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Rheumatology, 2024, **00**, 1–7
https://doi.org/10.1093/rheumatology/keae660
Advance access publication 4 December 2024
Original Article

Basic science

Dermal cellular senescence and EndMT in patients with systemic sclerosis undergoing cyclophosphamide or aHSCT treatment

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Abstract

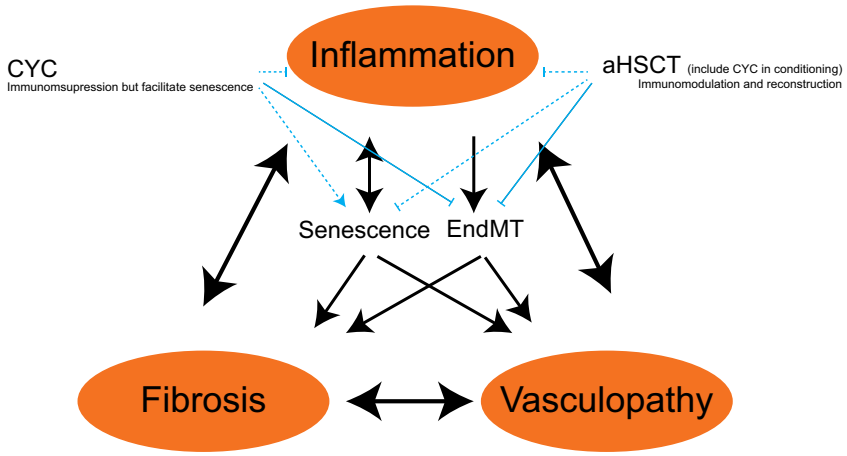
Objectives: Cellular senescence and endothelial-to-mesenchymal transition (EndMT) are profibrotic cellular processes involved in systemic sclerosis (SSc), but how they respond to treatment is largely unknown.

Methods: Skin biopsies from diffuse cutaneous SSc (dcSSc) patients who underwent either autologous haematopoietic stem cell transplantation (aHSCT) or cyclophosphamide pulse (iv CYC) treatment were collected before and 6 months after randomization in the Autologous Stem Cell Transplantation International Scleroderma trial. The extent of fibrosis, inflammation, senescence, EndMT and tissue remodelling were examined in histopathology.

Results: Fourteen pairs of skin biopsies were analysed. Decrease in modified Rodnan skin score was more pronounced in aHSCT-treated patients compared with iv CYC at 6 months (median change −14 [IQR −16 to −9] vs -6 [IQR −9 to −4], respectively, $P = 0.028$). Histologically, expression of urokinase-type plasminogen activator receptor (uPAR) on fibroblasts, P21 on vessels and EndMT decreased after treatment in both groups, yet the reduction was more pronounced in the aHSCT group. Poor skin response was associated with high baseline connective tissue growth factor (CTGF) on fibroblasts and low baseline P21 on vessels, with an odds ratio (OR) of 1.43 and 0.41, respectively. Furthermore, poor response was also seen in patients with a rise in CTGF on fibroblasts (OR 1.29) and P21 on vessels (OR 3.02) after treatment, $P < 0.001$.

Conclusion: Both aHSCT and iv CYC in dcSSc reduced skin thickening clinically and attenuated EndMT, but affected cellular senescence not significantly different. EndMT and uPAR were associated with fibro-remodelling activity, whereas senescence, CTGF, uPAR and vascularity were associated with treatment response.

Graphical abstract



This study investigated histological changes in the skin biopsies from diffuse cutaneous systemic sclerosis (dcSSc) patients treated with either autologous haematopoietic stem cell transplantation (aHSCT) or cyclophosphamide pulse (iv CYC). Six months after treatment, modified Rodnan skin score improved in both groups, and histological reduction in endothelial to mesenchymal transition (EndMT) was observed. The senescent marker on vessels changed numerically in an opposite way, with a dynamic difference between the aHSCT group and the iv CYC group. The blue dotted and solid lines indicate numerical and significant pathological effects, respectively.

