDs.

Variables	*n* events	Person-months	Incidence rate × 1000 person-months	Hazard ratio (CI 95%)
All patients	35	2611.0	13.4	
BMI				
0–84th percentile	22	1645.0	13.3	1
≥85th percentile	11	696.8	15.7	1.25 (0.6–2.6)
Age at puberty (years)				
0–10	8	268.7	29.7	1
≥11	9	538.8	16.7	0.58 (0.22–1.50)
ESR (mm/h)				
0–19	4	515.7	7.7	1
≥20	29	1925.2	15.0	1.77 (0.62–5.06)
CRP (mg/dL)				
0.0–0.49	7	799.01	8.76	1
≥0.50	26	1607.34	16.18	1.72 (0.75–3.98)
Number of active joints				
0–4	19	1407.9	13.5	1
≥5	16	1203.1	13.3	0.92 (0.47–1.82)
Number of active joints in lower limbs				
0–4	27	1910.8	14.1	1
≥5	5	500.3	9.9	0.68 (0.26–1.78)

Incidence rate × 1000 person-months and hazard ratio. Univariate analysis.

children (15.79) than in healthy weighted children (13.37). The HR was 1.25, CI 95% 0.6–2.6.

The Cox regression showed a higher HR of relapse rate in overweight/obese children compared to healthy weighted children, and in children with abnormal values of CRP compared to those with normal values. However, all HR estimates did not reach statistical significance (**Table 5**).

Forty-eight patients required a second-line treatment: 27 etanercept (13 in overweight/obese), 19 adalimumab (5 in overweight/obese), and 2 abatacept (all in healthy weighted children).

Remission rate with second-line therapy was 122.12/1,000 person-months, higher in healthy weighted children (146.21) than in obese children (87.45). The HR was 0.61, CI 95% 0.31–1.20.

Remission rates were lower in individuals with several active joints when compared with those with few active joints (>4 vs. 0–4). The corresponding HR were 0.59, CI 95% 0.32–1.08 for more than 4 active joints vs. 0–4, and 0.46, CI 95% 0.19–1.13 (>4 vs. 0–4 joints) considering only lower limbs (**Table 6**). The multivariate analysis confirmed the results of the univariate calculation (**Table 7**).

TABLE 5 | Relapse post-remission with classic DMARDS.

Variables		Hazard ratio	(CI 95%)
BMI (percentile)	0–84th	1	
	≥85th	1.35	0.61–2.98
CRP (mg/dL)	0.0–0.49	1	
	≥0.50	1.90	0.80–4.48
Number of joints involved	0–4	1	
	≥5	0.73	0.34–1.57

Multivariate analysis. Cox regression.

DISCUSSION

We investigated the negative effect exerted by obesity on JIA as suggested by data on adults affected by RA. Although JIA shares some features with RA, they should be considered distinct diseases. JIA represents the most common rheumatic disease of childhood with a prevalence of 16–150 cases per 100,000 children (Prakken et al., 2011). Regardless of the differences in the underlying pathogenesis of the various types of JIA, the immunopathology involves a predominant abnormality of the adaptive immune system with a consistent overproduction of pro-inflammatory cytokines, many of which, such as TNF-α, IL-1, and IL-6, have been shown to be increased also in the presence of adipose expansion.

We observed a very small number of obese subjects in our cohort of patients; therefore, we divided it into two groups, one comprising children with a "healthy weight" (BMI < 85th percentile) and the other comprising children with an "increased weight" (BMI ≥ 85th percentile). Overweight/obesity rate in our study population is 27%, reflecting the pediatric prevalence of this condition in our country. We also excluded patients affected by systemic subtype, for its clinical heterogeneity compared to the other JIA subtypes, and patients with uveitis, which may require further treatments despite a good arthritis control, to avoid confounding factors.

At baseline, we observed a negative influence of obesity/overweight on disease activity. Our JIA overweight/obese children showed a tendency to have a greater number of active joints, in particular those of lower limbs, and higher values of ESR and CRP compared to healthy weight subjects.

Few data are available about the impact of obesity on JIA. A first study by Pelajo et al. analyzed 154 JIA subjects, 18% of whom were obese and 12% were overweight; no association was found

TABLE 6 | Remission with second-line therapy (biologics).

Variables	*n* events	Person-months	Incidence rate × 1000 person-months	Hazard ratio (CI 95%)
All patients	43	352.1	122.1	
BMI				
0–84th percentile	27	184.6	146.2	1
≥85th percentile	14	160.1	87.4	0.61 (0.31–1.20)
Age at puberty (years)				
0–10	10	114.1	87.5	1
≥11	13	91.3	142.2	1.56 (0.66–3.68)
ESR (mm/h)				
0–19	13	96.3	134.9	1
≥20	22	216.5	101.6	0.81 (0.40–1.63)
CRP (mg/dl)				
0.0–0.49	20	155.93	128.26	1
≥0.50	15	155.04	96.75	0.76 (0.38–1.51)
Number of active joints				
0–4	20	119.3	167.5	1
≥5	23	232.7	98.8	0.59 (0.32–1.08)
Number of active joints in lower limbs				
0–4	31	200.0	154.9	1
≥5	6	67.9	88.3	0.46 (0.19–1.13)

Incidence rate × 1000 person-months and hazard ratio. Univariate analysis.

TABLE 7 | Remission with second-line therapy (biologics).

Variables		Hazard ratio	(CI 95%)
BMI (percentile)	0–84th	1.0	
	≥85th	0.49	(0.19–1.24)
Age at puberty (years)	0-10	1.0	
	≥11	1.93	(0.77–4.80)
Number of active joints	0–4	1.0	
	≥5	0.49	(0.20–1.20)

Multivariate analysis. Cox regression.

between obesity and disease activity in terms of Juvenile Arthritis Disease Activity Score 27, physician's assessment of disease activity, parent's assessment of child's well-being, CRP, ESR, and number of active joints (Pelajo et al., 2012).

On the other hand, Amine et al. (2011) in their series of 58 Moroccan JIA patients, characterized by high BMI prevalence (41.4% overweight and 22.4% obese), observed a correlation between overweight and functional limitation and severe joint pain.

In 2016, Makay et al. studied 72 patients with enthesitis-related arthritis, 27% of whom have a BMI ≥ 85th percentile. In subjects with increased BMI, they observed a higher frequency of tarsitis and ankle arthritis at baseline, while sacroiliac, hip, and entheses involvement as well as ESR values were similar to those with a BMI < 85th percentile (Makay et al., 2016).

Compared to adult RA, the correlation between disease activity and obesity seems to be less evident in JIA. Furthermore, data regarding the influence of BMI in treatment responses and outcome are still very poor in the pediatric age. In RA, obesity appears to be a strong predictor of worse treatment responses, decreasing the chances of achieving good disease control during the early phase, in which most of the patients receive DMARDs (Sandberg et al., 2014). Some recent studies have shown that the effect of TNF blockage also appears to be impaired in obese RA patients, while no significant difference between obese and non-obese were seen in patients treated with abatacept and tocilizumab (Klaasen et al., 2011; Gremese et al., 2013; Iannone et al., 2015; Kim et al., 2016; Shan and Zhang, 2018). The modification in the pharmacokinetics and pharmacodynamics of drugs related to the excess of fat has been hypothesized to influence the response to therapy. On the other hand, obesity may mask early identification of active joints, delaying the beginning of the correct treatment (Barnabe et al., 2014).

In pediatrics, only the study by Makay et al. (2016) evaluated the response to therapy in patients with enthesitis-related arthritis with different BMI, reporting a failure in achieving a clinically inactive disease in children with a higher BMI treated with DMARDs or anti-TNF drugs, suggesting a negative effect of obesity on disease outcome.

In our JIA population, obesity/overweight corresponds to a worse trend in the therapeutic response both to first-line treatments and to anti-TNF drugs, although without reaching statistical significance.

Despite the fact that our data do not identify obesity as an independent risk factor for JIA, they suggest a negative influence on disease activity at baseline and an unfavorable impact on the therapeutic response to conventional DMADs and anti-TNF drugs. These observations may take into consideration weight management with dietary and physical activity interventions as part of the treatment strategy for obese/overweight patients.

Limitations of our study include the fact that few of our patients were obese and that the sample size of our population was relatively small; this may have hampered the achievement of statistical significance of several comparisons. Nonetheless, our study is one of the few that have investigated such factors and deserves further investigations.

AUTHOR CONTRIBUTIONS

TG was in charge of project planning and paper writing. FT and IM were in charge of data collection. SM and MF performed the statistical analysis. GS and RC were the coordinators.

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MINI REVIEW
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Mini Review: New Treatments in Psoriatic Arthritis. Focus on the IL-23/17 Axis

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Psoriasis, an inflammatory skin disease, and psoriatic arthritis (PsA), an inflammatory arthritis, share clinical, genetic, and pathogenic factors and may be summed as one disease, the psoriatic disease. Interleukin (IL)-17 plays a major role in the development of both psoriasis and PsA. IL-23 is important in the proliferation and maintenance of IL-17, and therefore, cytokines of the IL-23/IL-17 axis attracted much interest as therapeutic targets in psoriasis and PsA. Therapeutic agents targeting the IL-23/IL-17 axis have been proven to be very effective in psoriasis and PsA, some are already in the therapeutic armamentarium and others are in the development. Some agents, target IL-23 and others IL-17 and include anti-IL-12/IL-23 p40 (ustekinumab, briankizumab), anti-IL-23p19 (guselkumab, tildrakizumab, risankizumab, brazikumab, mirikizumab), anti-IL-17A (secukinumab, ixekizumab), dual anti-IL-17A and anti-IL-17F (bimekizumab), or anti-IL-17 receptor (brodalumab) monoclonal antibodies. Janus tyrosine kinase(JAK) inhibitors also directly affect IL-23 and, thus, IL-17. After the first-generation pan-JAK inhibitors have been shown efficacy (tofacitinib, baricitinib), new-generation selective JAK inhibitors (filgotinib, upadacitinib) are under investigation in psoriasis and PsA.

