CLIC1 Expression in Skin Biopsies from Patients With Rheumatoid and Psoriatic Arthritis as a Potential Tool to Predict Therapy Response

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Abstract. *Background/Aim: Chloride intracellular channel protein 1 (CLIC1) activates inflammasomes in rheumatoid (RA) and psoriatic (PsA) arthritis. We studied CLIC1 expression in RA and PsA patients' skin with vasculitis and its variability depending on the therapy used. Materials and Methods: CLIC1 immunoexpression was evaluated in the vascular (CLIC1-V) and stromal (CLIC1-S) compartments of the RA and PsA skin biopsies of patients treated with methotrexate (MTX), leflunomid (LFN), corticotherapy (CT), or biological therapies. Results: MTX significantly reduced CLIC1-S expression (p=0.016), whereas LFN decreased CLIC1-V (p<0.001). LFN therapy duration also correlated with CLIC1-V (p<0.001). CT decreased CLIC1-S expression (p=0.006). CLIC1-S expression persisted in skin biopsies despite of erythrocyte sedimentation rate (ESR, p=0.018) and C reactive protein (CRP, p=0.0026) normalisation. For PsA, CLIC1-S expression significantly related to MTX (p<0.022). Both CLIC1-S (p<0.001) and CLIC1-V (p=0.007) decreased by biological therapies in RA. Conclusion: CLIC1 expression is strongly influenced by the therapy used. Our data strongly support the extensive evaluation of CLIC1 in RA as a potential marker of inflammation and tool to predict therapy response.*

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Chloride intracellular channel protein 1 (CLIC1) is a member of the CLICs family, and one of the most conserved proteins (1). It was first cloned due to its increased expression in activated macrophages (2). CLIC1, also known as NCC27, has an early and variable expression in human fetal tissues, and is found in low levels in human fetal brain but in high levels in the human fetal lung, kidney, and liver (3). During human adult life, expression variability persists among most human tissues (4). The intracellular chloride channels are membrane bound and participate in different pathological processes including inflammation associated with neurodegenerative diseases (5) atherosclerosis (6) ankylosing spondylitis (7) tumor progression, and metastasis of urinary bladder cancer (8) renal cell carcinomas (9) or hepatocellular carcinomas (10). CLIC1 has a high translocation ability between the cytoplasmic and nuclear compartments depending on the functional status and based on this translocation; CLIC1 functions are extremely versatile (11). These versatile functions of chloride channels such as CLIC1 include the regulation of cellular metabolism by acting at the

enzymatic level (12). The role of CLIC1 in inflammation associated with different pathologies is well known. In this context CLIC1 is highly expressed in macrophages, the key cells of adaptive and innate immunity. CLIC1 expression is not restricted only to macrophages, it is also observed in connective tissue fibroblasts and myofibroblasts, abundantly found in different inflammatory lesions. The effects of CLIC1 on stromal cells are mediated by the presence of transforming growth factor beta (TGFβ) and tumor necrosis factor alpha (TNF α). In rheumatic diseases, CLIC1 is poorly studied, and the experimental models mainly include macrophages or stromal fibroblasts isolated from mice (13).

In rheumatic diseases, CLIC1 is closely correlated with the induction of inflammasomes that support the inflammation. CLIC1 is not only over-expressed in stromal cells, but also in epithelial cells such as endothelial or epidermal cells (12).

The methods of evaluating conventional and targeted therapies in rheumatic diseases are limited and the reported results are often variable. Musculoskeletal ultrasonography and synovial biopsies could not correctly evaluate the effects of the applied therapies, which is why the use of other diagnostic methods and therapeutic evaluation tools are required in rheumatoid arthritis (RA) and psoriatic arthritis (PsA) (14). Changes in the skin induced by RA or PsA are scarcely studied (15, 16). Data from the literature regarding the reactivity of the skin in RA and PsA before or after therapy are also extremely limited (17). Moreover, there is no specific marker that clearly characterizes skin or synovial response to therapy. CLIC1 stimulates endothelial injury, vascular inflammation, and oxidative stress, basic components in the pathophysiology of RA or PsA (18). Recent data have shown that CLIC1 inhibition attenuates the accumulation of inflammatory cytokines and reduces oxidative stress (18). Currently, for human subjects, we have no strong evidence regarding the involvement of CLIC1 in the pathophysiology of RA and PsA and its response to targeted or conventional therapy. Moreover, there are no data indicating that CLIC1 expression in skin biopsies from patients with RA and PsA supports the use of skin biopsies as a method of evaluating therapy response. Therefore, the present study aimed to evaluate CLIC1 expression in skin biopsies from RA and PsA patients administered various first/second line treatments or biological therapies.