Received: 31 May 2024. **Accepted:** 15 November 2024

Rheumatology key messages

- Cellular senescence and EndMT are involved in the profibrotic pathogenesis in SSc and can be modulated by treatments.
- The early biological response to treatment was more pronounced in vessels than in fibroblasts.

Keywords: systemic sclerosis, fibrosis, scleroderma, senescence, pathology, autologous haematopoietic stem cell transplantation.

Introduction

Systemic sclerosis (SSc) is a rare and heterogeneous systemic autoimmune disease characterized by skin fibrosis and internal organ involvement, which is associated with high morbidity and increased mortality [1]. The extent of skin fibrosis is highly correlated with internal organ involvement and survival in SSc [2, 3]. Central to SSc pathogenesis is the interplay between immune dysregulation, vasculopathy and progressive fibrosis [4].

Both cellular senescence and endothelial-to-mesenchymal transition (EndMT) are profibrotic cellular processes believed to be involved in SSc pathogenesis [5–8]. Cellular senescence is a process of cell cycle arrest induced by ageing and cellular stress, including telomere attrition, cumulative deoxyribonucleic acid damage, oxidative stress and stimulation of inflammatory chemokines and cytokines [9]. Senescent cells undergo a complex process with metabolic, morphological and physiological transformation and produce pro-inflammatory and profibrotic molecules, known as the senescence-associated secretory phenotype [10]. In addition, EndMT is a biological process of endothelial cells expressing mesenchymal markers (e.g. alpha-smooth muscle actin [α-SMA]) with myofibroblast-like behaviour [11]. Endothelial cells generally express CD31 on the cell surface and erythroblast transformation-specific related gene (ERG) in the cytoplasm [12]. The mesenchymal transformed endothelial cells lose their polarity while increasing their motility and invasive properties as well as extracellular matrix protein production [11]. Our previous work demonstrated a correlation between fibroblast senescence, EndMT, inflammation and fibrosis in SSc skin biopsies [13].

Cyclophosphamide (CYC) is an immunosuppressive treatment in SSc and is included in the conditioning regimen for autologous haematopoietic stem cell transplantation (aHSCT). CYC can facilitate cell cycle arrest in human fibroblasts in a dose-dependent manner *in vitro* [14]. Although improvement in skin and lung fibrosis is observed in SSc patients receiving CYC and aHSCT [15–17], it is largely unknown how cellular senescence and EndMT respond to immunosuppressive treatments or regenerative treatments like aHSCT. In this study, we investigated the effect of intravenous (iv) CYC and aHSCT on senescence and EndMT in skin biopsies from active diffuse cutaneous SSc (dcSSc) patients.

Methods

Patients

Skin biopsies from patients who participated in the randomized controlled *Autologous Stem Cell Transplantation International Scleroderma* (ASTIS) trial were used for this study [18]. In the ASTIS trial, patients with dcSSc were included when meeting the 1980 American Rheumatism Association criteria, disease duration was ≤4 years, and lung, kidney or heart involvement was present in combination with a modified Rodnan skin score (mRSS) ≥15. Patients were randomly assigned to receive aHSCT with a non-myeloablative, high-dose chemotherapy conditioning regimen or monthly iv CYC pulses. In patients participating in the skin substudy, a skin punch biopsy (4 mm) of the forearm was obtained at baseline (time of randomization) and 6 months.

The ASTIS study protocol was approved by the institutional review board at each participating centre, and all patients provided written informed consent. For this study, clinical data were retrieved from the ASTIS trial dataset, including demographics, autoantibody status, and mRSS at baseline and 6 months. Poor skin response was defined as a decrease in mRSS of <25% [19, 20].