Keywords: anti-IL-17, cytokine, IL-17, monoclonal antibodies, psoriatic disease

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INTRODUCTION

Psoriatic arthritis (PsA) is an inflammatory joint disease associated with psoriasis, a common inflammatory skin disease with a prevalence 2% to 3% worldwide (Rachakonda et al., 2014). PsA occurs in a third of the patients with psoriasis (Mease et al., 2013) and apart from psoriasis, manifests with peripheral arthritis, enthesitis, dactylitis, spine involvement, and uveitis. The presence of enthesitis, which may be subclinical is very common in psoriasis and substantially increase the frequency of PsA in patients with psoriasis (Sakkas et al., 2019; Mease et al., 2019b) The pathogeneses of both PsA and psoriasis are incompletely understood, but innate and adaptive cells and proinflammatory cytokines are involved, particularly the interleukin(IL)-23/IL-17 axis (Sakkas and Bogdanos, 2017). The shared pathophysiological and clinical features of psoriasis and PsA have led some investigators to consider these two entities as one, the psoriatic disease. Recent studies, reporting autoreactive T cells recognizing LL37, ADAMTSL5, and PLA2G4D (phospholipase A2 group IVD) and producing interleukin(IL)-17 (Lande et al., 2014; Cheung et al., 2016; Hawkes et al., 2017) bring forward the autoimmune element in the pathogenesis of psoriatic disease.

IL-17 Cytokine

The nature of IL-17 has been described in detail elsewhere (Wright et al., 2008 and **Supplementary Text** in gray) The IL-17 family consists of six proteins that share homology among them, and are known as IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. Among IL-17 family members, IL-17F shares the strongest amino acid sequence with IL-17A whereas IL-17E (IL-25) is the most distant from IL-17A. The IL-17 receptor differs from other cytokine receptors and consist of five members, IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE (Chang and Dong, 2011; Veldhoen, 2017). IL-17A and IL-17F are secreted by the same cell types, as homodimers or IL-17A/IL-17F heterodimers and signal through the constitutively expressed IL-17RA paired with the inducible IL-17RC (Wright et al., 2008).

IL-17 is produced by T (Th17) cells, γδ T cells, natural killer T (NKT) cells, NK cells, and type 3 innate lymphoid cells, which also can produce IL-17F, and IL-22. Th17 cells are differentiated from naïve T cells by the action of any of these three cytokine combinations, IL-6 and TGFβ, IL-21 and TGFβ, or IL-6, IL-23, and IL-1β (Veldhoen, 2017). The expression of IL-22 can be regulated separately. IL-22 is induced by IL-23 and signals through the IL-22Rα/IL-10Rβ heterodimer. IL-23 consists of p40 (which is also a subunit of IL-12, IL-12p40) and p19 subunit (IL-23p19) and signals through its receptor IL-23R paired with IL-12Rβ1 and is required for the proliferation and survival of Th17 cells (Teng et al., 2015).

IL-17 *in vitro* can induce the production of proinflammatory cytokines, such as IL-6, IL-1, GM-CSF, G-CSF, and enhances the expression of several chemokines involved in chemoattraction of neutrophils, monocytes, and lymphocytes. IL-17A and IL-17F induce similar cytokine profiles with IL-17F being less effective in macrophage cytokine production and act in synergy with TNFα. However, they may also have distinct roles. In a colitis model caused by dextran sulfate sodium, IL-17A deficiency enhanced colitis, whereas IL-17F deficiency reduced colitis. In addition, in an asthma model, IL-17A deficiency reduced Th2 responses, whereas IL-17F deficiency enhanced Th2 responses (Yang et al., 2008).

IL-25(IL-17E) is produced by eosinophils, mast cells, basophils, epithelial cells, and signals through IL-17RA paired with IL-17RB to promote Th2 cell immune responses. IL-17C signals through the IL-17RA/IL-17RE complex in Th17 cells and promotes proinflammatory responses (Chang et al., 2011). IL-17D is preferentially expressed in skeletal muscles, adipose tissue, and brain and induces IL-8 and IL-6 production in endothelial cells but inhibits hemopoiesis (Starnes et al., 2002). IL-17B and IL-17C are proinflammatory cytokines, as they exacerbate collagen-induced arthritis in mice (Yamaguchi et al., 2007).

IL-23/IL-17 Axis in Psoriatic Disease

IL-17A and IL-17F as well as their receptor complex (IL17RA/IL-17RC) are expressed in psoriatic skin lesion (Johansen et al., 2009) and PsA synovial tissues (van Baarsen et al., 2014; Glatt et al., 2018).

IL-17-producing T (Th17) cells are major players in the pathogenesis of psoriasis because they are present in psoriatic lesions and can induce activation/proliferation of keratinocytes and endothelial cells (Lowes et al., 2013; Skepner et al., 2014; Sakkas and Bogdanos, 2017; Boutet et al., 2018). Furthermore, T cells recognizing skin autoantigens, such as LL-37, ADAMTSL5, and PLA2G4D, produce IL-17 (Lande et al., 2014; Cheung et al., 2016; Hawkes et al., 2017). IL-17 synergizes with TNFα to induce the production of proinflammatory cytokines IL-6, IL-8, IL-1β (Wang et al., 2013). A mice model with T cell-specific hyperactive STAT3 has augmented Th17 response and exhibits epidermal proliferation and synovial-entheseal inflammation which improve by abrogation of IL-17 and IL-22 cytokines (Yang et al., 2018). Similarly, mannan from *Saccharomyces cerevisiae* (Baker's yeast) administered by intraperitoneal injection to mice induces a PsA-like disease with joint inflammation and psoriasis-like skin lesions, which are prevented by neutralization of IL-17A

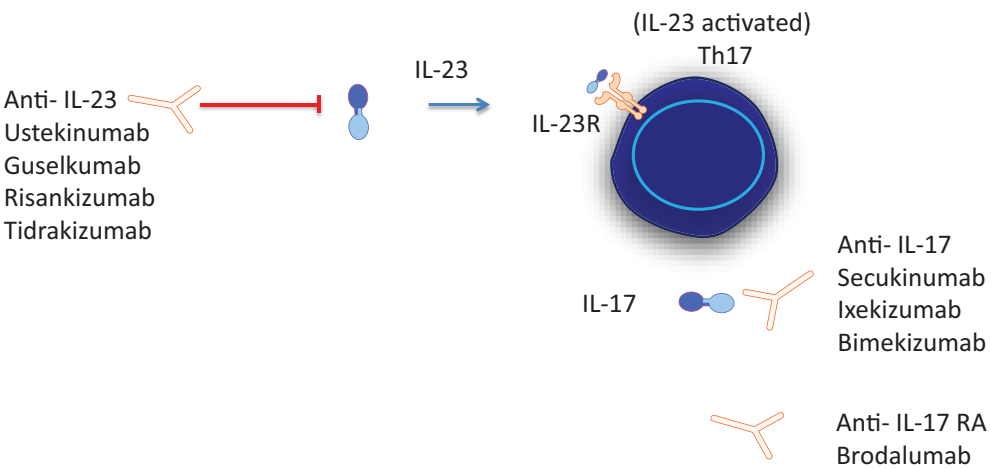

FIGURE 1 | IL-23/IL-17 axis is the focus of novel treatments targeting either cytokine or cytokine receptors.

(Khmaladze et al., 2014). IL-17- and IL-22-producing cells are present with different frequencies in various anatomical sites with IL-22 expression being very low in arthritic joints in PsA (Benham et al., 2013). IL-23 is produced locally at entheseal sites, and IL-23-induced IL-22 is found to be critical for the development of enthesitis (Sherlock et al., 2012).

Treatments Targeting the IL-23/IL-17 Axis

The first biologicals that target the IL-23/IL-17 axis approved for psoriatic disease were ustekizumab and secukinumab. List of all agents and an illustrated agents are provided in **Figure 1** and **Table 1**.

A. IL-12/IL-23p40 Inhibitors

Ustekinumab. Ustekinumab is a human IgG1 MoAb that binds with high affinity to the p40 subunit of IL-12 and IL-23 and is approved for the treatment of moderate to severe psoriasis and PsA. Ustekinumab has a mean half-life of 20 days but its effect stay much longer than expected from its half-life (Gottlieb et al., 2007). The dose is 45 mg (90 mg in persons >100 kg) given subcutaneously (SC) at weeks 0, 4, and then every 12 weeks. Ustekinumab showed efficacy in psoriasis in two phase III trials (PHOENIX-1 and PHOENIX-2) (Leonardi et al., 2008; Papp et al., 2008) and in two phase III trials in PsA (PSUMMIT-1 and PSUMMIT-2) (McInnes et al., 2013; Ritchlin et al., 2014). At week 24, ustekinumab achieved American College of Rheumatology (ACR) 50 in 26.4% of patients versus 8.7% of patients in the placebo group. In TNFα experienced patients ustekinumab achieved ACR20 in 35.6% of patients versus 14.5% in the placebo group. By week 52, PASI 75 and PASI 100 was achieved by 60.6% and 43.7% of patients, respectively. Dactylitis, enthesitis, and spondylitis were also improved, and radiographic joint damage was inhibited (Kavanaugh et al., 2014). Ustekinumab has a good safety profile, with nasopharyngitis being the most common adverse effect, whereas major cardiovascular events and carcinoma were rare events (Ghosh et al., 2019).

Briakinumab. Briakinumab is a fully human IgG1 MoAb that binds to the p40 subunit of IL-12 and IL-23 and has a terminal half-life of 9 days. In a dose ranging phase II trial in psoriasis,

TABLE 1 | Major clinical trials of trreatments targeting the IL-23/IL-17 axis and their main efficacy and serious adverse events characteristics in patients with psoriatic arthritis.