Materials and Methods

Patients and biopsies. Thirty skin biopsies were collected from patients aged between 20 and 60 years old previously diagnosed with RA (22 skin biopsies) and PsA (8 skin biopsies) who had clinical signs of vasculitis. An informed consent was obtained from each patient before the procedure. The study was performed by respecting all ethical issues regarding the use of human tissues and the manipulation of paraffin embedded specimens and approval was

obtained from the County Clinical Ethic Committee (Approval No. 2654/19.02.2019). All patients were previously treated for RA and PsA by using common treatments for these diseases associated or not with biological therapies. We selected 30 patients who received methotrexate (MTX), 20 mg/week as first line therapy (FLIT) followed by second line therapy (SLIT) with leflunomide (LFN), 20 mg/day. In addition, corticotherapy was used when necessary, with 7.5 mg/day prednisone, for at least 14 days in 10 out of 30 patients. Furthermore, 6 out of 30 patients received biological therapy with Humira 40 mg/bimonthly and Tofacitinib 10 mg/day. Therapies used for the treatment of RA and PsA were statistically evaluated with regard to CLIC1 immunoexpression in skin biopsy stromal and vascular compartments. Patients' data included age, diagnosis, therapy duration, associated corticotherapy and serum inflammatory parameters (ESR and CRP).

Institutional review board statement. The study was performed by respecting all ethical issues regarding the use of human tissues and the manipulation of paraffin embedded specimens, and ethical approval was obtained from the County Clinical Ethic Committee (Approval No. 2654/19.02.2019).

Skin biopsies primary processing and histopathologic diagnosis. Collected fresh skin biopsies were fixed in buffered formalin for 24-48 h followed by routine paraffin embedding protocol. Three μm thick sections were obtained from each block and mounted on glass slides. One slide from each case was stained with hematoxylin and eosin for histopathologic evaluation. Based on histopathology we selected slides for immunohistochemistry.

Immunohistochemistry was performed for the evaluation of CLIC1 expression on skin biopsies. All immunohistochemical procedures were performed by using the fully controlled automated immunohistochemistry workflow Bond Max Autostainer (Breckland, Linford Wood, UK). We used anti-CLIC1 (356.1) antibodies (Santa Cruz Biotechnology, SC 81873, Santa Cruz, CA, USA) with a dilution of 1:2000. One-hour incubation with CLIC1 primary antibody at room temperature was followed by applying the detection step using Bond Polymer Refine Detection Kit (Leica Microsystems). The final step of the immunohistochemical technique was visualization of the final product as a brown staining given by the use of 3, 3 diaminobenzidine as chromogen and haematoxylin for nuclear counterstaining.