Histopathology

The 3 μm formalin-fixed paraffin-embedded sections were stained with haematoxylin and eosin stain (HE), Masson's Trichrome (MTC), and antibodies to connective tissue growth factor (CTGF, Cell Signalling Technology 86641, 1:600 dilution), urokinase-type plasminogen activator receptor (uPAR, R&D Systems AF807, 1:1000 dilution), P16 (Immunologic VWRKILM0632-C05, 1:800 dilution [21]) and Ki-67 (Thermo Scientific RM9106S, 1:400 dilution) double staining, P21 (Cell Signalling Technologies 2947, 1:100 dilution), α-SMA (Sigma A2547, 1:8000 dilution) and CD31 (LSBio LS-B4737, 1:50 dilution) double staining, and α-SMA (Abcam ab5694, 1:1000 dilution) and ERG (Dako M7314, 1:200 dilution) double staining as previously described [13]. Autofluorescence was quenched with Vector TrueVIEW SP-8400.

The semiquantitative score was determined by the agreement of two researchers who were blinded for any patient information (M.R.D. and Y.H.C.). The severity of dermal fibrosis was scored in HE and MTC slides from one to three with validated classification criteria [13, 22]. Dermal inflammatory cell infiltration in HE-stained sections and the presence of other immunostaining markers on fibroblasts and endothelial cells were scored as absent (0), weak (1), or strong (2) [13]. The presence of senescence was defined as $P21^+$ or $P16^+$/Ki-67^-. The expression of EndMT was presented as the percentage of vessel cross-sections with EndMT features, including enclosure of ERG-positive endothelial nuclei by α-SMA stained cytoplasm in immunohistochemically double-stained (α-SMA-ERG positive) sections and co-localization of CD31 and α-SMA by immunofluorescence double-stained (α-SMA-CD31 positive) sections. Tissue remodelling activity was evaluated in CTGF and uPAR stained sections. The immunohistochemically stained slides were also scanned and imported in QuPath 0.4.4 for colour deconvolution and pixel classification [23]. The histochemical markers were evaluated in the whole dermis, and a spatial approach in the superficial and deep dermis was used.

Statistical analysis

The difference of continuous variables between groups was determined using the Wilcoxon rank-sum test and the Wilcoxon signed-rank test, as appropriate. The correlation between ordinal and/or continuous variables was examined using Spearman's correlation. The interaction between pathological markers and time points in predicting poor skin response was examined with the generalized linear mixed model. A P-value <0.05 was considered statistically significant. All statistical analyses were performed using R 4.3.2.

Results

Patient characteristics

Fourteen pairs of skin biopsies were analysed. Six patients received aHSCT, and eight received monthly iv CYC. The median age was 45 years (interquartile range [IQR] 40–54), and nine patients (64%) were female. Twelve patients (86%) were anti-topoisomerase-I (ATA, also known as anti-Scl-70) positive. The median disease duration was 15 months (IQR 9–32). The baseline characteristics are listed in Table 1.

Clinical response to treatment at 6 months

In both treatment groups, skin thickening improved at 6 months, and the decrease of mRSS was more pronounced in patients who underwent aHSCT compared with CYC-treated patients (median −14 [IQR −16 to −9] vs -6 [IQR −9 to −4], respectively, P 0.028). There were five (83%) skin responders (mRSS reduction $\geq$ 25% at 6 months) in the aHSCT group and five (63%) in the CYC group, P 0.580.

Dermal histological response to treatment

Inflammation and fibrosis

In all baseline samples, dermal fibrosis was observed, yet inflammation was relatively low (Supplementary Figs S1 and S2, available at Rheumatology online). The median baseline fibrosis score was 2 (IQR 2–3) in CYC-treated patients and 3 (IQR 2–3) in aHSCT, P 0.479. The median baseline inflammatory score was 1 in both treatment arms. Collectively, the post-treatment dermal fibrosis and inflammatory scores at 6 months were not significantly different from those at baseline.

