

ORIGINAL ARTICLE

CD21^{low} B cells are predictive markers of new digital ulcers in systemic sclerosis

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Summary

The objective of this study was to evaluate the predictive role of CD21^{low} B cells as markers of new digital ulcers in systemic sclerosis patients. Peripheral blood B cell subpopulations and clinical assessments have been evaluated in 74 systemic sclerosis patients at baseline and after a 12-month follow-up. After a 12-month follow-up, 23 (31.1%) systemic sclerosis patients developed new digital ulcers. The median percentage of CD21^{low} B cells was significantly higher in patients with than without new digital ulcers [10.1 (4.3–13.6) versus 4.8 (3.5–7.4); $p < 0.01$]. The 10% cut-off shows good diagnostic accuracy [area under the curve (AUC) = 0.732, confidence interval (CI) = 0.587–0.878; $P = 0.01$]. Kaplan–Meier curves show a significantly reduced free survival from new digital ulcers in systemic sclerosis patients with CD21^{low} B cells $\geq 10\%$ ($p < 0.0001$). In multivariate analysis, CD21^{low} B cells $\geq 10\%$, modified Rodnan skin score (mRSS) and systolic pulmonary arterial pressure (sPAP) are associated with the development of new digital ulcers. We hypothesize that CD21^{low} B cells are a predictive marker of new digital ulcers in systemic sclerosis patients.

KEYWORDS

B cells, CD21^{low}, connective tissue diseases, digital ulcers, major vascular complications, raynaud's phenomenon, systemic sclerosis

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease characterized by dysregulation of the immune system, endothelial dysfunction, vascular damage and fibrosis of skin and internal organs (1,2). Several studies have shown that in SSc patients there is an impairment of the homeostasis of B cells, and the efficacy of B cell depletion with rituximab further supports the involvement of B cells in disease development or progression (3–7). Recently, an

increased percentage of B cells with reduced expression of CD21 (CD21^{low} B cells) has been identified in SSc patients. Forestier *et al.* did not find a significant difference in percentage of CD21^{low} B cells between patients with diffuse (dcSSc) or limited (lcSSc) cutaneous SSc. In a cohort of 74 SSc patients, CD21^{low} B cells were significantly increased in SSc patients compared to healthy controls. The percentage of CD21^{low} B cells is increased in SSc patients with impaired angiogenesis, increased systolic pulmonary arterial pressure (sPAP), reduction

Marcella Visentini and Chiara Pellicano contributed equally to this study.

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of diffusion lung of carbon monoxide (DLco) and major vascular manifestations (4). Digital ulcers (DUs) are the main vascular complication of SSc and are reported in approximately 35–40% of SSc patients (8,9). Several risk factors for the onset of new DUs have been identified, such as high modified Rodnan skin score (mRSS), presence of anti-topoisomerase I antibody, dcSSc subset, higher value of sPAP, increased intrarenal arterial stiffness and late capillaroscopic pattern (10–17). Sebastiani *et al.* identified a new capillaroscopic predictive index of new DU onset. The capillaroscopic skin ulcer risk index (CSURI) is a new prognostic tool for DUs development in SSc patients (14).

The aim of this study was to evaluate the predictive role of CD21^{LOW} B cells in the development of new DUs after 12 months of follow-up in SSc patients.

METHODS

Subjects

Seventy-four SSc patients [$F = 64$, median age = 54 years, interquartile range (IQR) = 46–63], fulfilling the American College of Rheumatology/European League Against Rheumatism Collaborative Initiative criteria for SSc, were enrolled into this study. Thirty-four (45.9%) patients had dcSSc, according to Le Roy *et al.* Demographic and clinical features of SSc patients are shown in Table 1.

Exclusion criteria were: hematological malignancies, pulmonary and cardiovascular diseases not related to SSc, diabetes, peripheral arterial disease, thrombophilia, secondary anti-phospholipid syndrome, smoke, pregnancy, breastfeeding and treatment in the last 6 months with

TABLE 1 Demographic and clinical features of SSc patients

Female, n (%)	64 (86.5)
dcSSc, n (%)	34 (45.9)
SSc-specific autoantibodies	
Anti-topoisomerase I, n (%)	29 (39.2)
Anti-centromere, n (%)	21 (28.4)
Anti-RNA polymerase III, n (%)	1 (1.4)
None, n (%)	23 (31.1)
Nailfold capillaroscopic pattern	
Early, n (%)	16 (21.6)
Active, n (%)	17 (23)
Late, n (%)	41 (55.4)
New digital ulcers, n (%)	23 (31.1)
CD21 ^{LOW} B cells \geq 10% at baseline, n (%)	20 (27)
CD21 ^{LOW} B cells \geq 10% at follow-up, n (%)	21 (28)

Abbreviations: dcSSc, diffuse cutaneous systemic sclerosis; SSc, systemic sclerosis.

immunosuppressive agents and corticosteroids at an equivalent dose of prednisone \geq 10 mg/day.