Microscopic evaluation and data analysis. Haematoxylin and eosin-stained slides were evaluated for the identification of persistent histopathologic changes and to select slides for immunohistochemistry. Our study focused on the identification of CLIC1 expression in two compartments of the dermis: stromal compartment (especially on CLIC1 expression in stromal fibroblasts and inflammatory cells, CLIC1-S) and vascular compartment (CLIC1 expression on endothelial cells of dermal blood vessels, CLIC1-V). Based on CLIC1 expression on endothelial cells, we calculated the microvessel density, which was subsequently correlated with therapy type and other clinicopathologic parameters. All slides were scanned by using Desk Pannoramic Scanner (3D Histech, Budapest, Hungary) and they were stored in the Histology Department Digital Slides Library Case Center. Slide evaluation, picture capture, and processing were performed by using Pannoramic Viewer program (3D Histech). Evaluation of CLIC1 expression in stromal fibroblasts was performed by using the facilities of Cell Quant, at Quant Center (kindly provided
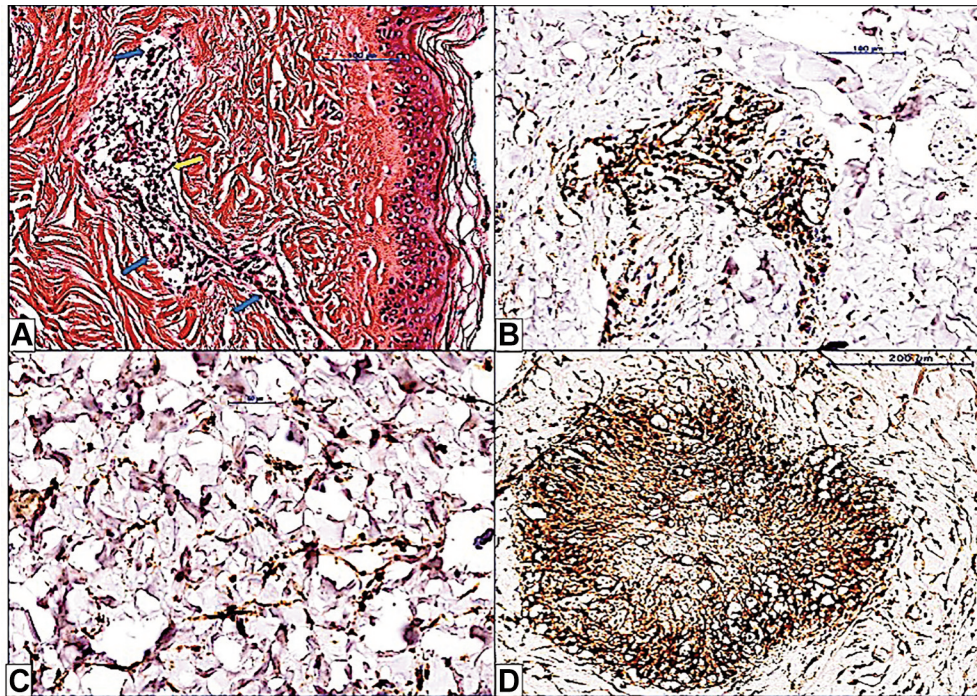
Figure 1. *Skin biopsies from patients with rheumatoid arthritis (RA). (A) Microscopic view of vasculitis in skin biopsies of patients with RA. Note the branched capillaries network (blue arrows) and the massive inflammatory infiltrate (yellow arrow) surrounding it. (B) CLIC1 immunohistochemical expression in the endothelium of capillaries and inflammatory cells from vasculitis lesions. (C) CLIC1 positive stromal fibroblasts. (D) Rheumatoid nodule positive for CLIC1. Note the cytoplasmic and nuclear expression of CLIC1 in the outer part of the rheumatoid nodule, while the center is less stained.*

by 3DHistech). The evaluation score of this program is given by combination of intensity of CLIC1 expression and number of CLIC1 positive fibroblasts previously selected and defined by the pathologist. Also, CLIC1 expression in the endothelium of vessels was quantified by using the same semiautomated method of the Quant Center by counting CLIC1 positive blood vessels from skin biopsies.

Statistical analysis. Statistical analysis was performed by using JAMOVI software version 1.2.27.0. A *p*-value of <0.05 was considered statistically significant, while a *p*-value of <0.001 was considered strongly statistically significant. Charts and correlation tables included as part of the results section in this study were automatically generated by JAMOVI software.

Results

Histopathology and CLIC1 immunoexpression in RA and PsA skin biopsies. Vasculitis microscopic changes (Figure 1A) and classical rheumatoid nodules were observed on hematoxylin and eosin-stained skin biopsies. Microscopically, all selected cases were positive for CLIC1 by immunohistochemistry. CLIC1 was positive in the endothelium of skin blood vessels, stromal fibroblasts, and in the outer part of rheumatoid nodules. Inflammatory cells surrounding skin capillaries also expressed CLIC1 (Figure