Agent	Biologic target	Trial	ACR50 Week12/16	ACR50 Week24	SAEs (%)	Reference
Ustekinumab	Anti-IL-12/23 p40 MoAb	PSUMMIT 1 Phase III		90 mg 27.9% 45 mg 24.9% Placebo 8.7%	1.5% 2% 2%	McInnes et al., 2013
Ustekinumab	Anti-IL-12/23 p40 MoAb	PSUMMIT 2 Phase III		90 mg 22.9% 45 mg 17.5% Placebo 6.7%	1.9% 0% 4.8%	Ritchlin et al., 2014
Guselkunab	Anti-IL-23 p19 MoAb	Phase II		100 mg 34% Placebo 10%	6% 0%	Deodhar et al., 2018
Secukinumab	Anti-IL-17A MoAb	FUTURE-1 Phase III		150 mg 34.7% 75 mg 30.7% Placebo 7.4%	11.5% 7.4% 5%	Mease et al., 2015
Secukinumab	Anti-IL-17A MoAb	FUTURE-2 Phase III		300 mg 35% 150 mg 35% Placebo 7% Anti-TNFIR 300 mg 27% Anti-TNFIR 150 mg 19% Placebo 9% Anti-TNFnaive 300 mg 39% Anti-TNFnaive 150 mg 44% Placebo 6%	6.4% 5.1% 8.6%	McInnes et al., 2015
Secukinumab	Anti-IL-17A MoAb	FUTURE-5 Phase III	300mg with loading dose 39.6% 150mg with loading dose 35.9% 150mg without loading dose 32% Placebo 8.1%		3.2% 4.1% 2.7% 3.6%	Mease et al., 2018b
Ixekizumab	Anti-IL-17A MoAb	SPIRIT-P1 Phase III	q2w:39.8% qw4:33.6% Placebo:4.7% Adalimuab:29.7%	q2w:46.6% q4w:40.2% Adalimumab:38.6%	q2w:4.9% q4w:3.7% Placebo:1.9%	Mease et al., 2017b
Ixekizumab	Anti-IL-17A MoAb	SPIRIT-P2 Phase III		q2w:33% q4w:35% placebo:5%	Q2w:7% Q4w:3% Placebo:2%	Nash et al., 2017
Brodalumab	Snti-IL17RA MoAb	Phase II	140mg:14% 280mg:14% Placebo:4%		140mg:2% 280mg:4% Placebo:2%	Mease et al., 2014

IL-17RA, interleukin 17 receptor A; MoAb, monoclonal antibody; q2w, once every 2 weeks; q4w, once every 4 weeks; SAEs, serious advese events.

briakinumab at week 12 achieved PASI75 in ~90% of patients versus 3% in the placebo group, but caused adverse effects in 36% of patients versus 10% in the placebo group (Kimball et al., 2008).

B. IL-23p19 Inhibitors

Tildrakizumab. Tildrakizumab is a humanized IgG1κ MoAb that binds to the p19 subunit of IL-23 with high affinity and inhibits downstream signaling of IL-23. After SC injection, its half-life is 25 days (Khalilieh et al., 2018). It has been approved for the treatment of moderate-to severe plaque psoriasis. In two phase III trials (reSURFACE 1 and reSURFACE 2), patients with psoriasis were randomized to receive Tildrakizumab 200 or 100 mg SC at weeks 0, 4, and 16, or placebo (reSURFACE-1), and tildrakizumab 200 mg, or 100 mg at weeks 0,1, and 16, or placebo or etanercept (reSURFACE-2). At 12 weeks in the reSURFACE-1 trial, 62% of the tildrakizumab 200 mg group, 64% of the tildrakizumab 100 mg group, and 6% of the placebo achieved PAS75. In the reSURFACE-2 trial, the respective figures were 66%, 61%, 6%, and 48% of the etanercept group. Tildrakizumab was well-tolerated with a low frequency of adverse effects (Reich et al., 2017). At 28 weeks, 78% of patients achieved PASI75, 58% achieved PASI90, and 29% achieved PASI100 (Sinclair and Thirthar Palanivelu, 2018).

Guselkumab. Early reports in mice with collagen-induced arthritis mice showing that a loss of IL-23 gene($p19^{-/-}$) was protective whereas a loss of IL-12 gene($p35^{-/-}$) exacerbated arthritis (Murphy et al., 2003) lead to efforts to neutralize only IL-23 without affecting IL-12. Guselkumab is a human IgG1λ MoAb that binds to IL-23p19 subunit and inhibits the downstream signaling of IL-23. Its mean half-life is 12 to 19 days (Zhuang et al., 2016). Guselkumab proved to be efficacious treatment for psoriasis in phase III trials (VOYAGE-1 and VOYAGE-12). Patients with psoriasis were randomized to receive guselkumab 100 mg SC at weeks 0, 4, and then every 8 weeks, or placebo or adalimumab 80 mg at week 0, 40 mg at week 1, and then 40 mg every 2 weeks. At week 16, 73.3% of patients achieved PASI90 and 37.4% of patients achieved PASI100 in the guselkumab group compared with 2.9% and 0.6%, respectively, in the placebo group. Adalimumab achieved PASI90 in 49.7% and PASI100 in 17.1% of patients (Blauvelt et al., 2017b). Guselkumab was superior to adalimumab through week 48 (Blauvelt et al., 2017b). Nonresponders to adalimumab at week 28 switched to guselkumab, and 66.1% of them achieved PASI90 at week 48 (Reich K, JAAD 2017;76:418). Guselkumab has a good safety profile and has been approved for the treatment of moderate to severe psoriasis at a dose of 100 mg SC at weeks 0, 4, and then every 8 weeks.

In PsA, guselkumab also appears to be very effective. In a phase II trial, patients with PsA received guselkumab 100 mg SC at weeks 0, 4, and then every 8 weeks. At week 24, guselkumab achieved ACR50/70 in 34%/14% of patients versus 10%/2% in the placebo group and PASI75/90 in 79%/66% of patients versus 13%/6% in the placebo group. Neutropenia was found in 5% of patients as in other anti-IL-17 agents, secukinumab, ixekizumab, and brodalumab (Deodhar et al., 2018).

Risankizumab. Risankizumab is a humanized IgG1κ MoAb that targets the IL-23p19 subunit and selectively inhibits IL-23. Its half-life is 27 days (Suleiman et al., 2019). Risankizumab has been tried in psoriasis with impressive efficacy, and a trial in PsA is underway. In a dose ranging trial in psoriasis risankizumab (90 or 180 mg) or ustekinumab were given SC at weeks 0, 4, and 16. At week 12, risankizumab (90 and 180 mg grouped together) achieved PASI90 in 77% of patients compared with 40% of patients in the ustekinumab group (Papp et al., 2017). In two-phase III trials, patients with psoriasis were randomized to receive risankizumab 150 mg, placebo, or ustekinumab. At week 16, risankizumab achieved PASI90 in 75% of patients, placebo in 2% to 4.9% and ustekizumab in 42% to 47% of patients (Gordon et al., 2018). Nasopharyngitis was the most common adverse effect, whereas basal cell carcinoma and acute myocardial infarction have been reported (Papp et al., 2017).

C. IL-17 Inhibitors

Secukinumab. Secukinumab is a fully human IgG1κ MoAb that selectively binds to IL-17A with high affinity and reduces inflammation. It also quickly restores serum Dkk-1 (Wnt signaling antagonist) in PsA (Fassio et al., 2019). A large body of evidence from many studies including phase III trials FUTURE-1 (Mease et al., 2015) and FUTURE-2 (McInnes et al., 2015) have shown that secukinumab is very effective treatment in patients with PsA, both TNFα naïve and TNFα experienced. It is approved for the treatment of psoriasis, PsA, and ankylosing spondylitis. Dose for PsA: 150 mg SC at week 0, 1, 2, 3, 4, and then every 4 weeks. In PsA with coexistent moderate to severe psoriasis, the dose is 300 mg given as above. If a patient continues to have active arthritis, a dose of 300 mg may be considered.

In a randomized study, secukinumab proved superior to ustekinumab in clearing psoriasis. At week 16, secukinumab achieved PASI90 in 79% and PASI100 in 44.3% of patients compared with 57.6% and 28.4% of patients, respectively, of the ustekinumab group (Thaci et al., 2015). Analysis of results from the FUTURE-2 trial revealed that at week 16, PASDAS remission plus low disease activity in TNFα naive patients was achieved with secukinumab 300 and 150 mg in 46.2% and 42.9% of patients, respectively, versus 17.5% of patients in the placebo group. In TNFα-experienced patients, the corresponding figures were 22.6% and 19.4% versus 13.3% in the placebo group. Furthermore, remission/low disease activity (LDA) was sustained through 2 years in the secukinumab group (Coates et al., 2018). Secukinumab in TNFα naive PsA patients and clazakizumab (an anti-IL-6 MoAb) appear to be the most effective treatments among biologicals in treating dactylitis (Sondag et al., 2019). A recent FUTURE 5 study of secukinumab in PsA to compare the 300 mg with 150 mg dose on clinical and radiographic response revealed that the 300 mg dose with loading dose achieved the numerically highest efficacy in all end points, particularly in psoriasis improvement. A 150-mg dose without loading dose provided comparable results, although resolution of dactylitis and enthesitis was not significant at 16 weeks (Mease et al., 2018b).

There was no difference in the ACR20/50 response between secukinumab and infliximab during the first 16 weeks of treatment in PsA, but later secukinumab achieved higher responses than infliximab (Strand et al., 2019). Also, secukinumab achieved higher ACR responses than adalimumab through 1 year (Nash et al., 2018b).

In one systematic review and meta-analysis, secukinumab was superior to ustekinumab in TNFα naïve but not in TNFα-experienced PsA patients (Kawalec et al., 2018). Secukinumab greatly improved nail psoriasis, as assessed by NAPSI (Reich et al., 2018). Secukinumab was a cost-effective biological in the treatment of PsA with the highest net monetary benefit than other biologicals in Finland (Purmonen et al., 2018).

Ixekizumab. Ixekizumab is a recombinant IgG4κ MoAb antibody that binds, with high affinity to and neutralizes IL-17A. Its half-life is 13 days. In two trials (UNCOVER-1 and UNCOVER-2) patients with chronic plaque psoriasis were randomized to receive ixekizumab 80 mg SC every 2 weeks(q2w) or every 4 weeks(q4w) after a starting dose of 160 mg, or placebo or etanercept. At 12 weeks, PASI 75 was achieved by 89.7% of patients in the ixekizumab q2w group, 77.5% of patients in the ixekizumab q4w group, 2.4% of patients in the placebo group, and 41.6% of patients in the etanercept group. Ixekizumab was well tolerated, and upper respiratory infections and injection site reactions were the most frequently reported adverse effects (Griffiths et al., 2015). Ixekizumab was also well tolerated and exhibited sustained efficacy through week 108 (Blauvelt et al., 2017a).