The subjects' written consent was obtained according to the Declaration of Helsinki and the study was approved by the ethics committee of Sapienza University (no. 5435).

Evaluation of B cell subpopulation

Immunophenotyping of peripheral blood samples at baseline and after 12-month follow-up was performed with combinations of fluorochrome-labeled monoclonal antibodies (Becton-Dickinson Biosciences, San Jose, CA, USA) to CD19-PC5.5, CD21-phycoerythrin (PE), CD27-allophycocyanin (APC) and immunoglobulin (Ig)D-fluorescein isothiocyanate (FITC). Flow cytometry analysis was performed on a BD fluorescence activated cell sorter (FACS)Calibur system and data files were acquired and analysed using CELLQuest version 3.3 (Becton Dickinson, Mountain View, CA, USA) and FlowJo (TreeStar, Inc., Ashland, OR, USA) software. We chose a threshold of 10% for CD21^{LOW} B cells. This value has been chosen as media \pm 2 standard deviations (s.d.) of the CD21^{LOW} B cells percentage of healthy controls, according to our previous study (4).

Clinical assessments

The main clinical indices were assessed for all SSc patients at baseline and every 6 months for a follow-up period of 12 months. Skin involvement was assessed by mRSS (18). The activity and severity of disease were assessed by disease activity index (DAI) (19) and disease severity scale (DSS) (20), respectively. The scleroderma pattern (early, active, late) was evaluated with nailfold videocapillaroscopy (NVC) equipped with a \times 500 optical probe (21). DUs were defined according to Amanzi *et al.* (22). The timing of first new DUs was reported and the patient was censored.

Statistical analysis

All results are expressed as mean \pm s.d. and median and IQR, as appropriate. SPSS version 25.0 software was used for statistical analysis. The coefficient of skewness and the Shapiro–Wilk test were used to evaluate normal distribution of data. Receiver operating characteristic (ROC) curve analysis was performed to analyse the prognostic accuracy of CD21^{LOW} B cells for the development of new DUs at follow-up. All time-to-event end-points were estimated by the Kaplan–Meier method and analysed with the log-rank test. Hazard ratios with 95% confidence intervals (CI) were calculated by use of Cox regression models. Multivariate analysis was applied for the estimation of CD21^{LOW} B cells with clinical features.

Group comparisons were made by Mann–Whitney *U*-test. The Wilcoxon signed-rank test was used for comparison of the average of two dependent samples. Spearman's rank correlation coefficient was used to test for associations between numerical variables. The χ^2 test or Fisher's exact test, as appropriate, were used to compare categorical variables. *p* values < 0.05 were considered significant.

RESULTS

CD21^{low} B cells in SSc patients are stable during the long-term follow-up

The mean percentage of B cells of total lymphocytes was unchanged from baseline ($8.8 \pm 4.5\%$ versus $9.4 \pm 5.7\%$), as were percentages of naive ($65.4 \pm 20.9\%$ versus $61.4 \pm 22.7\%$), IgM⁺ memory ($12.2 \pm 10\%$ versus $12.1 \pm 9.9\%$), switched memory (16.5 ± 12.2 versus $18.6 \pm 13.3\%$) and double-negative (DN) B

cells ($6.5 \pm 4.4\%$ versus $6.5 \pm 5\%$) (Figure 1a). CD21^{low} B cells were slightly increased over time ($6.7 \pm 4.3\%$ versus $8.6\% \pm 5$; *p* < 0.05) (Figure 1b), but when the two subgroups of SSc patients, SSc-CD21⁺ and SSc-CD21^{low}, were analyzed separately, we found no differences in the percentages of CD21^{low} B cells after follow-up (Figure 1b). Only one patient (male, 47 years) changed from CD21⁺ to CD21^{low} SSc subgroup because of an increase of CD21^{low} B cells above 10% of total B cells (5.3% at baseline and 15% at follow-up), confirming that CD21⁺ and CD21^{low} SSc patients are distinct subsets of patients.