1B-D). The inflammatory infiltrate was different from one case to another; it varied from scattered inflammatory cells with a heterogeneous distribution to a massive inflammatory infiltrate associated with groups of small skin capillaries lined by cells with the morphology of activated endothelial cells, which were positive for CLIC1. Based on this positivity in endothelial cells, we were able to quantify CLIC1 expression by performing counting of CLIC1 positive vessels, noted as CLIC1-V, and assessed their relationship to clinical, paraclinical, and therapeutic data. CLIC1 was expressed in blood vessel endothelium but not in the lymphatic endothelium of the skin. When we analysed CLIC1 expression in the vascular compartment of skin biopsies from RA and PsA patients, we observed that small blood vessels with CLIC1 positive endothelium had a heterogeneous morphology highly suggestive of an intense angiogenic process (we were able to detect microscopically angiogenic sprouts, intussusceptive angiogenesis, tube formation, and capillary like structures) also in the absence of inflammatory infiltrate. Most of the CLIC1 positive blood vessels had a branched morphology with a lumen lined by CLIC1 positive endothelial cells. The CLIC1 expression pattern was both nuclear and cytoplasmic (Figure 2).
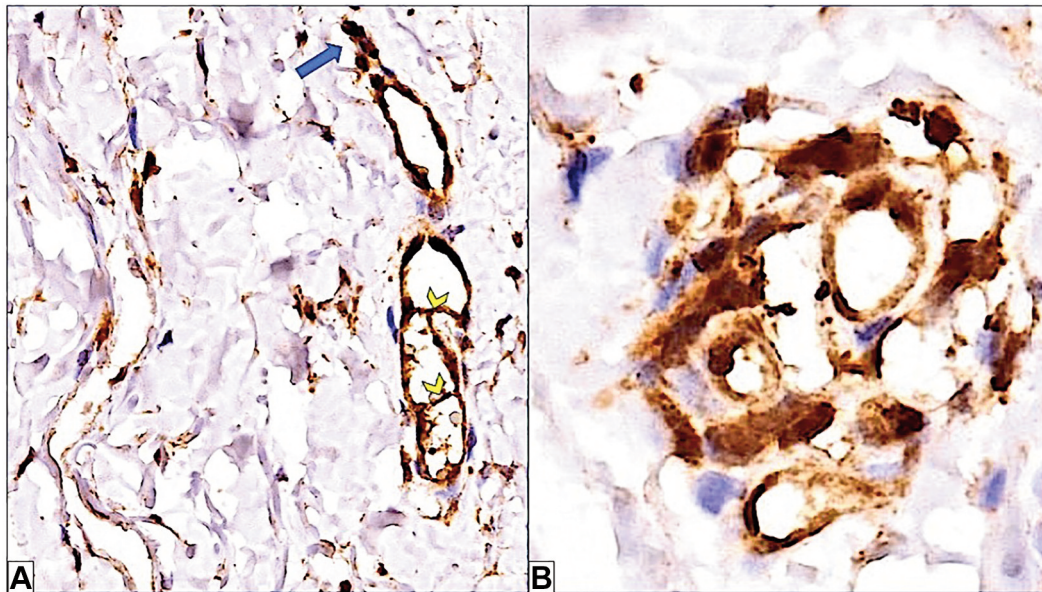
Figure 2. *CLIC1 expression in the vascular compartment of the skin from patients with rheumatoid arthritis (RA). (A) CLIC1 positive vessels with morphology suggestive of activated endothelial cells with nuclear and cytoplasmic expression organized as sprouts emerging from pre-existing blood vessels (blue arrow). Vascular lumen split by CLIC1 positive intraluminal pillars (yellow arrowhead) highly suggestive for the presence of intussusceptive angiogenesis. (B) Groups of CLIC1 positive small capillaries arranged in a pseudo-glomerular pattern mimicking glomeruloid bodies usually found in highly angiogenic tissues but here in the skin biopsies from patients with RA. Note, also the nuclear and cytoplasmic expression of CLIC1 in endothelial cells.*

Table I. *Correlation matrix showing age-dependent CLIC1 immunoexpression in the stromal compartment (CLIC1-S) of the rheumatoid arthritis affected skin despite of the therapy received.*

		Age
CLIC1-S	Pearson's r	0.737**
	p-value	0.001
	95% CI Upper	0.903
	95% CI Lower	0.380
	Spearman's rho	0.932***
	p-value	<0.001
	Kendall's Tau B	0.843***
	p-value	<0.001

CLIC1-S: Chloride intracellular channel protein 1 expression in stromal compartment. **$p<0.01$, ***$p<0.001$, one-tailed.

Table II. *An inverse correlation was found between CLIC1 immunoexpression in stromal (CLIC1-S) and vascular (CLIC1-V) compartments.*

		CLIC1-V
CLIC1-S	Pearson's r	−0.544*
	p-Value	0.018
	Spearman's rho	−0.135
	p-Value	0.315
	Kendall's Tau B	−0.220
	p-Value	0.126

CLIC1-S: Chloride intracellular channel protein 1 expression in stromal compartment; CLIC1-V: chloride intracellular channel protein 1 expression in vascular compartment. *$p<0.05$, one-tailed.

Sprouting phenomenon with tip cells intensely positive for CLIC1 and intraluminal CLIC1 positive pillars were the main microscopic features indicating intense angiogenesis in skin biopsies from RA and PsA patients (Figure 2A and B).