Endothelial to mesenchymal transition

Baseline EndMT abundance, both α-SMA-ERG positive and α-SMA-CD31 positive vessels, did not differ between treatment arms. After treatment, expression of EndMT decreased, and a larger reduction was observed in patients treated with aHSCT compared with the iv CYC group (Fig. 1). At baseline, a median of 4.13% (IQR 1.82–7.45) of vessels was α-SMA-CD31 positive, which decreased to 0.87% (IQR 0.09–2.85) at 6 months, P 0.002. The median change in α-SMA-CD31 positive vessels was −5.89% (IQR −6.11 to −2.75) in the aHSCT group and −1.30% (IQR −2.95 to −0.75) in the CYC group, P 0.043. A median of 5.86% (IQR 4.41–11.01) of vessels was α-SMA-ERG positive at baseline compared with 1.90% (IQR 0.80–5.87) at 6 months, P 0.002. The median change of α-SMA-ERG positive vessels was −5.59% (IQR −5.90 to −2.48) in patients who underwent aHSCT and −3.72% (IQR −4.83 to −0.98) in the iv CYC group, P 0.181. These data suggest that EndMT was attenuated after both treatments, but to a greater extent in the aHSCT group.

Table 1. Patient characteristics

	All, $n=14$	CYC, $n=8$	aHSCT, $n=6$	P value
Age	45 (40–54)	50 (44–56)	40 (35–44)	0.070
Female	9 (64%)	5 (63%)	4 (66%)	1
Disease duration	15 (9–32)	19 (8–34)	14 (11–23)	0.950
Baseline mRSS	21 (16–28)	20 (16–26)	25 (17–31)	0.399
ATA$^+$	12 (86%)	8 (100%)	4 (67%)	0.165
Baseline FVC	80 (72–89)	84 (76–93)	75 (71–82)	0.271
Baseline DLCO	54 (48–70)	50 (48–62)	66 (45–78)	0.437

CYC: cyclophosphamide; AHSCT: autologous haematopoietic stem cell transplantation; mRSS: modified Rodnan skin score; ATA: anti-topoisomerase-I; FVC: the percentage of predicted forced vital capacity; DLCO: the percentage of predicted diffusing capacity for carbon monoxide.

Cellular senescence

There was no distinct change in senescent markers (p16 and p21 positive) on fibroblasts and vessels. P21 positive pixels were 0.0077% (IQR 0.0022–0.0162) at baseline and 0.0046% (IQR 0.0021–0.0084) at 6 months in the whole group, P 0.096. The change in P21 positive pixels was −0.0008% (IQR −0.0063 to 0.0024) in the aHSCT group and −0.0006% (IQR −0.0147 to 0.0034) in iv CYC group, $P = 0.802$. There were subtle changes in P21 scores on vessels in the superficial and deep dermis, with a mean change of -0.2 ± 0.8 in patients undergoing aHSCT and $+0.4 \pm 0.9$ in CYC (P 0.049). Taken together, P21 expression on vessels increased in the CYC group and decreased in the aHSCT group.

Tissue remodelling markers

The expression of uPAR on fibroblasts decreased after treatment without a significant difference between the two treatment arms. The median score of uPAR on fibroblasts was 1 (IQR 1–1) at baseline and 0 (IQR 0–1) at 6 months, P 0.041. At 6 months, responders had numerically higher uPAR scores on vessels with a median of 2 (IQR 1–2) compared with 1 (IQR 1–1) in those with poor skin response, P 0.077. There was no significant change in the expression of CTGF on fibroblasts, vessels, and positive pixels before and after treatment or between treatment groups. The representative immunohistochemistry figures are shown in Supplementary Fig. S3, available at Rheumatology online.

Change in mRSS and histological markers

The correlation matrix of change in mRSS and histological markers is shown in Fig. 2. Although the change in mRSS did not correlate with the histological fibrotic score, it did correlate with the change in EndMT (α-SMA-CD31 positive vessels, r 0.547 P 0.043) and reversely correlated with uPAR expression on fibroblasts (r −0.645 P 0.013) and vessels (r −0.651 P 0.012). Improvement in skin thickening was observed in patients with persistent uPAR expression, especially in CYC-treated patients (Supplementary Fig. S4, available at Rheumatology online). Histological change in dermal inflammation was associated with the change in CTGF-positive pixels (r 0.623 P 0.017), especially CTGF expression on fibroblasts (r 0.549 P 0.042). In addition, change in mRSS was associated with baseline vascularity (r 0.573, P 0.032) and uPAR expression on vessels at baseline (r 0.571, P 0.033). In conclusion, change in mRSS was associated with change in EndMT and uPAR expression on both fibroblasts