CD21^{low} B cells and DUs

At baseline, 20 (27%) SSc patients had CD21^{low} B cells $\geq 10\%$ of total B cells and 44 (59.5%) had history of DUs. We did not observe a linear correlation between CD21^{low} B cells and mRSS or changes of capillaroscopic pattern in all patients at follow-up. Table 1 shows clinical findings of SSc patients.

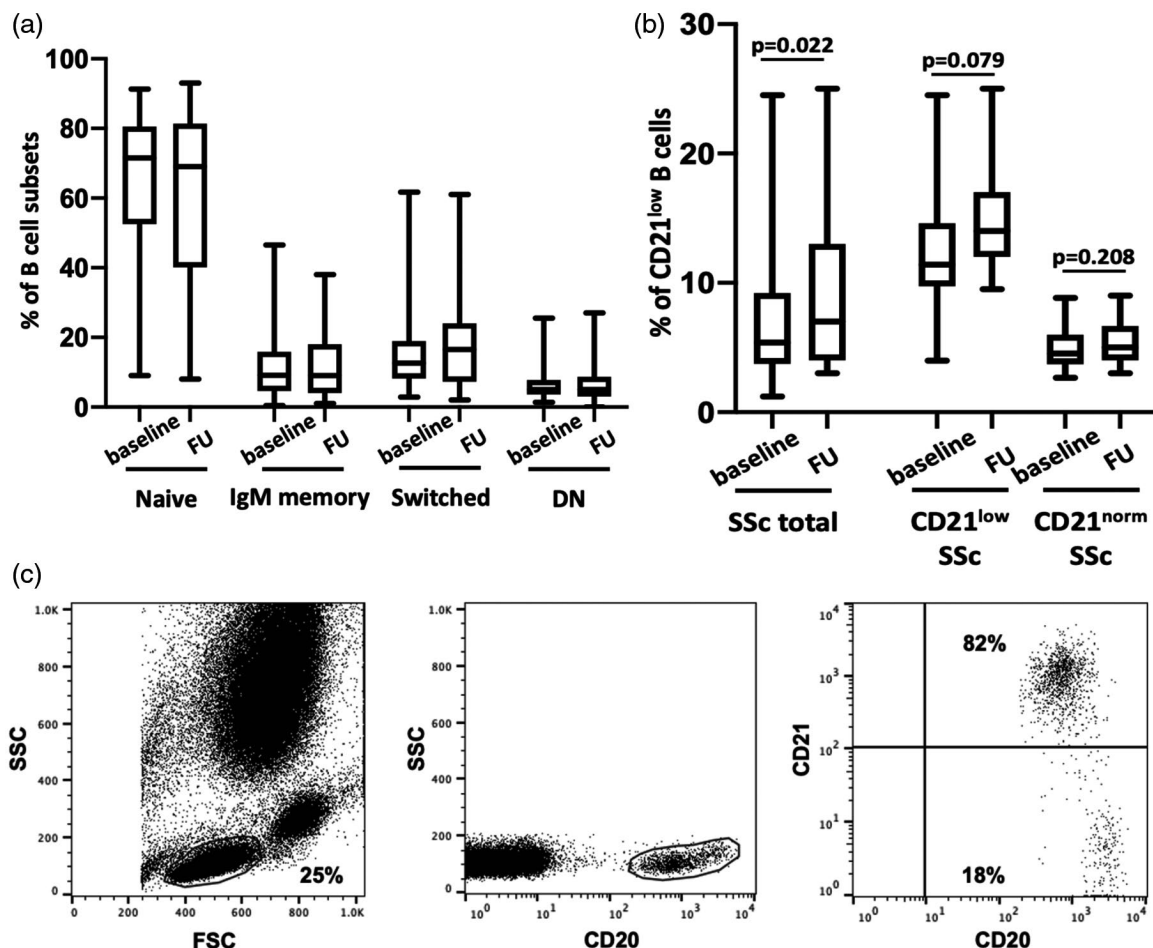


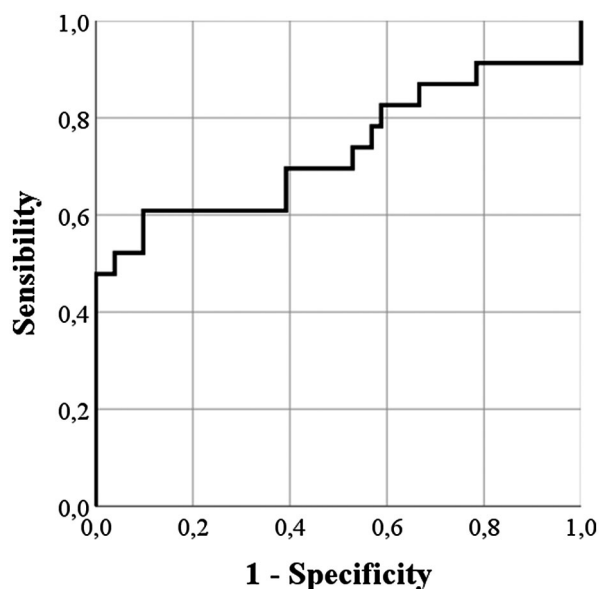
FIGURE 1 B cell subpopulation distribution in systemic sclerosis (SSc) patients at baseline and follow-up. Percentages of naive, immunoglobulin (Ig)M⁺ memory, switched memory and double-negative (DN) B cells in SSc patients at baseline and at the end of follow-up (a); percentages of CD21^{low} B cells in SSc patients and in CD21^{low} and CD21⁺ SSc patients at baseline and at the end of follow-up (b); gating strategy of CD21^{low} B cells in one representative SSc CD21^{low} patient (c)

TABLE 2 Median values and IQR of CD21^{low} B-cells, sPAP, FVC and DLco in SSc patients at baseline and after 12-month follow-up

	Baseline	Follow-up	<i>p</i>
CD21 ^{low} B cells, %, median and IQR	5.3 (3.7–9.1)	7 (4–13)	<0.05
sPAP, mmHg, median and IQR	28.5 (26–33)	28 (25–31)	>0.05
FVC, % predicted, median and IQR	96 (85–110)	100.5 (91–115)	<0.05
DLco, % predicted, median and IQR	71 (64–80)	73.5 (63–82)	>0.05
mRSS, median and IQR	9.5 (6–16)	9 (6–16)	>0.05
DAI, median and IQR	1.5 (0.6–2.4)	1.5 (1–3)	>0.05
DSS, median and IQR	5 (4–6)	5 (4–6)	>0.05

