Cumulative endogenous estrogen exposure is not associated with severity of peripheral microangiopathy in patients with systemic sclerosis

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ABSTRACT

Objective. *To determine whether cumulative endogenous estrogen exposure (CEEE) is associated with severity of microvascular damage or with presence of clinical characteristics in women with systemic sclerosis (SSc).*

Methods. *The population was composed of female SSc patients from the Leiden CCISS (combined care in SSc) cohort. Reproductive life history was investigated through structured questionnaires and CEEE was calculated with a mathematical equation. Demographic, laboratory and clinical characteristics were available for all patients. The most recent nailfold videocapillaroscopy (NVC) was used to semiquantitatively score microangiopathy parameters.*

Results. *We included 97 patients, with a mean age of 59.6±14 years and a mean CEEE of 9±5.5 years. Ordinal logistic regression using CEEE as independent variable failed to demonstrate an association with loss (OR 1.05, 95% CI 0.97-1.14), dilated capillaries (OR 1.05, 95% CI 0.96-1.14), giants (OR 1.03, 95% CI 0.95-1.12) and ramifications (OR 0.99, 95% CI 0.92-1.07). Binary logistic regression did not show an effect of CEEE on presence of scleroderma pattern vs. non-scleroderma pattern, (OR 0.99, 95% CI 0.89-1.1) or of late scleroderma pattern vs. non-late patterns (OR 0.96, 95% CI 0.88-1.05) at NVC. Furthermore, no association was found between CEEE and presence of interstitial lung involvement (OR 0.98, 95% CI 0.88-1.08) but a trend for occurrence of digital ulcers (OR 1.09, 95% CI 0.99-1.19) was observed.*

Conclusion. *In SSc patients, CEEE is not associated with the extent of microvascular derangement. No associations between CEEE and organ involvement were found.*

Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterised by dysregulation of the immune system, vasculopathy, and progressive fibrosis of skin and internal organs (1). Vasculopathy is the most frequent presenting symptom, with Raynaud's phenomenon (RP) described as the earliest sign of SSc in up to 97% of patients (2). Nailfold videocapillaroscopy (NVC) is now widely used to assess presence and degree of microvascular impairment in patients with RP. To confirm their relevance, both RP and NVC abnormalities have been included in the 2013 ACR/EULAR classification criteria of SSc (3), and have also been proposed as part of the VEDOSS (very early diagnosis of systemic sclerosis) criteria (4) for the identification of patients with early disease or at risk of progression toward definite SSc (5). Typical changes observed in the nailfold capillaries of patients with SSc have been grouped under the term "scleroderma pattern", and further categorised in "early", "active", and "late" (6). This ideally defines a continuum from mild to advanced capillary derangement, that in clinical practice is reflected in the highly variable impairment of microvascular array encountered in patients with different SSc severity and different phases of the disease course. Patients with longstanding mild forms of SSc can present with minor capillaroscopic changes even after years, whereas patients with aggressive and rapidly progressive disease subsets frequently show severe microangiopathy since the earliest stages. This outlines the heterogeneity of SSc, contributed by a complex and poorly understood pathogenesis, in which an interplay of genetic and environmental factors has been hypothesised (1, 7). In consideration of the strong female pre-

ponderance, with a female to male ratio reported between 3:1 and 14:1 (7), a role of sex hormones has been proposed (8), but it is not supported by strong evidence. Estrogens, the main female sex hormones, act as enhancers of the immune system and of cell proliferation (9), with a profibrotic role demonstrated in cultures of cells harvested from skin biopsies of SSc patients (10, 11), but their effects on the vascular system are largely unknown. In women with SSc, hormone replacement therapy (HRT) might be protective against the risk of pulmonary arterial hypertension (PAH) (12) but, in post-menopausal women without SSc, unopposed estrogen replacement therapy was associated with increased RP (13). Moreover, two studies (14, 15) demonstrated that, at macrovascular level, short- and long-term administration of conjugated estrogens, induced flow-mediated dilatation in the brachial artery of SSc patients. However, little is known about the effects of estrogens on microangiopathic features that can be visualised through NVC in patients with SSc. The limited available evidence on the contribution of sex hormones to the pathogenesis and clinical manifestations of the disease mostly derives from studies addressing the role of exogenous estrogen administration. The effects of cumulative endogenous estrogen exposure (CEEE), to the best of our knowledge, have never been studied in SSc, neither in other rheumatic diseases. CEEE is determined by several aspects of the reproductive history and is defined as the length of exposure to endogenous estrogens experienced by a woman during her lifetime, which can be estimated through a mathematical equation. We therefore decided to assess a possible association between CEEE and the severity of microangiopathy evaluated through NVC in women with SSc. A secondary aim of our study was to investigate whether a different length of CEEE is associated with the presence of disease manifestations.