Age and CLIC1 immunoexpression in skin biopsies from RA and PsA patients. Age of the patients seems to differentially influence CLIC1-V and CLIC1-S immunoexpression in the skin of patients treated for RA. A strong correlation has been found between age and high CLIC1-S immunoexpression ($p<0.001$) for patients aged over 50 years compared with younger ones (Table I). Also, when we analyzed cases according to age, we observed that CLIC-S expression was partial and negatively correlated with CLIC-V immunoexpression ($p<0.018$) in the dermal compartment of patients with RA (Table II). These findings suggest that older patients retain CLIC1 positive stromal cells in their skin but not CLIC1 positive blood vessels, independently of the therapy received. CLIC1-S persistence in

Table III. *Methotrexate (MTX) and leflunomide (LFN) therapy influence on CLIC1 expression in skin biopsies from rheumatoid arthritis patients (a) MTX therapy was significantly correlated with CLIC1-S but not with CLIC1-V. MTX therapy duration did not influence CLIC1 expression.*

		CLIC1-S	CLIC1-V	MTX
CLIC1-V	Pearson's r	–0.084	—	
	p-Value	0.741	—	
	95%CI Upper	0.399	—	
	95%CI Lower	–0.530	—	
	Spearman's rho	–0.387	—	
	p-Value	0.113	—	
	Kendall's Tau B	–0.324	—	
	p-Value	0.080	—	
MTX	Pearson's r	0.556*	0.301	—
	p-Value	0.016	0.225	—
	95%CI Upper	0.812	0.673	—
	95%CI Lower	0.121	–0.193	—
	Spearman's rho	0.652**	–0.131	—
	p-Value	0.003	0.605	—
	Kendall's Tau B	0.565**	–0.115	—
	p-Value	0.007	0.589	—
MTX-Months	Pearson's r	–0.191	0.371	0.156
	p-Value	0.449	0.130	0.536
	95%CI Upper	0.303	0.714	0.581
	95%CI Lower	–0.604	–0.116	–0.335
	Spearman's rho	–0.365	0.419	0.137
	p-Value	0.137	0.083	0.587
	Kendall's Tau B	–0.312	0.316	0.127
	p-Value	0.111	0.110	0.571

CLIC1-S: Chloride intracellular channel protein 1 expression in stromal compartment; CLIC1-V: chloride intracellular channel protein 1 expression in vascular compartment; MTX: methotrexate therapy; MTX-MONTHS: therapy duration (months) with MTX. *$p<0.05$, **$p<0.01$.

Table IV. *Significant correlation between LFN dose and therapy duration and the decrease in CLIC1-V expression in skin biopsies from rheumatoid arthritis patients.*

		CLIC1-S	CLIC1-V	LFN
CLIC1-V	Pearson's r	–0.084	—	
	p-Value	0.741	—	
	95%CI Upper	0.399	—	
	95%CI Lower	–0.530	—	
	Spearman's rho	–0.387	—	
	p-Value	0.113	—	
	Kendall's Tau B	–0.324	—	
	p-Value	0.080	—	
LFN	Pearson's r	0.082	–0.766***	—
	p-Value	0.746	<0.001	—
	95%CI Upper	0.529	–0.465	—
	95%CI Lower	–0.400	–0.908	—
	Spearman's rho	0.225	–0.770***	—
	p-Value	0.370	<0.001	—
	Kendall's Tau B	0.159	–0.678***	—
	p-Value	0.415	<0.001	—
LFN.Months	Pearson's r	0.148	–0.712***	0.635**
	p-Value	0.559	<0.001	0.005
	95%CI Upper	0.575	–0.367	0.850
	95%CI Lower	–0.343	–0.885	0.240
	Spearman's rho	0.622**	–0.810***	0.703**
	p-Value	0.006	<0.001	0.001
	Kendall's Tau B	0.605**	–0.706***	0.611**
	p-Value	0.002	<0.001	0.003

CLIC1-S: Chloride intracellular channel protein 1 expression in stromal compartment; CLIC1-V: chloride intracellular channel protein 1 expression in vascular compartment; LFN: leflunomide therapy; LFN-MONTHS: therapy duration (months) with LFN. **$p<0.01$, ***$p<0.001$.

skin biopsies from older patients with RA may be considered as a poor prognostic factor. No significant correlation was detected between age, CLIC1-S ($p=0.777$), and CLIC1-V ($p=0.702$) for patients with PsA.