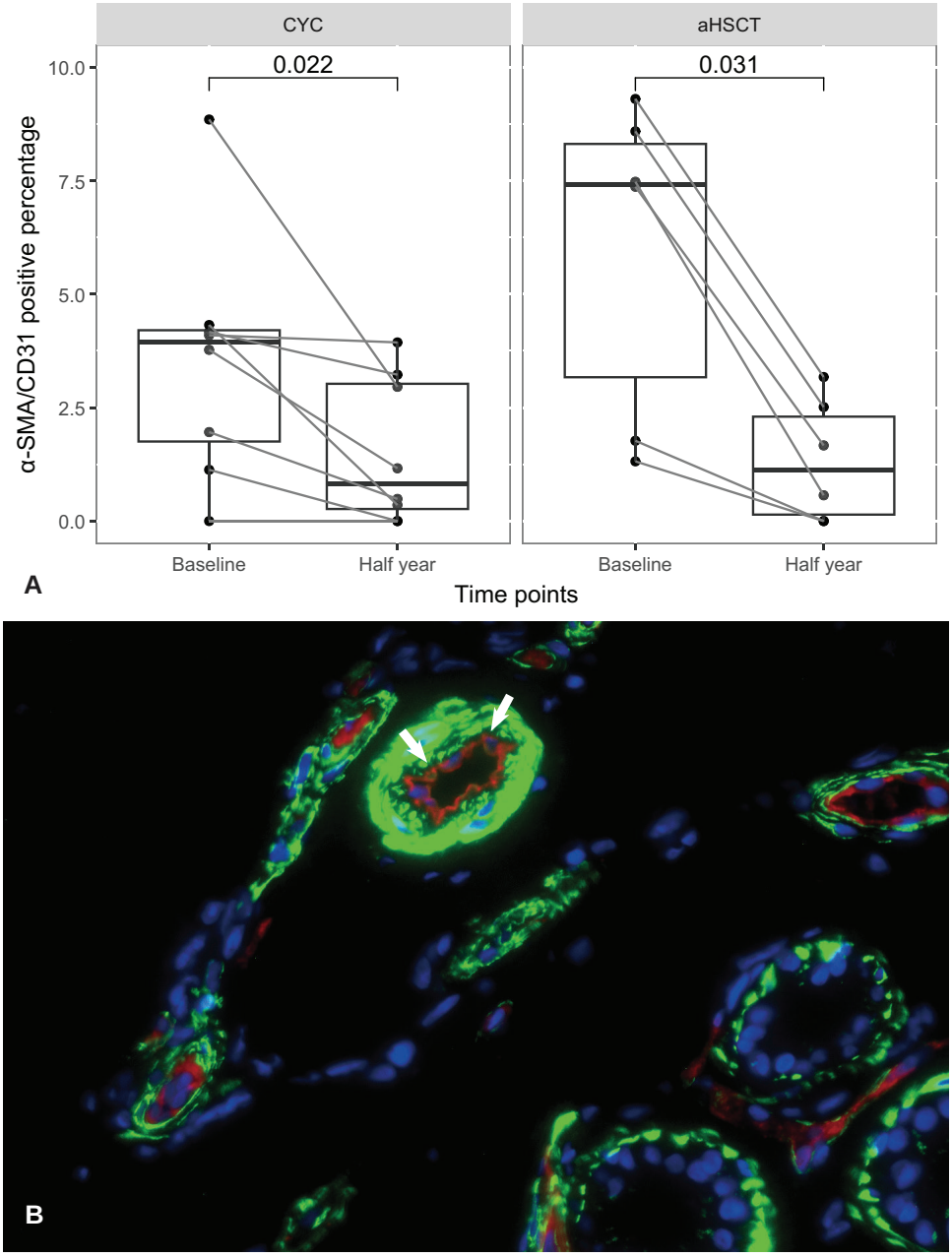


Figure 1. (**A**) Endothelial-to-mesenchymal transition, as measured by co-localization of CD31 and alpha-smooth muscle actin on double immunofluorescence staining (α-SMA/CD31 positive), was reduced at 6 months in both groups of patients undergoing autologous haematopoietic stem cell transplantation ($n = 6$) and cyclophosphamide ($n = 8$). The histopathology was examined in a single forearm skin biopsy of each patient at baseline and 6 months after randomization. (**B**) Immunofluorescent staining of α-SMA (green) and CD31 (red) was taken with the 20× objective lens. Cell nuclei were stained with DAPI (blue). Co-localization of α-SMA and CD31 (arrow) showed yellow in the endothelial cytoplasm

and vessels, as well as baseline skin vascularity and uPAR expression on vessels.

Predicting skin response

Poor skin response was associated with a high baseline CTGF score on fibroblasts with an odds ratio (OR) of 1.43 [95% confidence interval (CI) 1.42–1.44] and with an increase of CTGF expression on fibroblasts after treatment, OR 1.29 (95% CI 1.28–1.30), $P < 0.001$.

Improvement of mRSS was seen in patients with high baseline P21 expression on vessels, OR 0.41 (95% CI 0.41–0.42), whereas a rise in P21 expression on vessels after treatment was associated with poor skin response, OR 3.02 (95% CI 2.99–3.05), $P < 0.001$. Overall, response was associated with low CTGF expression on fibroblasts and high expression of P21 on vessels at baseline. An increase in CTGF expression on fibroblasts and P21 on vessels after treatments was associated with poor skin response.

Discussion

Our study provides more insight into the profibrotic pathophysiology in response to treatment with iv CYC or aHSCT in dcSSc. Treatment response reflected by mRSS was