The efficacy of ixekizumab in PsA was assessed in two phase III trials (SPIRIT-P1 and SPIRIT-P2). In SPIRIT-P1, PsA patients with inadequate response to csDMARDs were randomized to receive ixekizumab 80 mg SC every 2 weeks (q2w), ixekizumab 80 mg q4w, placebo, or adalimumab 40 mg q2w. Both ixekizumab regimens received 160 mg loading dose (Mease et al., 2017b). At week 12, both ixekizumab regimens achieved complete clearing of psoriasis (PASI100) in more patients (q2w:40.7%, q4w: 31.5% of patients) than adalimumab (14.7% of patients), whereas the effect on joints, as assessed by ACR50 was comparable between ixekizumab (q2w, 39.8%; q4w, 33.6% of patients) and adalimumab (29.7% of patients). The effect on nail psoriasis, as assessed by NAPSI, was also comparable between ixekizumab (q2w, 27%; q4w, 20% of patients) and adalimumab (19.7% of patients) (Mease et al., 2017b). In biological-naive PsA patients from the SPIRIT-P1 trial, ixekizumab q2w or q4w achieved comparable ACR50 and ACR70 responses and delayed joint structural damage at 24 weeks irrespective of concomitant csDMARD or MTX use. However, structural joint damage progression was less in patients treated with concomitant csDMARD or MTX (Coates et al., 2017). Similarly, the incidence of moderate/severe AEs was similar to placebo regardless of concomitant csDMARD or MTX use (Coates et al., 2017). In the SPIRIT-P2 trial, ixekizumab was found to be effective in TNFα inhibitor inadequate response PsA patients. At 24 weeks ixekizumab both the q2w and the q4w regimens achieved ARC50 response in 35% of patients compared with 5% of patients in the placebo group (Nash et al., 2017). Similarly, ixekizumab achieved high response rates in psoriasis score (PASI 90:44% in q4w and 50% in q2w regimens, and 12% in placebo) (Nash et al., 2017). In PsA patients with inadequate response or intolerant to TNFα inhibitor, at 24 weeks ixekizumab achieved comparable responses regardless of concomitant csDMARD use (Nash et al., 2018a). Data from SPIRIT-P1 and SPIRT-P2 trials showed that ixekizumab significantly improved dactylitis (ixekizumab q2w, 78%; ixekizumab q4w, 65%; placebo, 24% of patients). The effect on enthesitis was less impressive (Gladman et al., 2019). Injection site reactions and mucocutaneous Candida infection were frequent adverse effects (Mease et al., 2019a).

In treating enthesitis and dactylitis, secukinumab, ustekinumab, or ixekizumab may have similar efficacy as TNFα inhibitors (Mourad and Gniadecki, 2019). However, in treating arthritis secukinumab has the highest efficacy in PsA compared with ixekizumab, and ustekinumab, whereas ustekinumab has the lowest probability for severe adverse effects (Wu et al., 2018).

Bimekizumab. Bimekizumab is an IgG1κ humanized MoAb that selectively and potently binds to and neutralizes both IL-17A and IL-17F. Bimekizumab inhibition of both IL-17A and IL-17F suppressed proinflammatory cytokine production and neutrophil chemotaxis *in vitro* more effectively than blockade of either IL-17A or IL-17F alone (Glatt et al., 2018). Its mean half-life is 20 days (Glatt et al., 2017). In a phase IIb dose-ranging trial in patients with psoriasis, bimekizumab was given at doses 64 to 480 mg SC every 4 weeks. At 12 weeks, bimekizumab 160 mg (with 320 mg loading dose) achieved impressive improvement of psoriasis with PASI90 in 75% and PASI100 in 60% of patients (Papp et al., 2018). Adverse effects had no apparent relationship to dose. Mucocutaneous fungal infections were reported in 4.3% of bimekizumab-treated patients and transient grade 2 (nonserious) neutropenia in 2.4% of patients (Papp et al., 2018).

In a proof of concept trial in 39 patients with PsA, multiple doses of bimekizumab at weeks 0, 3, and 6 resulted in a rapid and profound joint and skin responses at week 8 that sustained or improved through week 20. In particular, ACR50 response was 40% at week 8 and 56.7% at week 20 compared with 8.3% and 18.2%, respectively, in the placebo group. Similarly, PASI100 was achieved by 86.7% of patients at week 8 and 73.3% at week 20, compared with 0% in the placebo group (Glatt et al., 2018). Two fungal infections were reported in the bimekizumab group, one oropharyngitis and one vulvovaginitis after bimekizumab infusion (Glatt et al., 2018).

Brodalumab. Brodalumab is a human IgG2 anti-IL-17 receptor A (IL-17RA) MoAb that inhibits IL-17A, IL-17F, and IL-17E (IL-25). In a phase II dose-ranging trial in psoriasis, at 12 weeks brodalumab at a dose of 140 mg and 210 mg at weeks 0, 1, 2, and then every 2 weeks achieved PASI75 in 77% and 82% of patients, respectively, compared with 0% in the placebo group. The respective percentages for PASI100 were 38% and 62% (Papp et al., 2012). In two phase III trials (AMAGINE-2 and AMAGINE-3) brodalumab at a dose 210 mg was more effective than ustekinumab at week 12: PASI100 in 37% to 44% versus 19% to 22% of patients (Lebwohl et al., 2015).

In a phase II trial in PsA, brodalumab 140 or 280 mg given SC at week 1, 2, and then every 2 weeks, at 12 weeks achieved ACR50 response in 14% of patients compared with 4% in the placebo group and ACR70 response in 5% of patients (Mease et al., 2014). In the open extension study ACR50 response increased to 33% (Mease et al., 2014). Brodalumab appears to be ineffective for dactylitis (Sondag et al., 2019).

Brodalumab has been approved for the treatment of moderate to severe psoriasis at a dose of 210 mg SC at weeks 0, 1, 2, and

then every 2 weeks. Brodalumab increases the risk of infections, and, therefore, vaccinations according to local guidelines are recommended before initiation of brodalumab. It may decrease neutrophil count and can cause suicidal ideation.

JAK Inhibitors

Janus tyrosine kinase (JAK) inhibitors are small molecules, taken orally, that target JAK and block intracellular cytokine pathways. There are four members of the JAK family JAK1, JAK2, JAK3, and TYK2 that form heterodimers and transmit signals from cytokine receptors of the cell membrane. Tofacitinib, a JAK1/JAK3 inhibitor has shown efficacy in PsA (Gladman et al., 2017; Mease et al., 2017a). Baricitinib, a JAK1/JAK2 inhibitor, in a dose-ranging phase IIb trial in psoriasis at week 12 at a dose 8 mg or 10 mg achieved PASI75 in 43% and 54% of patients, respectively, compared with 17% in the placebo group (Papp et al., 2016).

In a phase II trial (EQUATOR) in PsA filgotinib, a selective JAK1 inhibitor at a dose of 200 mg orally once a day at week 16 achieved ACR50 in 55%, LDA(DAPSA ≤ 14) in 49%, and PASI75 in 45% of patients. The respective percentages in the placebo group were 12%, 15%, and 15%. (Mease et al., 2018a). Other (baricitinib (JAK1/JAK2) and selective (upadacitinib [JAK1] JAK inhibitors are being evaluated in PsA.

CONCLUSION

IL-23/IL-17 axis cytokines are important players in the pathogenesis of psoriasis and PsA. Inhibition of IL-23 and IL-17 with MoAbs is a very effective therapy for both psoriasis and PsA. The numbers of these agents are increasing.

AUTHOR CONTRIBUTIONS

LS and DB scripted the original manuscript. EZ scripted significant parts of the manuscript. DB designed the artwork. All authors approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00872/full#supplementary-material

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Effectiveness and Tolerability of Repeated Courses of Viscosupplementation in Symptomatic Hip Osteoarthritis: A Retrospective Observational Cohort Study of High Molecular Weight *vs*. Medium Molecular Weight Hyaluronic Acid *vs*. No Viscosupplementation

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Background: Nonsurgical management of symptomatic hip osteoarthritis needs real-world evidence. We evaluated the effectiveness and tolerability of US-guided intra-articular treatment of two hyaluronic acids (HAs) commercially available in Italy and investigated predictors of response.

Methods: Outpatient records including three cohorts: 122 subjects treated with medium (1,500–3,200 kDa; Hyalubrix®) molecular weight (MW) or high (hylan G-F20; Synvisc®) MW HAs and 20 controls taking NSAIDs/analgesics on demand were retrospectively analyzed. Pain VAS score, WOMAC, NSAID/analgesic consumption, and causes of suspension were available at 1, 6, 12, and 24 months after first administration. As selection bias usually affects observational retrospective studies, a quasi-randomization process was attained by performing propensity score approach.

Results: Propensity score adjustment successfully allowed comparisons among balanced groups of treatments. VAS and WOMAC considerably decreased over time in treated groups independently of the radiological grade ($p<0.001$). On the other hand, the control group showed only a slight and rather uneven variation in VAS. Mean score changes were comparable in both HA cohorts from the earliest stages ($\Delta VAS(HA1,500–3,200kDa)_{T1vsT0}$ = –20%; $\Delta VAS(hylan\ G\text{-}F20)_{T1vsT0}$ = –23%/$\Delta WOMAC(HA1,500–3,200kDa)_{T1vsT0}$ = –17%; $\Delta WOMAC(hylan\ G\text{-}F20)_{T1vsT0}$ = –19%), reaching a further substantial reduction after

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12 months (ΔVAS(HA1,500–3,200kDa)$_{T12vsT0}$ = –52%; ΔVAS(hylan G-F20)$_{T12vsT0}$ = –53%/ ΔWOMAC(HA1,500–3,200kDa)$_{T12vsT0}$ = –45%; and ΔWOMAC(hylan G-F20)$_{T12vsT0}$ = –47%). Almost 11% (=13/122) of ineffectiveness and few moderate local side effects 3% (=4/122) were detected.