Effects of conventional therapies, corticotherapy, and biological therapies on CLIC1 expression in the skin stromal and vascular compartments from patients with RA and PsA. Methotrexate was used as first line therapy for treating patients with RA. Following MTX therapy, the increased expression of CLIC1 in the stromal compartment of the skin of RA patients was maintained (CLIC1-S, $p=0.016$), while CLIC1-V was not influenced by MTX therapy ($p=0.255$) (Table III). Also, MTX therapy duration had no impact on both CLIC1-S ($p=0.449$) and CLIC1-V ($p=0.130$). Leflunomide (LFN) therapy in RA patients did not influence CLIC1-S but significantly reduced MVD as assessed by CLIC1-V immunoexpression ($p<0.001$). Compared with MTX therapy whose duration did not influence CLIC1 expression, LFN therapy duration strongly decreased CLIC-V immunoexpression ($p<0.001$) (Table IV).

Associated corticotherapy reduced CLIC1-S expression in a dose- and time-dependent manner ($p=0.006$), but it did not influence dermal CLIC1-V. For skin biopsies derived from PsA patients, MTX therapy had a behaviour similar to RA; significantly correlated with CLIC1-S expression ($p=0.022$) but had no influence on CLIC1-V expression. No other therapies influenced CLIC1-S and CLIC1-V immunoexpression in the skin from patients with PsA, which was sustained; no significant correlations were found. Biological therapies (humira and tofacitinib) had a dual impact on both CLIC1-S and CLIC1-V expression in skin biopsies from RA patients. Both therapies reduced not only CLIC1-S ($p<0.001$) but also CLIC1-V ($p=0.007$) immunoexpression (Table V).

Serum inflammatory parameters such as erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) were usually normalized during the therapy applied. The correlation of serum inflammatory parameters, (usually used in RA as part of the pre- and post-therapy evaluation) with CLIC1-S and CLIC1-V was examined. We found that, despite of theESR and CRP normalisation an inverse

Table V. *Correlation matrix showing significant effects of biological therapies on CLIC1 immunoexpression in both vascular and stromal compartments of skin biopsies.*

		CLIC1-V	CLIC1-S
CLIC1-V	Pearson's r	—	
	p-Value	—	
	95%CI Upper	—	
	95%CI Lower	—	
	Spearman's rho	—	
	p-Value	—	
	Kendall's Tau B	—	
	p-Value	—	
CLIC1-S	Pearson's r	0.984***	—
	p-Value	<0.001	—
	95%CI Upper	0.998	—
	95%CI Lower	0.859	—
	Spearman's rho	1.000***	—
	p-Value	<0.001	—
	Kendall's Tau B	1.000*	—
	p-Value	0.012	—
Biol therapy	Pearson's r	–0.982***	–0.933**
	p-Value	<0.001	0.007
	95%CI Upper	–0.839	–0.502
	95%CI Lower	–0.998	–0.993
	Spearman's rho	–0.866*	–0.866*
	p-Value	0.026	0.026
	Kendall's Tau B	–0.816	–0.816
	p-Value	0.053	0.053

CLIC1-S: Chloride intracellular channel protein 1 expression in stromal compartment; CLIC1-V: chloride intracellular channel protein 1 expression in vascular compartment; Biol Therapies: Biological Therapies (Humira, Tofacitinib). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table VI. *ESR and CRP variability related to CLIC1-S and CLIC1-V in the skin of patients with rheumatoid arthritis. Note that CLIC1-S expression showed a significant negative correlation with both ESR and CRP.*

		CLIC1-V	CLIC1-S	ESR
CLIC1-V	Pearson's r	—		
	p-Value	—		
	95%CI Upper	—		
	95%CI Lower	—		
	Spearman's rho	—		
	p-Value	—		
	Kendall's Tau B	—		
	p-Value	—		
CLIC1-S	Pearson's r	–0.084	—	
	p-Value	0.741	—	
	95%CI Upper	0.399	—	
	95%CI Lower	–0.530	—	
	Spearman's rho	–0.387	—	
	p-Value	0.113	—	
	Kendall's Tau B	–0.324	—	
	p-Value	0.080	—	
ESR	Pearson's r	–0.137	–0.552*	—
	p-Value	0.587	0.018	—
	95%CI Upper	0.352	–0.115	—
	95%CI Lower	–0.568	–0.810	—
	Spearman's rho	–0.052	–0.313	—
	p-Value	0.839	0.206	—
	Kendall's Tau B	–0.031	–0.182	—
	p-Value	0.874	0.342	—
CRP	Pearson's r	–0.093	–0.523*	0.740***
	p-Value	0.715	0.026	<0.001
	95%CI Upper	0.391	–0.075	0.897
	95%CI Lower	–0.536	–0.796	0.417
	Spearman's rho	0.218	–0.478*	0.622**
	p-Value	0.384	0.045	0.006
	Kendall's Tau B	0.191	–0.414*	0.562*
	p-Value	0.368	0.049	0.010