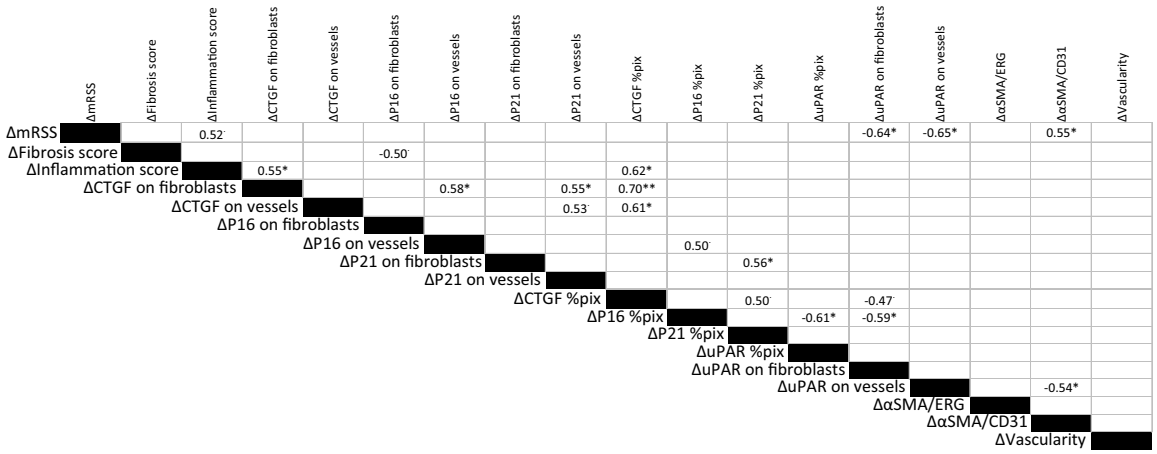


Figure 2. The correlation matrix of changes (Δ) in clinical skin score and pathological scores in 14 pairs of skin biopsies performed at baseline and 6 months after autologous haematopoietic stem cell transplantation or intravenous cyclophosphamide. The change in mRSS was correlated with the change in the percentage of α-SMA and CD31 co-localized vessels and negatively correlated with the change in scores of uPAR on fibroblasts and vessels. *P* values of ≤0.01, ≤0.05, ≤0.1 were marked with **, * and ., respectively. mRSS: modified Rodnan skin score; CTGF: connective tissue growth factor; %pix: percentage of positively stained pixels for respective markers; uPAR: urokinase-type plasminogen activator receptor; α-SMA/ERG: percentage of vessels with enclosure of ERG positive endothelial nuclei by smooth muscle alpha-actin (α-SMA) stained cytoplasm; α-SMA/CD31: percentage of vessels with co-localization of CD31 and α-SMA on immunofluorescence double-staining

associated with skin vascularity, uPAR and P21 expression on vessels and CTGF on fibroblasts at baseline. Improvement in mRSS and changes in fibroinflammatory markers were observed at 6 months but were not correlated with the dermal fibrotic score on forearm skin biopsies. Interestingly, the early histological response to aHSCT and CYC at 6 months was more pronounced in vessels than in fibroblasts, which may underscore the role of vascular pathology in SSc.

Clinical and histological differences between treatment groups were observed in dcSSc in our study. Clinically, we observed a decline in mRSS at 6 months in both treatment groups, with a more profound response in the transplanted patients, which aligns with the findings of the ASTIS and the Scleroderma: Cyclophosphamide or Transplantation trials [18, 20]. Histologically, the expression of P21 on vessels decreased more in patients treated with aHSCT compared with iv CYC at 6 months. In addition, baseline histological markers were associated with treatment response in our study. Higher baseline CTGF expression on fibroblasts and lower P21 expression on vessels were associated