Conclusions: Viscosupplementation in a real-life setting seems to provide a sound alternative in pain management in comparison to oral NSAIDs/analgesics, guaranteeing a reduced intake of pain killer medications. Analgesic effectiveness, functional recovery, and reduced joint stiffness extend and improve over 12 and 24 months, suggesting that repeated administrations achieve an additive effect.

Keywords: hyaluronic acid, hip osteoarthritis, viscosupplementation, VAS, WOMAC, hylan G-F 20, joint injection, ultrasound

INTRODUCTION

Osteoarthritis (OA) is the most common cause of coxofemoral pain in adults, especially in elderly subjects (Hoaglund and Steinbach, 2001; Castell et al., 2015). First-line treatments are analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) (Hochberg et al., 2012), along with rehabilitative physical therapy (Pisters et al., 2007; Hernandez-Molina et al., 2008). When patients do not benefit any more from conservative treatments, total joint arthroplasty is the last option. Surgical treatment is challenging, requires prolonged rehabilitation, and is burdened by serious risks of complications (infection, instability, deep vein thrombosis, etc.) (Tsertsvadze et al., 2014). Viscosupplementation is the injection of hyaluronic acid (HA) inside the ill joint in order to restore the physiological articular environment.

Despite the lack of evidence of HA efficacy in hip OA in 2000, in the following years, there were emerging evidence that it could be a treatment option (Migliore et al., 2003; Tikiz et al., 2005). The latest recommendations of Osteoarthritis Research Society International (OARSI) for the management of hip OA suggest intra-articular therapy with steroids and HA in addition to the standard therapy (Zhang et al., 2010). The hip is a difficult joint to inject, and many studies in the literature show greater safety and precision when the procedure is guided by ultrasound (US) (Migliore et al., 2003; Migliore et al., 2006b). Usually, many HAs with different characteristics are used (Migliore et al., 2016), but a clear priority is given to high molecular weight (MW) HA (Tikiz et al., 2005; van den Bekerom et al., 2008). Whether MW differences are associated with different therapeutic effects or durability, it is still to be clarified.

Aim of this study is to evaluate the effectiveness and tolerability of US-guided intra-articular treatment of symptomatic hip OA with two different HA commercially available in Italy (Synvisc® 2 ml, a high MW HA, and Hyalubrix® 2 ml, an intermediate MW HA) compared to standard analgesic/NSAID administration and to evaluate whether there are predictive parameters of response to treatment.

MATERIALS AND METHODS

This is a retrospective observational cohort study based on the patients' database of a degenerative joint disease outpatient clinic.

Consecutive patients with hip OA who had received intra-articular HA injection were retrospectively reviewed.

Selection Criteria

Patients were eligible for inclusion if they met the following criteria: symptomatic hip OA according to the American College of Rheumatology criteria (Altman et al., 1991); radiological grades II, III, and IV according to the Kellgren-Lawrence classification (Kellgren and Lawrence, 1957) evaluated by standard hip X-rays not older than 6 months before baseline (all the X-rays were interpreted by the same expert reader); and hip pain for at least 1 year. The included patients underwent viscosupplementation (treatment cohorts) or NSAID/analgesic administration on demand (control cohort) in case they rejected viscosupplementation (not willing to do it, fear of needles) with a minimum of 6-month follow-up between January 2006 and April 2014. Exclusion criteria were: patently secondary OA (after acetabular or cephalic fracture, avascular necrosis, developmental dysplasia, slipped capital femoral epiphysis, Legg-Calvè-Perthes disease, primary inflammatory rheumatic diseases), corticosteroid injection of the target hip in the previous 3 months, HA injection of the target hip in the previous year, previous open or arthroscopic surgery of the target hip, and ongoing systemic corticosteroid therapy. The patients were classified into three cohorts: HA 1,500–3,200 kDa, hylan G-F 20, and controls, depending on which HA was administered to them, if any.

Hyaluronic Acid Features

HA 1,500–3,200 kDa 2ml (Hyalubrix®, Fidia Farmaceutici, Abano Terme [PD], Italy) is a sterile nonpyrogenic solution of HA sodium salt (15 mg/ml sodium HA) with a MW ranging between 1,500 and 3,200 kDa (medium MW). Hylan G-F 20 2ml (Synvisc® Sanofi, Paris, France) is a sterile nonpyrogenic solution composed by chemically cross-linked hyaluronans (polyanionic form of hyaluronate), ranging between 4,000 and 6,000 kDa (high MW), termed hylans (hylan A soluble + hylan B insoluble gel).

Viscosupplementation Dosing and Injection Procedure

Each patient of viscosupplementation cohorts received three hip injections with HA 1,500–3,200 kDa 2 ml or hylan G-F 20 2 ml, once a month for three consecutive months, then further maintenance injections with the same HA were administered every 6 months for 2 years. All injections were carried out using a standardized technique, under US guidance. Through a 6–18 MHz linear transducer (Esaote MyLab 70) with a sterile guide attached, the hip joint was visualized using an anterior parasagittal scan, lateral to the femoral vessels. Intra-articular injection was performed by inserting a 18-gauge needle (15 cm long) in the sterile guide with an antero-inferior approach (**Figure 1A**) aiming at the top of the femoral head. Correct intracapsular positioning of the needle was monitored in real time by direct US visualization (**Figure 1B**).

Data Collection

At baseline demographic data (age, gender, height, weight, and body mass index) duration of disease (calculated as time passed between the onset of symptoms and the pre-treatment data collection, termed "baseline" or t_0), Kellgren-Lawrence radiological grade of OA, degree of hip pain reported on visual analogue scale (0–100 mm), WOMAC score (based on the three domains pain, stiffness, and joint function), and number of days of NSAID/analgesic consumption in the last month before visit for hip pain were collected.

At given time points after first injection (1, 6, 12, and 24 months), we gathered information about VAS score, WOMAC, number of days of NSAID/analgesic consumption, adverse events, and causes of treatment discontinuation.

Data Analysis

Statistical analyses were performed with R software, version 3.0.2 for Windows [RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/]. Descriptive statistics were used to summarize data. Overall, age, disease duration, and BMI were also coded as categorical factors (age: 0, if < 65 years; 1, otherwise; duration of disease: 0, if <5 years; 1, otherwise; BMI: 0, if <25; 1, otherwise). Associations and differences among the three treatments groups were assessed by chi-squared or Fisher's exact tests and one-way ANOVA or Kruskal–Wallis tests, respectively. Cochrane–Armitage trend test was applied to compare percentage of patients using NSAIDs over time and among groups of treatment. Day-per-month consumption of NSAIDs/analgesics over time within the same group of treatment was assessed using Friedman test for repeated measurements and Dunn's test for multiple comparisons adjustment. A p-value less or equal to 0.05 was considered statistically significant.

Quasi-Randomization Process

As observational studies can be affected by selection bias, propensity score (PS) approach (Rosenbaum and Rubin, 1983) was used for correcting the analysis of the nonrandomized design (D'Agostino, 1998). After generating a score based on the propensity for each patient of receiving a specific treatment given a set of baseline characteristics, the PS was included as covariate in the model further used to analyze repeated measures (Rosenbaum and Rubin, 1984). PS was calculated for each patient using logistic regression with treatment (HA or NSAIDs/analgesics) as dependent variable and baseline characteristics as independent variables. Covariates used for calculating PS were selected based on the method of standardized differences (Flury and Riedwyl, 1986) or considering characteristics at baseline that clearly differed among groups. A threshold of 0.10 of standard difference was considered as sign of important covariate imbalance.

Modeling Repeated Measures Over Time

The change over time of VAS and WOMAC scores, was assessed by the approach of generalized estimating equations (GEE) (Liang and Zeger, 1986) for repeated measurements with an exchangeable working correlation matrix. The variables of radiological grade of OA, administration timing, and therapy were included in the model in order to clarify their degree of association with outcomes and adjusted through PS.

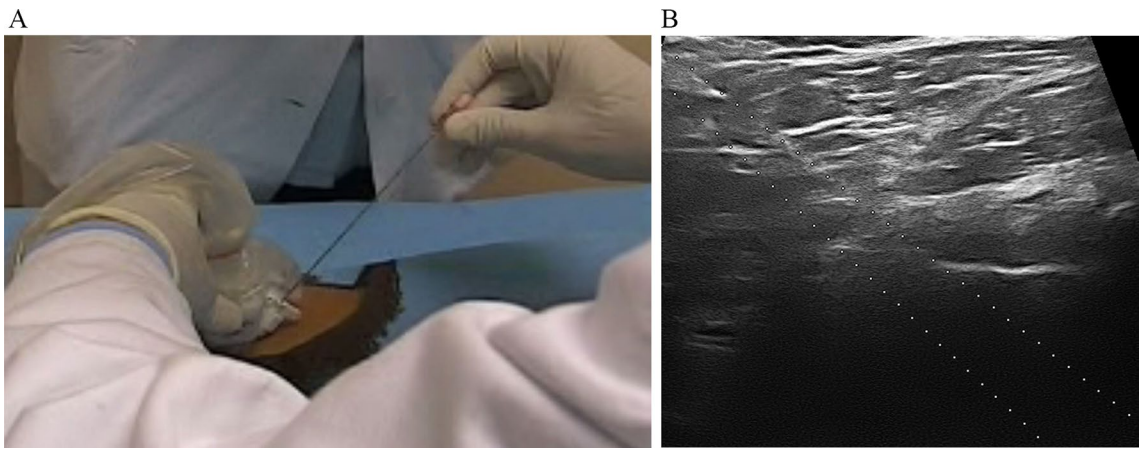

FIGURE 1 | (A) Intra-articular injection performed by inserting a 18-gauge needle in the sterile guide with an antero-inferior approach; **(B)** intracapsular positioning of the needle was monitored in real time by direct US visualization of the needle on the screen of the ultrasound machine.

Considering the small size of control group, bias-corrected estimates for the regression coefficients were calculated using a bias formula as provided by Lunardon et al. (Lunardon and Scharfstein, 2017).