Abbreviations: DAI, disease activity index; DLco, diffusing capacity of the lung for monoxide carbon; DSS, disease severity scale; FVC, forced vital capacity; IQR, interquartile range; mRSS, modified Rodnan skin score; PAP, systolic pulmonary artery pressure.

Significant *p*-values are reported in bold type.

**FIGURE 2** Receiver operating characteristic (ROC) curve analysis performed to analyze the prognostic accuracy of CD21^{low} B cells

After a 12-month follow-up period, 23 (31.1%) SSc patients developed new DUs. Sixteen of these 23 (21.6%) SSc patients had a percentage of CD21^{low} B cells \geq 10% of total B cells (Table 1).

Table 2 shows the median value of CD21^{low} B cells and clinical variables at baseline and after 12-month follow-up. The median values of CD21^{low} B cells at follow-up were significantly ($p < 0.05$) higher than at baseline [7 (4–13) versus 5.3% (3.7–9.1); $p < 0.05$]. Median values of CD21^{low} B cells at baseline [10.1 (4.3–13.6) versus 4.8% (3.5–7.4); $p < 0.01$] were significantly higher in patients with new DUs than patients without new DUs.

ROC curve analysis demonstrated that CD21^{low} B cells predict new DUs [area under the curve (AUC) = 0.732 (95% CI = 0.587–0.878); $p < 0.05$](Figure 2).

Kaplan–Meier analysis comparing new DUs free survival in SSc patients with CD21^{low} B cells \geq 10% or CD21^{low}

B cells $<$ 10% demonstrated that those with CD21^{low} B cells \geq 10% develop new DUs (log-rank test $<$ 0.0001) (Figure 3).