with poor skin response. Regardless of the baseline expression of CTGF on fibroblasts and P21 on vessels, an increase of both markers after treatment was associated with poor skin response. In previous studies, CYC has been shown to have pro-senescent effects in multiple cell types, which may explain the small differences in the P21 dynamic [14, 24]. Although CYC is also part of the conditioning regimen in aHSCT, less senescence was observed in the skin of patients treated with aHSCT. A possible explanation could be that fibro-inflammation signatures are normalized after immune reconstitution in aHSCT, which may counterbalance the pro-senescence effect of CYC [25].

P16 and P21 are pivotal markers in identifying senescence [26–28]. Compared with previous studies, we found relatively low expression of P16 in our biopsies. Patients in the ASTIS trial were all diagnosed with dcSSc, whereas our previous study included both diffuse cutaneous and limited cutaneous disease subsets [13]. The expression of P16 seems higher in skin biopsies from limited cutaneous SSc (lcSSc) than dcSSc [13]. Notably, P16 and P21 are involved in different stages of cellular senescence. P21 is activated early in senescence evolution, while P16 maintains cellular senescence [29]. Therefore, in pre-senescent pathology, P21 is more abundant than P16 [13, 26, 27]. Although previous studies could not distinguish dcSSc and lcSSc transcriptomically due to heterogeneity and change over time [30, 31], the dynamic equilibrium of cell cycle regulation may contribute to the phenotype of skin involvement in SSc. In that respect, it is noteworthy that skin involvement (local skin score on physical examination) at the biopsy site was also associated with fibroinflammatory gene expression and resulted in transcriptomic heterogeneity [30]. Importantly, P16 is present not only in senescence but also in other conditions where the cell cycle needs to be attenuated, e.g. development and malignancy [32]. To increase sensitivity and specificity in detecting senescence, we used both P21 and P16 while excluding Ki-67 positive cells, which are active in the cell cycle [27].