Compliance With Ethical Standards

The study was conducted according to the Declaration of Helsinki and approved by the local responsible Ethics Committee (Comitato Etico Milano Area B, protocol n° 125_2017). In this retrospective study, no written informed consent was required. Patient records/information were anonymized and de-identified prior to data analysis.

RESULTS

Characteristics of the Study Population at Baseline

One hundred twenty-two patients (50 males and 72 females) with symptomatic hip OA met all the inclusion criteria: 117 with unilateral hip OA and 5 with bilateral hip OA who underwent bilateral viscosupplementation. Then, a total of 127 hips were treated. A cohort of 20 subjects (22 hips) managed with only NSAIDs/analgesics on demand served as controls.

The baseline characteristics of study population stratified by treatment are summarized in **Table 1**. In general, the three groups of patients turned out to be homogeneous in all demographic and clinical characteristics. Regarding disease scores, both HA groups showed a statistically significant difference compared to control group having a slightly higher WOMAC score, as shown in **Table 2** (left-hand column). On the other hand, the three groups did not differ in pain VAS score.

Tolerability of Viscosupplementation

Overall, treatment with HAs was suspended in 20 out of 122 patients (16.4%), and the rate of suspension was similar ($\chi^2 = 0.7$, p = 0.400) between the two formulations (18.6% (8/43) *vs.* 15.2% (12/79), in HA 1,500–3,200 kDa and hylan G-F 20 groups, respectively). Reasons for HA 1,500–3,200 kDa suspension were related to side effects (n = 4 temporary worsening of hip pain recovering from 2 to 10 days) or ineffectiveness (n = 4), whereas reasons for hylan G-F 20 suspension were indicated as ineffectiveness (n = 9) or not reported (n = 3). Three out of four patients suspending because of side effects stopped the medication after 6 months while the last one complained hip pain after 1 year. Inefficacy occurred after 6 months or 1 year in 8 or 5 patients, respectively.

TABLE 1 | Demographic and clinical characteristics of patients stratified by treatment, at time of recruitment. Continuous variables are summarized as mean ± SD, categorical variables as percentage (absolute frequency).

Baseline	Hyaluronic acid 1,500–3,200 kDa	HYLAN G-F 20	CTRLS	Overall p-value
	(N = 43)	(N = 79)	(N = 20)	
Age (years)	61.2 ± 14.8	63.0 ± 13.1	60.4 ± 11.1	0.647
Age% (>65ys)	39.5 (17)	50.6 (40)	40.0 (8)	0.429
Gender F %(N)	51.2 (22)	62.0 (49)	55.0 (11)	0.492
Weight (kg)	76.8 ± 12.7	72.6 ± 13.1	73.6 ± 10.2	0.215
Height (cm)	169.6 ± 9.9	166.2 ± 8.8	169.2 ± 7.4	0.105
BMI (Kg/m²)	26.6 ± 3.3	26.2 ± 3.6	25.7 ± 2.7	0.546
BMI% (>25 Kg/m²)	60.5 (26)	55.7 (44)	70.0 (14)	0.498
Disease duration% (≥5ys)	20.9 (9)	27.8 (22)	25.0 (5)	0.703
K-L rating score %(N)				0.322
Grade 2	32.6 (14)	22.8 (18)	15.0 (3)	
Grade 3	58.1 (25)	57.0 (45)	60.0 (12)	
Grade 4	9.3 (4)	20.3 (16)	25.0 (5)	

TABLE 2 | Differences in VAS score and WOMAC index (mean ± SD) between patients treated with HA or controls at baseline before and after propensity score (PS) adjustment.

Comparison	Not adjusted difference	Difference after PS adjustment
Hyaluronic acid 1,500–3,200 kDa *vs.* controls		
Pain VAS	–1.3 ± 4.8 *(p = 0.783)*	–6.4 ± 4.8 *(p = 0.187)*
WOMAC index		
Total	8.2 ± 4.5 *(p = 0.07)*	2.7 ± 4.3 *(p = 0.537)*
Pain	2.0 ± 1.1 *(p = 0.07)*	0.9 ± 1.1 *(p = 0.435)*
Stiffness	1.4 ± 0.5 *(p = 0.008)*	0.7 ± 0.4 *(p = 0.071)*
Function	4.3 ± 3.5 *(p = 0.216)*	1.1 ± 3.4 *(p = 0.746)*
HYLAN G-F 20 *vs.* NSAIDs		
Pain VAS	1.8 ± 3.7 *(p = 0.638)*	–5.1 ± 4.0 *(p = 0.201)*
WOMAC index		
Total	10.3 ± 3.4 *(p = 0.002)*	2.6 ± 3.5 *(p = 0.466)*
Pain	2.5 ± 0.9 *(p = 0.006)*	0.9 + 1.0 *(p = 0.376)*
Stiffness	1.2 ± 0.5 *(p = 0.009)*	0.3 ± 0.3 *(p = 0.320)*
Function	6.6 ± 2.6 *(p = 0.013)*	1.3 ± 2.8 *(p = 0.634)*

Variation in NSAID/Analgesic Consumption During Follow-Up

At baseline, almost 80% of patients took NSAIDs/analgesics with an average consumption of about 10 days/month; the frequency of using it at baseline was comparable in the three groups of patients. During the 24 months of treatment with HA, a significant reduction in the use of NSAIDs/analgesics for pain control was observed both in terms of number of patients (HA 1,500–3,200 kDa *vs.* Ctrls, z = 3.64, p < 0.001; hylan G-F 20 *vs.* Ctrls, z = 4.25, p < 0.0001) (**Table 3**) and dosage (**Table 4**). In particular, HA-treated patients showed a significant decrease in day-per-month usage from the first month of therapy whereas, except for an increased consumption during the first month (p<0.01), controls maintained the same dosage over time.

Propensity Score

Based on the standardized difference approach, the treatment groups were unbalanced in terms of age (st.diff. = 0.19), BMI (st. diff. = 0.24), baseline WOMAC pain (st.diff = 0.46), stiffness (st.diff. = 0.58), and function (st.diff. = 0.36), whilst balanced in terms of disease duration (st.diff. = 0.04) and VAS (st.diff. = 0.006). Then, PS was calculated using age, BMI, and all the baseline WOMAC domains as observed covariates. **Table 2** (right-hand column) shows improvement in the balance with regard to baseline WOMAC domains after PS adjustment.

TABLE 3 | Percentages and relative frequencies (in brackets) of patients taking NSAID/analgesic medication in each group of treatment and observation period.

Therapy	Baseline	1 mth	6 mths	12mths	24 mths
Hyaluronic acid 1,500–3,200 kDa	83.7% (36/43)	53.5% (23/43)	58.1% (25/43)	38.9% (14/36)	17.1% (6/35)
Hylan G-F 20	77.2% (61/79)	57.0% (45/79)	50.6% (40/79)	44.2% (19/71)	48.0% (12/70)
Controls	75.0% (15/20)	70.0% (14/20)	85.0% (17/20)	80.0% (16/20)	90.0% (18/20)

TABLE 4 | Mean values and related standard errors (SE) of NSAID/analgesic consumption (days/month) within each group of treatment.

Therapy	Mean (SE)	Overall p-value	p-value (t_i *vs.* t_0)
Hyaluronic acid 1,500–3,200 kDa		<0.0001	
Baseline	9.7 (1.4)		–
1 mth	5.0 (1.1)		0.003
6 mths	4.5 (1.1)		0.0001
12 mths	3.2 (0.9)		<0.0001
24 mths	3.6 (1.0)		<0.0001
Hylan G-F 20		<0.0001	
Baseline	9.1 (1.1)		–
1 mth	5.7 (1.0)		0.001
6 mths	3.7 (0.7)		<0.0001
12 mths	3.5 (0.7)		<0.0001
24 mths	3.9 (0.7)		<0.0001
Controls		0.06	
Baseline	8.0 (1.8)		–
1 mth	15.9 (2.4)		<0.01
6 mths	11.5 (1.6)		0.157
12 mths	11.4 (1.6)		0.152
24 mths	11.7 (1.3)		0.091

Variation of Pain VAS During Follow-Up

Patients treated with HAs clearly showed a different pathway in pain score when compared to controls during follow-up (**Figure 2**). **Table 5** shows the PS adjusted estimates [95% CI] of VAS scores at each time visit and stratified by radiological grade as resulted from GEE approach. Accordingly, patients undergoing HA treatments significantly decreased VAS score compared to baseline conditions since the first month of therapy and independently of radiological grade (i.e., the 95% CI of VAS variation for each K-L grade never include the null value). The progression in the decrease still occurred over time reaching a statistical significance after 12 months for both HAs (ΔVAS(HA 1,500–3,200 kDa)$_{T12vsT1}$ = −24.2 [95% CI: −14.4 to −34.0], p<< 0.001; ΔVAS(hylan G-F 20) $_{T12vsT1}$: −23.8 [95% CI: −15.1 to −32.4], p<< 0.001) and was stable after 24 months as shown in **Figure 2**. When compared to controls, the effect of HA treatment was always significantly more relevant than NSAIDs/analgesics in all K-L grade subgroups (ΔVAS at 1-month follow-up: HA 1,500–3,200 kDa *vs.* Ctrls: −11.5 [95% CI: −18.9 to −4.1], p = 0.002; hylan G-F 20 *vs.* Ctrls: −13.7 [95% CI: −19.9 to −7.6], p<< 0.001). Overall, the trend of the two HAs overlapped almost perfectly.

As expected, we observed a significant slight effect of NSAIDs/analgesics on VAS score but only in patients reporting a K-L grade 2 (−5.1 [95% CI: −9.8 to −0.4], p = 0.03); such a change was gradually reduced over time as late as 12 months from the first visit (−2.9 [95% CI: −5.7 to −0.1], p = 0.04), then disappeared after 24 months (−0.9 [−4.2 to 2.3], p = 0.584).