Using univariate regression analysis, CD21^{low} B cells \geq 10% [hazard ratio (HR) = 0.413 (95% CI = 0.272–0.625); $p < 0.0001$], dcSSc [HR = 1.627 (95% CI = 1.058–2.5); $p < 0.05$], late capillaroscopic pattern [HR = 0.373 (95% CI = 0.203–0.685); $p < 0.01$], mRSS [HR = 1.053 (95% CI = 1.012–1.095); $p < 0.05$], sPAP [HR = 1.155 (95% CI = 1.074–1.242); $p < 0.0001$] and DLco [HR = 0.965 (95% CI = 0.943–0.988); $p < 0.01$] predicted new DUs (Table 3). Variables included in the multivariate model were those statistically significant by univariate analysis. The final multivariate model included CD21^{low} B cells \geq 10% [HR = 0.392 (95% CI = 0.245–0.627); $p < 0.0001$], SSc subset [HR = 0.726 (95% CI = 0.388–1.356); $p > 0.05$], late capillaroscopic pattern [HR = 0.612 (95% CI = 0.294–1.277); $p > 0.05$], mRSS [HR = 1.074 (95% CI = 1.004–1.149); $p < 0.05$], sPAP [HR = 1.116 (95% CI = 1.026–1.514); $p < 0.05$] and DLco [HR = 0.987 (95% CI = 0.958–1.017); $p > 0.05$] (Table 3).

DISCUSSION

In our cohort of SSc patients we found an increased percentage of CD21^{low} B cells compared to the data reported in the literature concerning the general population. In SSc patients, the percentage of CD21^{low} B cells \geq 10% represents a predictive marker of new DUs during a 12-month follow-up.

Forestier *et al.* showed an increased percentage of CD21^{low} B cells in SSc patients compared to health controls, but they did not find an association of this subset of B cells with any disease feature (3). Marrapodi *et al.* demonstrated that CD21^{low} B cells are increased and are less prone to apoptosis in SSc patients. Moreover, the authors observed a positive correlation between percentage of CD21^{low} B cells and sPAP or renal resistive index; conversely, a negative correlation

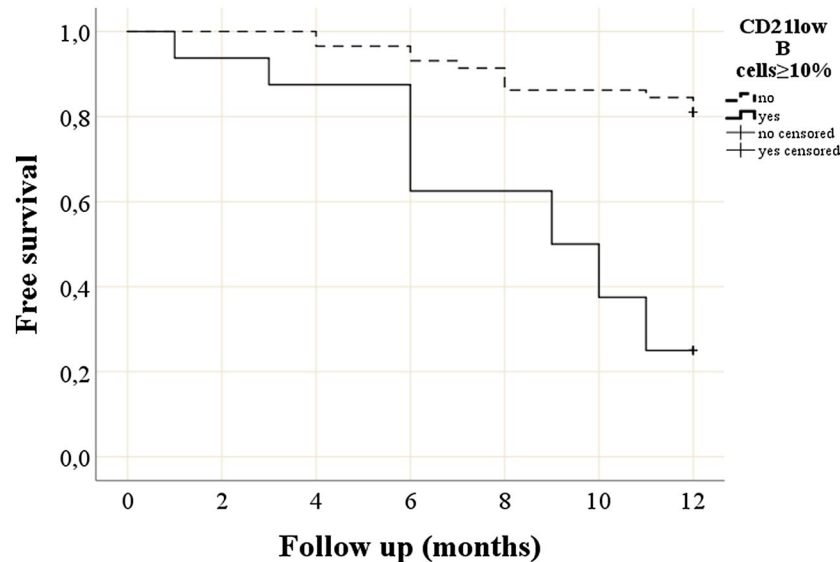


FIGURE 3 Kaplan–Meier curves for the onset of new digital ulcers during a 12-month follow-up period in systemic sclerosis patients with normal or increased CD21^{low} B cells

TABLE 3 Univariate and multivariate analysis with hazard ratio (HR) and confidence interval (CI) for the development of new digital ulcers in SSc patients with CD21^{low} B cells $\geq 10\%$

	Univariate analysis		Multivariate analysis	
	HR (CI)	<i>p</i>	HR (CI)	<i>p</i>
SSc subset (dcSSc)	1.627 (1.058–2.500)	<0.05	0.726 (0.388–1.356)	>0.05
Nailfold capillaroscopic pattern (late)	0.373 (0.203–0.685)	<0.01	0.612 (0.294–1.277)	>0.05
SSc-specific autoantibodies (Scl70)	0.656 (0.336–1.282)	>0.05	–	–
CD21 ^{low} B cells	0.413 (0.272–0.625)	<0.0001	0.392 (0.245–0.627)	<0.0001
mRSS	1.053 (1.012–1.095)	<0.05	1.074 (1.004–1.149)	<0.05
sPAP	1.155 (1.074–1.242)	<0.0001	1.116 (1.026–1.514)	<0.05
DLco	0.965 (0.943–0.988)	<0.01	0.987 (0.958–1.017)	>0.05

Abbreviations: dcSSc, diffuse cutaneous systemic sclerosis; DLco, diffusing capacity of the lung for monoxide carbon; mRSS, modified Rodnan skin score; sPAP, systolic pulmonary artery pressure; SSc, systemic sclerosis.