We observed that change in mRSS was inversely correlated with change in uPAR expression, and patients with a profound mRSS reduction had sustained or even higher uPAR expression after treatment. As a glycosyl phosphatidyl inositol anchored protein, uPAR functions as a cell surface-associated proteolytic complex with its ligand, urokinase-type plasminogen activator (uPA), and converts plasminogen to plasmin, which is associated with tissue remodelling by interfering with cell adhesion, migration, proliferation, reorganization of the actin cytoskeleton and angiogenesis [33]. UPAR expression is associated with cellular senescence and has been investigated as a target for senolytic chimeric antigen receptor (CAR) T cell therapy in fibrotic diseases [34]. Interestingly, uPAR expression is increased in SSc fibroblasts and microvascular endothelial cells, but uPAR seems functionally impaired compared with healthy skin [35–37]. The uPAR-dependent endothelial invasion, proliferation, capillary morphogenesis, cytoskeletal rearrangements and cell motility were less effective in SSc than in healthy endothelial

cells [36, 38]. Furthermore, the secreted soluble uPAR in plasma is associated with microvascular abnormalities and the extent of fibrosis in SSc [39]. Thus, uPAR expression may indicate tissue remodelling activity in the skin and is crucial in maintaining fibroinflammatory homeostasis, which may be functionally normalized after aHSCT.

The strength of this study is the well-standardized approach, with all tissue being obtained from the same location (forearm) with punch biopsy by protocol and the inclusion of skin biopsies before and after treatment for all patients. However, the forearm skin may not be the most affected site in some patients; therefore, fibrosis in the forearm dermis may not fully reflect the systemic burden.

Our study has some limitations. The post-treatment biopsies were obtained at 6 months, which may be too early to observe full tissue remodelling. Nonetheless, the skin biopsies at 6 months can reflect early mechanistic steps of tissue remodelling. Second, the number of skin biopsies analysed was relatively small compared with the main ASTIS trial, which included 156 patients. Therefore, this study is focused on the histopathological changes after treatment instead of the safety and efficacy of treatments, which have been reported in the main ASTIS trial. Interestingly, an improvement in mRSS at 6 months was not correlated with the histological dermal fibrosis score, contrary to previous studies [22]. There may be possible explanations. The physical examination of skin thickness with the mRSS may not only reflect stiffness of the dermis but also oedema and fibrosis of the hypodermis, subcutaneous tissue and deep fascia, which cannot be thoroughly evaluated in skin punch biopsies [40, 41].

In summary, this study investigated histological changes in the skin in dcSSc patients treated with either aHSCT or iv CYC. Six months after treatment, mRSS improved in both groups, and histological reduction in EndMT was observed. However, the change of senescent markers on vessels differed between the aHSCT and iv CYC groups. Moreover, the early biological response to treatment appeared more pronounced in vessels than in fibroblasts. EndMT and uPAR expression may be valuable biomarkers for monitoring fibrotic tissue remodelling activity, whereas the expression of senescence, CTGF, uPAR and vascularity may be prognostic biomarkers for predicting treatment response.

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

Data and materials are available from the corresponding author on request.

Contribution statement

All authors contributed to the conception and design of the study. M.R.D. and Y.H.C. evaluated the histopathology slides and performed semiquantitative scoring. Y.H.C. performed the experiments and the statistical analysis and drafted the manuscript. All authors interpreted the data and revised the manuscript for important intellectual content. All authors approved the final version of the manuscript for publication.

Funding

This study is partly funded by Stichting Het Sclerodermie Fonds and a student grant from the Government of Taiwan (Y.H.C.).

Disclosure statement: J.S. has received a research grant from Boehringer Ingelheim and honoraria from Boehringer Ingelheim and Janssen; all payments were made to the institution UMCU. J.M.v.L. has received honoraria from Abbvie, BMS, Boehringer Ingelheim, Eli Lilly, Galapagos, and research grants from Astra Zeneca, Boehringer Ingelheim, Roche, and Thermofischer. J.K.d.V.-B. received research grants from Galapagos, Janssen, Roche; and honoraria from Boehringer Ingelheim, Janssen and Abbvie; all payments were made to the institution LUMC.

Acknowledgements

The authors want to thank Roel Broekhuizen for technical assistance in the experiments and the tissue facility of the Pathology department, UMC Utrecht, for MTC staining, slide scanning and biobank management.

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Rheumatology, 2024, 00, 1–7
https://doi.org/10.1093/rheumatology/keae660
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