Variation of WOMAC During Follow-Up

The trend over time of WOMAC was investigated in terms of total score and its components, pain, stiffness and joint function

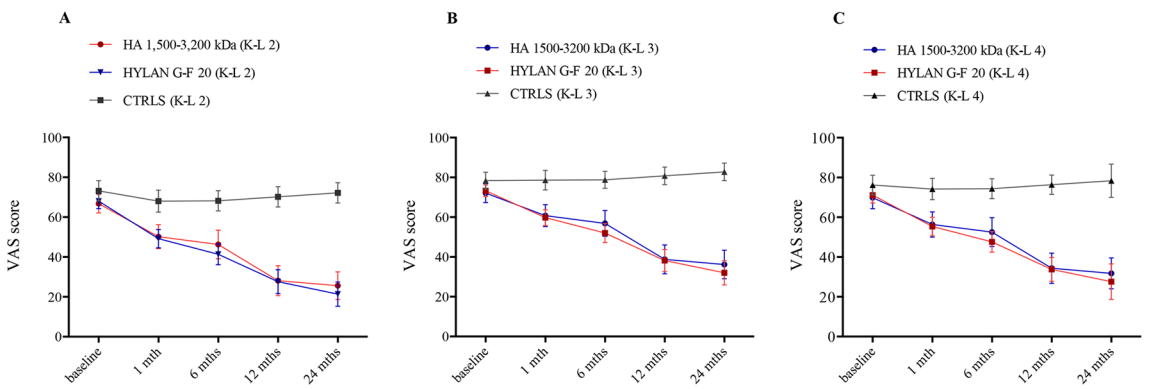

FIGURE 2 | Time trend of VAS score over the course of follow-up (baseline and after 1, 6, 12, and 24 months) stratified by treatment (hyaluronic acid [HA] 1,500–3,200 kDa, hylan G-F 20, NSAIDS as controls [CTRLS]) and radiological grades II, III, and IV according to the Kellgren-Lawrence classification (**A**, K-L 2; **B**, K-L 3; **C**, K-L 4). Bars refer to standard error.

TABLE 5 | Follow-up variations of VAS score (mean [95% CI]) compared to baseline setting and stratified by radiological grade and therapy. All the estimates resulted by a quasi-randomization process obtained including propensity scores in the model for repeated measures (GEE model).

Timing x K-L grade	Hyaluronic acid 1,500–3,200 kDa	HYLAN G-F 20	Ctrls
Grade II			
Baseline	66.8 [57.5 to 75.9]	68.0 [60.6 to 75.4]	73.1 [63.3 to 83.1]
1 mth vs. baseline	−16.6 [−22.3 to −10.9] p< < 0.001	−18.8 [−22.6 to −15.0] p< < 0.001	−5.1 [−9.8 to −0.4] p = 0.03
6 mths vs. baseline	−20.5 [−29.7 to −11.3] p< < 0.001	−26.6 [−32.0 to −21.2] p< < 0.001	−4.9 [−8.5 to −1.2] p = 0.01
12 mths vs. baseline	−38.7 [−48.7 to −28.7] p< < 0.001	−40.4 [−47.7 to −33.1] p< < 0.001	−2.9 [−5.7 to −0.1] p = 0.04
24 mths vs. baseline	−41.2 [−51.3 to −31.1] p< < 0.001	−46.6 [−54.0 to -39.2] p< < 0.001	−0.9 [−4.2 to 2.3] p = 0.584
Grade III			
Baseline	72.1 [62.8 to 81.4]	73.3 [67.6 to 79.0]	78.4 [69.9 to 86.9]
1 mth vs. baseline	−11.3 [−18.5 to −4.1] p = 0.004	−13.5 [−20.8 to −6.2] p = 0.001	0.2 [−7.6 to 8.0] p = 0.398
6 mths vs. baseline	−15.2 [−24.6 to −5.8] p = 0.003	−21.3 [−29.7 to −12.9] p< < 0.001	0.4 [−6.7 to 7.5] P = 0.397
12 mths vs. baseline	−33.3 [−43.7 to −22.9] p< < 0.001	−35.1 [−45.0 to −25.2] p< < 0.001	2.4 [−4.4 to 9.2] 0.315
24 mths vs. baseline	−35.9 [−46.7 to −25.1] p< < 0.001	−41.3 [−50.8 to −31.8] p< < 0.001	4.4 [−2.5 to 11.3] p = 0.184
Grade IV			
Baseline	69.9 [59.0 to 80.8]	71.1 [63.6 to 78.6]	76.2 [66.6 to 85.8]
1 mth vs. baseline	−13.5 [−22.6 to −4.4] p = 0.006	−15.7 [−24.5 to −6.9] p = 0.001	−2.0 [−11.1 to 7.1] p = 0.364
6 mths vs. baseline	−17.4 [−28.4 to −6.4] p = 0.003	−23.5 [−32.8 to −14.2] p< < 0.001	−1.8 [−10.8 to 7.2] p = 0.369
12 mths vs. baseline	−35.5 [−47.1 to −23.9] p< < 0.001	−37.3 [−48.1 to −26.5] p< < 0.001	0.2 [−8.2 to 8.6] p = 0.399
24 mths vs. baseline	−38.1 [−50.0 to −26.2] p< < 0.001	−43.5 [−54.4 to −32.6] p< < 0.001	2.2 [−6.7 to 11.1] p = 0.355

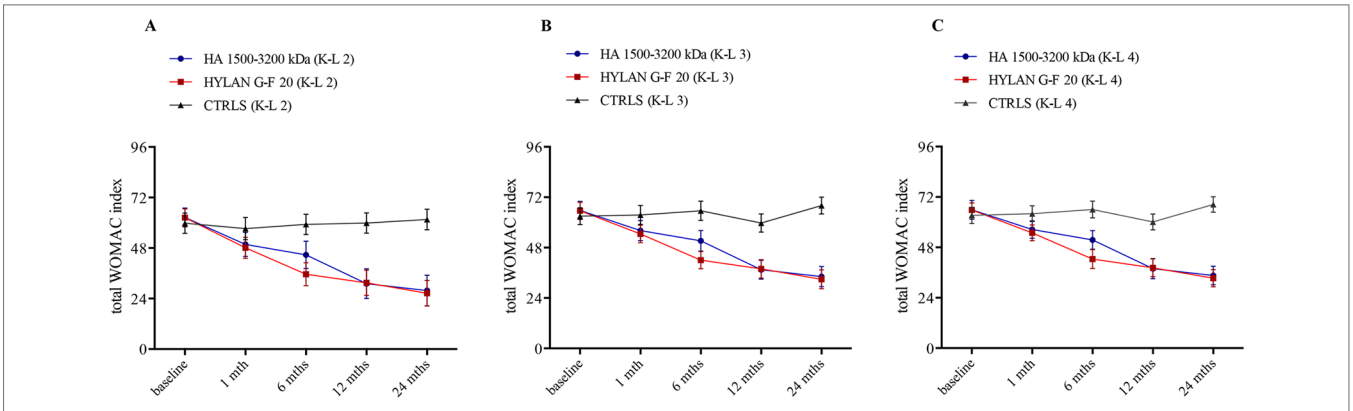

FIGURE 3 | Time trend of total WOMAC index over the course of follow-up (baseline and after 1, 6, 12, and 24 months) stratified by treatment (hyaluronic acid [HA] 1,500–3,200 kDa, hylan G-F 20, NSAIDS as controls [CTRLS]) and radiological grades II, III, and IV according to the Kellgren-Lawrence classification (**A**, K-L 2; **B**, K-L 3; **C**, K-L 4). Bars refer to standard error.

domains. **Figure 3** illustrates the follow-up variations in total WOMAC stratified by treatment and K-L grade, and the related estimates are reported in **Table 6**. Both HAs significantly improved the total score compared to baseline conditions since the first month of therapy and independently of radiological grade (i.e., the 95% CI of VAS variation for each K-L grade never include the null value). An additional significant variation was found after 1 year of therapy (ΔWOMAC(HA 1,500–3,200 kDa)$_{T12vsT1}$: −21.3 [95% CI: −12.6 to −30.0], p<< 0.001; ΔWOMAC(hylan G-F 20) $_{T12vsT1}$: −19.2 [95% CI: −10.8 to −27.6], p < 0.001) before finally setting in at the end of follow-up (**Figure 3**). Overall, the two HAs produced a similar trend

TABLE 6 | Follow-up variations in WOMAC score (mean [95% CI]) compared to baseline setting and stratified by radiological grade and therapy. All the estimates resulted by a quasi-randomization process obtained including propensity scores in the model for repeated measures (GEE model).

Timing x K-L grade	Hyaluronic acid 1,500–3,200 kDa	HYLAN G-F 20	Ctrls
Grade II			
Baseline	62.5 [53.7 to 71.3]	62.4 [54.2 to 70.6]	59.8 [50.4 to 69.2]
1 mth vs. baseline	−12.8 [−17.9 to −7.7] p < 0.001	−14.3 [−19.4 to −9.2] p< < 0.001	−2.6 [−5.7 to 0.5] p = 0.100
6 mths vs. baseline	−17.7 [−25.0 to −10.4] p< < 0.001	−26.8 [−32.9 to −20.7] p< < 0.001	−0.6 [−2.8 to 1.6] p = 0.593
12 mths vs. baseline	−31.4 [−40.9 to −21.9] p< < 0.001	−30.9 [−38.2 to −23.6] p< < 0.001	+0.1 [1.3 to 0.0] p = 0.913
24 mths vs. baseline	−34.7 [−44.7 to −24.7] p< < 0.001	−35.9 [−43.4 to −28.4] p< < 0.001	+1.8 [1.8 to 1.0] p = 0.312
Grade III			
Baseline	65.7 [57.3 to 74.1]	65.6 [57.7 to 73.5]	63.0 [55.2 to 70.8]
1 mth vs. baseline	−9.6 [−16.4 to −2.8] p = 0.009	−11.1 [−19.1 to −3.1] p = 0.010	0.6 [−6.0 to 7.2] p = 0.393
6 mths vs. baseline	−14.5 [−22.7 to −6.3] p = 0.001	−23.6 [−33.2 to −14.0] p< < 0.001	+2.6 [−3.3 to 8.5] p = 0.274
12 mths vs. baseline	−28.2 [−38.6 to −17.8] p< < 0.001	−27.7 [−37.4 to −18.0] p< < 0.001	−3.3 −9.5 to 2.9] p = 0.233
24 mths vs. baseline	−31.5 [−43.3 to −19.7] p< < 0.001	−32.7 [−41.1 to −24.3] p< < 0.001	+5.0 [−1.5 to 11.5] p = 0.129
Grade IV			
Baseline	65.9 [57.1 to 74.7]	65.8 [59.1 to 72.5]	63.2 [55.5 to 70.9]
1 mth vs. baseline	−9.4 [−16.8 to −2.0] p = 0.019	−10.9 [−19.2 to −2.6] p = 0.014	+0.8 [−6.4 to 8.0] p = 0.389
6 mths vs. baseline	−14.3 [−23.1 to −5.5] p = 0.002	−23.4 [−33.0 to −13.8] p< < 0.001	+2.8 [−4.0 to 9.6] p = 0.287
12 mths vs. baseline	−28.0 [−38.3 to −17.7] p< < 0.001	−27.5 [−37.5 to −17.5] p< < 0.001	−3.1 [−10.1 to 3.9] p = 0.273
24 mths vs. baseline	−31.3 [−42.4 to −20.2] p< < 0.001	−32.5 [−42.6 to −22.4] p< < 0.001	+5.2 [−2.0 to 12.4] p = 0.145

in WOMAC change also after 6 months of therapy where hylan G-F 20 seemed to be more effective than HA 1,500–3,200 kDa (ΔWOMAC: −9.1 [95% CI: −18.7 to +0.4], p = 0.06).