Significant *p*-values are reported in bold type.

was found between the percentage of CD21^{low} B cells and vascular endothelial growth factor (VEGF) or DLco. The authors hypothesized that CD21^{low} B cells play a role in the vascular manifestations of SSc (4).

In this study we demonstrate for the first time, to our knowledge, that the percentage of CD21^{low} B cells $\geq 10\%$ is a predictive marker of new DUs. In SSc patients with new DUs the percentage of CD21^{low} B cells is increased. In univariate analysis we demonstrated that well-known risk factors of DUs (mRSS, late capillaroscopic pattern, dcSSc, Scl-70, sPAP) were predictive variables of onset of new DUs. In multivariate analysis, only CD21^{low} B cells, mRSS and sPAP represent predictive markers of new DUs. In several studies, late capillaroscopic pattern, dcSSc subset, anti-topoisomerase I antibody and high mRSS have been shown to be strong predictors for DUs (10–17).

Smith *et al.* found an association between worsening of capillaroscopic pattern and severe peripheral vascular involvement at 18 and 24 months. The late capillaroscopic pattern is associated with future severe peripheral vascular disease (13). Sebastiani *et al.* demonstrated that the capillaroscopic skin ulcers risk index (CSURI) is a predictive marker of onset of new DUs, with a sensitivity and a specificity of 92.98 and 81.4%, respectively, at the cut-off value of 2.96 (14). In a recent study, Marrapodi *et al.* hypothesized a possible interference of CD21^{low} B cells with proangiogenic mechanisms (4).

In addition, SSc patients with higher mRSS experienced earlier DU occurrences (10). Walker *et al.*, in a report from the European League Against Rheumatism (EULAR) Scleroderma Trials and Research group database, showed a predictive marker of new DU-positive anti-topoisomerase I

antibodies (15). In a *post-hoc* analysis of a registry evaluating the prevalence of pulmonary arterial hypertension (PAH) in SSc patients, DUs were associated with dcSSc and positive anti-topoisomerase I antibodies. Tiev *et al.* did not find any association of DUs with increased sPAP (11). In a recent study of 189 consecutive SSc patients with an incident DUs diagnosis identified from the EUSTAR database, increased sPAP at diagnosis was associated with the presence of any DUs at the prospective visit (16).

Rosato *et al.* demonstrated that renal resistive index is a predictive marker of onset of new DUs (17). The authors conclude that increased intrarenal stiffness is a major vascular complication, such as DUs, PAH and scleroderma renal crisis.

CD21^{low} B cells are associated with visceral vascular complications and impaired angiogenesis in SSc patients. In this study, the authors hypothesized a role of CD21^{low} B cells in major vascular complications such as DUs (4).

This study has some limitations, such as the absence of evaluation of regulatory T cell (T_{reg}) reductions, functional defects or increased numbers of plasmablasts.

We hypothesize that CD21^{low} B cells may be a possible predictive marker of new DUs. The association of this parameter with other well-known markers could indicate patients to be evaluated more frequently because they are more prone to develop new DUs. Further studies are needed to confirm these findings.

ETHICS APPROVAL STATEMENT

The study was approved by the ethics committee of Sapienza University (no. 5435), Rome (Italy).

ACKNOWLEDGEMENTS

None.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception or design of the work and acquisition of the data. All authors contributed to the critical review and revision of the manuscript and approved the final version. M. V., C. P., G. L., R. M., S. C., A. G., M. C. and E. R. acquired and analyzed the data, analyzed the results and prepared the manuscript; M. V., C. P., G. L., R. M. and S. C. performed the final data analyses; M. V., C. P., G. L., R. M., S. C., A. G., M. C. and E. R. wrote the manuscript; E. R. supervised the study.

DATA AVAILABILITY STATEMENT

All data are presented in the main manuscript.

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