CLIC1-S: Chloride intracellular channel protein 1 expression in stromal compartment; CLIC1-V: chloride intracellular channel protein 1 expression in vascular compartment; ESR: erythrocytes sedimentation rate; CRP: C reactive protein. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

correlation was detected between CLIC1-S and ESR *(p=0.0018)* and CRP *(p=0.026)* but not for CLIC1-V. This finding suggests that ESR and CRP normalization did not give a fully objective evaluation of the inflammation status in RA and the persistence of elevated CLIC1-S in patients' skin with RA may be considered (Table VI).

Discussion

RA is an autoimmune disease primarily affecting the joints but also other organs including the skin. Psoriasis, a well-known skin lesion, may be accompanied by PsA. Both diseases induce a general inflammatory response, developed through different mechanisms involving inflammasomes. Inflammasomes are stimulus-induced multimeric protein complexes of innate immunity (19), which have a high rate of polymorphism in RA and psoriasis (20, 21); they trigger an inflammatory response following their activation through heterogeneous complex mechanisms. *NLRP3* rs35829419 or *CARD8* rs2043211 polymorphisms support a proinflammatory state in about 8% of patients at the time of diagnosis, whereas the *CARD8*

rs2043211 TT genotype seems to be strongly involved in resistance to therapy by continuing to promote a proinflammatory state even 6 months after the beginning of therapy (20). CLIC1 seems to be one of the most important factors involved in NRLP3 inflammasome activation reported to be highly responsible for the inflammatory events in RA (12, 22). The involvement of CLIC1 in arthritis was indirectly shown by Jiang *et al.* (2), who created CLIC1$^{-/-}$ mice having macrophages with an elevated phagosome pH. Because of this peculiarity, such mice were not able to develop macrophage-dependent immune-complex mediated arthritis (2). Here, we report for the first time the presence and expression of CLIC1 in human rheumatoid nodules. This microscopic evidence

supports CLIC1 involvement in the pathogenesis of RA in humans most probably through NRLP3 activation. This is in accordance with the previous findings of Heruth *et al.*, who demonstrated that CLIC1 is exclusively expressed in human rheumatoid arthritis fibroblasts but not in human normal synovial fibroblasts (23). Based on this observation and together with the inflammatory events found in the RA patients' skin, we may hypothesize that CLIC1 positive skin fibroblasts (noted in our study with CLIC1-S) may be considered as an indicator of active disease and may be used to assess the impact of different therapies received by our patients during disease progression on CLIC1 expression.

The inflammation milieu induces endothelial cell activation and triggers angiogenic processes. CLIC1 translocation in endothelial cells has been observed in malignant tumors such as glioblastoma (24), suggesting that it may be involved in angiogenesis, tumor progression, and metastasis (25, 26). Immunohistochemical expression of CLIC1 in the tumor blood vessel endothelium has been recently reported by Raica *et al*. (27). Our team assessed MVD by using for the first time CLIC1 endothelial immunoexpression as a marker of activated endothelium in newly formed breast cancer blood vessels. We applied the same model for assessing CLIC1 positive blood vessels (noted here with CLIC1-V) in skin biopsies from RA and PsA patients, considering that endothelial cells are highly activated by inflammation if they express CLIC1.

It is well known that RA and PsA patients have no favorable response to the same drugs during disease progression. Thus, for most of them, there is a need to implement second line therapy associated or not with corticotherapy, and the use of antibody-based targeted therapies. Most of the RA and PsA patients are treated with methotrexate (MTX) as first line therapy, as in our study. Our results showed that MTX had no influence on CLIC1-S expression in skin biopsies from RA and PsA patients. This finding suggested that CLIC1 may be a potential factor of development of chemoresistance to MTX, which may explain in part why patients become unresponsive to MTX during RA and PsA disease progression. Recent data confirmed CLIC1 involvement in the development of MTX chemoresistance by tumor cells (28) and highlighted also the molecular mechanism of CLIC1-induced chemoresistance (29).