When compared to controls, the effect of HA treatment was always significantly more relevant than NSAIDs/analgesics in all K-L grade subgroups (ΔWOMAC at 1-month follow-up: HA 1,500–3,200 kDa *vs*. Ctrls: −10.2 [95% CI: −16.0 to −4.4], p < 0.001; hylan G-F 20 *vs*. Ctrls: −11.7 [95% CI: −17.6 to −5.9], p< < 0.001).

On the other hand, NSAIDs/analgesics did not show any effect at any time points and at any radiological grade (right-hand column of **Table 6**).

About WOMAC domains, the general trend of total score was confirmed by each component. In summary, patients undergoing HA treatment showed a remarkable effect since the first month of therapy in comparison with NSAID/analgesic group (*pain*: HA 1,500–3,200 kDa, T1 *vs*. T0: −2.3 [95% CI: −3.7 to −0.9], p = 0.001; hylan G-F 20, T1 *vs*. T0: −2.6 [95% CI: −4.0 to −1.2], p < 0.001; *stiffness*: HA 1,500–3,200 kDa, T1 *vs*. T0: −0.8 [95% CI: −1.4 to −0.1], p = 0.031; hylan G-F 20, T1 *vs*. T0: −0.7 [95% CI: −1.3 to −0.1]; *function*: HA 1,500–3,200 kDa, T1 *vs*. T0: −7.1 [95% CI: −11.3 to −2.9], p < 0.001; hylan G-F 20, T1 *vs*. T0: −8.5 [95% CI: −12.8 to −4.1], p < 0.001). The baseline scores of the three K-L grades were comparable within each domain (*pain*: K-L 3 *vs*. K-L 2: +1.2 [95% CI: −0.2 to 2.6], p = 0.09, and K-L 4 *vs*. K-L 2: +1.0 [95% CI: −0.8 to 2.8], p = 0.271; *function*: K-L 3 *vs*. K-L 2: +1.8 [95% CI: −2.8 to 6.4], p = 0.445 and K-L 4 *vs*. K-L 2: +2.0 [95% CI: −4.0 to 8.0], p = 0.515) except for stiffness where patients with radiological grade 4 reported a slightly greater WOMAC score compared to patients with radiological grade 2 (K-L 3 *vs*. K-L 2: +0.3 [95% CI: −0.2 to 0.8], p = 0.228, and K-L 4 *vs*. K-L 2: +0.8 [95% CI: 0.1 to 1.4] score units, p = 0.021).

Furthermore, the pattern of pain domain in controls was consistent with the VAS score variations since they showed a mild, even if not significant, improvement during the first two visits (month 1: −0.7 [95% CI: −1.6 to 0.1] score units, p = 0.085; month 2: −0.6 [−1.2 to 0.06], p = 0.075).

DISCUSSION

Comments on the Effectiveness and Safety

The primary endpoint of this study was to evaluate the effectiveness of viscosupplementation expressed as a reduction in VAS and WOMAC scores. Our data confirmed the analgesic effectiveness of viscosupplementation in a statistically significant way after every administration for both HAs. The percentage of VAS reduction was around 20–30%, in accordance with the literature (Migliore et al., 2011b). After the first month of treatment, patients treated with HAs reported a lower VAS score than patients treated with NSAIDs/analgesics alone. This reduction was not

only maintained over time but was further reduced upon each following administration leading to a decrease of 50–70% at the end of follow-up. To the best of our knowledge, this information is novel for hip OA; this finding further supports the reported ability of retreatment to consolidate single injections highlighted so far in knee OA (Navarro-Sarabia et al., 2011; Raman et al., 2018).

The overall effectiveness of the therapy over 2 years of treatment with high MW HA was similar to that obtained with intermediate MW HA: this confirms the observations of the studies published thus far comparing HAs with different MW (Tikiz et al., 2005; van den Bekerom et al., 2008).

HA safety profile confirms the positive data already present in the literature (Migliore et al., 2011a) and indicates that HA injections reduce NSAID/analgesic intake, possibly avoiding the most frequent complications linked to these drugs (such as gastrointestinal/renal complications and increased cardiovascular mortality) (Garcia Rodriguez and Hernandez-Diaz, 2001; Crofford, 2013).

Confounding Factors

Direct involvement in pain perception has not been demonstrated for any demographic and clinical variables; however, these factors may have a confounding role, and therefore, it is essential to take them into consideration estimating the VAS and WOMAC variation under HA treatments. Indeed, a different distribution of these characteristics among groups of treatment can be source of unbalanced designs in retrospective studies. Thus, we successfully performed a PS analysis to address selection bias due to non-random assignment of patients to treatment (**Table 2**).

Furthermore, radiological grade was investigated as prognostic factor of response to treatment. Presumably, the highest radiological grade may lead to an even poorer response to treatment because it is more difficult to treat chronic pain due to adaptive circuits of the nervous system (Lee et al., 2011). However, in our cohort, radiological grade was not mainly involved in response to treatment, and the effectiveness of HAs was always relevant independently of severity of disease both in terms of VAS and WOMAC scores.

Our data, together with the encouraging results obtained in various studies on the reduction of progression toward total hip arthroplasty (Migliore et al., 2012a; Migliore et al., 2012b; Tsertsvadze et al., 2014), support the use of viscosupplementation also in patients with severe hip OA when total hip arthroplasty is not feasible or refused.

Merits and Flaws of the Study

This study has several strengths. The sample size is rather large compared to other similar studies, and the observation period is longer than the majority of other studies in the literature, which usually have a follow-up ranging between 6 and 18 months (Migliore et al., 2006a; Migliore et al., 2006b; Migliore et al., 2011b; Rivera, 2016). Recently, data from ANTIAGE registry including more than 1,000 hip OA patients were analyzed and published (Migliore et al., 2017), but the study lacks completely a control group that is a significant criterion for conducting a study in evidence-based medical research.

Furthermore, we achieved balanced groups of treatment because of the PS approach; therefore, we reduced bias due to confounding factors and correctly estimated the effect of therapy by accounting for the covariates that predict the receiving treatment. PS analysis resulted particularly efficient since our population was naturally quite homogeneous for most demographic and clinical characteristics. Indeed, control group included patients that spontaneously rejected viscosupplementation, meaning that treatment allocation was not a clinician's choice.

On the other hand, the limitations of this statistical analysis should not be forgotten. In fact, PS does not correct for unobservable or unmeasured variables.

Finally, it should be highlighted that the treatment effect was estimated performing a GEE model that handles missing responses due to treatment discontinuation (side effects or ineffectiveness).

A limitation of this study consists in the few numbers of patients treated with NSAIDs/analgesics. Undeniably, control group was numerically less represented than HA groups. In order to address this issue, we applied a correction to regression coefficients of GEE model. However, the choice and the enrolment of a control group in clinical practice of hip pain management is challenging as reported also in the mini-review of Migliore and Anichini (2017).

Finally, we observed an improved pain score as measured by standardized and feasible tools such as VAS and WOMAC indexes. Although these outcomes allow a direct feedback as reported by patients themselves, additional efforts should be made to accomplish a whole evaluation, as recently suggested by Migliore and colleagues (2015).

CONCLUSIONS

US-guided hip injection technique allows us to act safely and accurately, without exposing the patient to ionizing radiation. High MW and medium MW HAs are both effective in the reduction of VAS and WOMAC at all the time intervals considered in patients with hip OA.

Our findings suggest that US-guided intra-articular HA injection is an effective and well tolerated treatment by patients with symptomatic hip OA. The benefits, such as pain reduction, functional recovery, and reduced joint stiffness, extend and improve over 12 months from the first injection, suggesting that repeated administrations display an additive effect. Moreover, we showed that HA injection is effective against pain beginning with the first administration.

Interestingly, a reduction in NSAID/analgesic intake is confirmed with viscosupplementation, with further potential benefits for general health of patients, tapering NSAID consumption and lowering classical drug side effects (Garcia Rodriguez and Hernandez-Diaz, 2001; Crofford, 2013).

Larger and longer prospective studies are needed to better estimate the effect of HA therapy in OA patients with different demographic and clinical characteristics. Unbiased outcomes as X-ray performed at the end of the treatment or the measure of the time to prosthesis should be considered (Migliore et al., 2015).

ETHICS STATEMENT

The study protocol was planned according to the Declaration of Helsinki and was approved by the local responsible Ethics Committee (protocol n° 125_2017).

AUTHOR CONTRIBUTIONS

Substantial contributions to study conception and design: OL, AM. Substantial contributions to acquisition of data: OL, LMP, EVe, CC, EVa, LP, DC, AM. Substantial contributions to analysis and interpretation of data: FP, OL, EVe, PL, PM, AM. Drafting the article or revising it critically for important intellectual content: all the authors. Final approval of the version of the article to be published: all the authors.

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