Leflunomide therapy negatively influenced CLIC1-V expression in our study. Indirect evidence, which may explain our results, is found in the literature but none of them are related to RA or PsA. Leflunomide is a NF-κB and TNF-α inhibitor, two factors involved in inflammation-induced angiogenesis in RA and PsA. NF-κB is positively regulated by CLIC1 (30), which also induces TNF-α, expression in endothelial cells promoting vascular inflammation (18). *In vitro*, the strongest activator of NF-κB in endothelial cells appears to be TNFα (31).

CLIC1-V was significantly decreased in leflunomide-treated patients, most probably due to inhibition of NF-κB and TNF-α, which are usually found in high levels in these diseases.

Biological therapies used in our study act on both the stromal and vascular compartment in skin biopsies from RA patients. Adalimumab (humira) exerts anti TNF-α effects, whereas tofacitinib acts on JAK/STAT3 pathway. Both drugs reduced CLIC1-S and CLIC1-V expression in skin biopsies from RA patients but no evidence of interaction of CLIC1 with JAK/STAT3 has been reported. The decrease in the expression of CLIC1-V and CLIC1-S following tofacitinib treatment may be explained by an indirect mechanism of lymphocyte inhibition and a subsequent change in the inflammatory microenvironment.

The presence of CLIC1 in the endothelium of blood vessels where we were able to observe sprouting and/or intussusceptive angiogenesis microscopic patterns suggested that CLIC1 may promote the angiogenesis. There has been indirect evidence of CLIC1 involvement in endothelial cell activation from blood vessels found in tumor and non-tumor conditions (32-34). *In vitro* studies performed by Tung and Kitajewski have shown that CLIC1 is involved in most steps of the angiogenic process by promoting endothelial cell migration, proliferation, and their ability to form capillary-like structures and branches (32). These data have not yet been validated on human tissues. Our observations regarding the presence of blood vessels having CLIC1 positive sprout-like structures and also CLIC1 positive intraluminal pillars are in concordance with previous *in vitro* findings by Tung and Kitajewski and are the first microscopic evidence of CLIC1 involvement in inflammation-induced angiogenesis in skin biopsies of patients with RA and PsA. Although there is only indirect evidence on the involvement of CLIC1 in angiogenesis, TNF-α and JAK/STAT3 pathway (the main targets for biological therapies in RA and PsA) are known to be key players in the angiogenic process (35, 36) by endothelial cell activation. For patients with RA enrolled in our study and treated with biological therapies (adalimumab as anti-TNF-α agent and tofacitinib as anti-JAK/STAT3 pathway) we found a significant decrease in CLIC1 immunoexpression in the vascular compartment followed by a decrease in microvessel density. Our data support a strong interrelation between CLIC1, TNF-α, and the JAK/STAT3 pathway in RA, however, further studies are needed to fully elucidate the mechanism of such interrelation.

Conclusion

The present study is the first report on CLIC1 expression in rheumatoid nodules, and stromal and vascular compartments in skin biopsies from patients with RA and PsA. Both diseases are characterised by inflammation involving several organs, including the skin, and it seems that CLIC1 is deeply involved

in the inflammatory skin lesions of patients with RA and PsA. We report here that RA and PsA therapeutic agents may differentially influence CLIC1 skin expression, and its dynamic changes are independent of serum inflammatory markers. Thus, we may conclude that CLIC1 assessment in skin biopsies from patients with RA and PsA may be used as a potential tool for predicting the general inflammatory state and therapy response. Currently, no data regarding CLIC1 serum evaluation are available for RA and PsA despite of its function as one of the potent factors in promoting inflammation. Our data strongly support extensive evaluation of CLIC1 in RA as a potential new tissue and serum marker of inflammation assessment and predictive tool for therapy response.

Funding

Conflicts of Interest

The Authors declare no conflicts of interest. The funders had no role in the design of the study; the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Authors' Contributions

Conceptualization, L.B. and M.C.; methodology, O.S.C.; validation, A.M.C., investigation, L.B. and A.M.C.; writing—original draft preparation, L.B. and M.C.; writing—review and editing, A.M.C.; supervision, M.R., CA- investigation. All Authors have read and agreed to the published version of the manuscript.

Acknowledgements

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