Materials and methods

Patients

The population was composed of female patients enrolled in the CCISS cohort (16). To be included in the study, all patients had to fulfil the 2013 ACR/EULAR criteria for classification of SSc (3), have received a clinical diagnosis of SSc, and have reasonable quality NVC images recorded for scoring. Finally, patients in which CEEE could not be reliably estimated from questionnaires, as described below, were excluded. Research on the CCISS cohort is approved by the Ethics Committee of Leiden University Medical Centre (approval number P09.003) and all patients gave written informed consent. The study was conducted in conformity with the principles of the Declaration of Helsinki.

Cumulative endogenous estrogen exposure

The patients' reproductive history was studied using a structured questionnaire, proposed to all women in the CCISS cohort and collected between January and February 2019. Through the questionnaire we recorded information about surgical procedures on reproductive organs, age at menarche, age at menopause, number of pregnancies (including livebirths and miscarriages/abortions), months of breastfeeding, years of oral contraceptive use, average duration and regularity of menstrual cycles. CEEE was calculated as previously described (17). The reproductive life span was determined taking the age at menarche from the age at menopause, or from the age at the last NVC examination for women in pre- or peri-menopausal status. Then, being interested in the effect of unopposed estrogen exposure, we subtracted from the reproductive life span all periods in which estrogens are counteracted by high progesterone levels. These are pregnancies, breastfeeding, oral contraceptive use, and post-ovulatory phases of menstrual cycles. We took 9 months for each livebirth pregnancy, 12 weeks for each miscarriage/abortion which occurred before 3 months, and 16 weeks for miscarriages/abortions which occurred after the third month of gestation, when not otherwise specified. The average length of menstrual cycles was investigated offering 4 options: <25 days, 25-29 days, 30-34 days, >34 days. In the equation we computed 24, 27, 32, or 35 days, respectively. Based on the length of menstrual cycles, we calculated the days of unopposed estrogen exposure, or pre-ovulatory phase, for each cycle, subtracting the 14 days of luteal phase. Finally, CEEE was obtained multiplying the number of days of pre-ovulatory phase of each menstrual cycle for the number of cycles per year, and then for the years of reproductive life span after subtracting pregnancies, breastfeeding and oral contraceptive use.

Capillaroscopy

NVC is performed on annual basis on all patients in the CCISS cohort. The second to fifth fingers of both hands are examined in each patient, after resting at room temperature for 15-20 minutes. The most recent available NVC was chosen for scoring. The same operator scored all recorded images, acquired with a videocapillaroscope equipped with x200 magnification optical probe (before 2018 Videocap, DS Medi-Group, Milan, Italy; since 2018 Inspectis, Inspectis Optical Systems, Solna, Sweden) and connected to image analysis software. NVCs were qualitatively classified as "non-scleroderma pattern", or as "early", "active", or "late" "scleroderma pattern" (6). Then, for scoring, the following parameters were assessed: capillary density reduction, dilated capillaries, giant capillaries, and ramified capillaries. Dilated capillaries were defined as homogenously or irregularly enlarged capillaries with diameter above 20 microns. Giant capillaries were defined as capillaries with homogeneous widening of the apical loop above 50 microns (18). The reference number of normal capillary density was 9/mm. Each parameter was scored semiquantitatively, based on the method reported by Sulli *et al*. (18) for the derivation of Microangiopathy Evolution Score (MES). In each finger, 3 to 4 sequential nailfold areas were examined and scored. For each parameter, the aggregate mean score was obtained summing the mean scores of each finger, and dividing it by 8. Fingers with poor quality images were excluded from calculation. Finally, for each parameter, a scale from 0 to 3 was adopt-

ed (0: no abnormalities/reduction; 1: <33% abnormalities/reduction; 2: 33-66% abnormalities/reduction; 3: >66% abnormalities/reduction). The NVC scoring investigator was not aware of the CEEE of the patients.

Statistical analysis

Statistical analysis was performed using SPSS version 23. Descriptive statistics were used for demographic, clinical, and laboratory characteristics, as well as for reproductive data and capillaroscopic evaluations. Mean and standard deviation, or median and interquartile range, are reported when appropriate. With the aim of investigating whether CEEE exerted an influence on one or more specific aspects of capillary alterations, ordinal logistic regression was used to assess the effect of CEEE on the parameters of microvascular damage, each entered consecutively as a single ordinal dependent variable. This analysis was adjusted for age and disease duration since onset of RP. Binary logistic regression was used to investigate presence of clinical characteristics in relation to CEEE used as independent variable, and to study the effect of CEEE on NVC patterns, considered as the qualitative evaluation of microangiopathy. Presence/absence of SSc pattern, and presence/absence of late SSc pattern, indicative of the worst degree of microvascular damage, were entered as dichotomous dependent variables. Analysis on clinical characteristics was adjusted for time since onset of RP, disease subset and autoantibody positivity. Odds ratio (OR) and 95% confidence intervals (CI) are reported.

Results

Patients' characteristics

One hundred and fifty women replied to the questionnaire and 97 met the inclusion criteria for the study. Epidemiological, clinical, and laboratory data are reported in Table I. Mean age at the scored NVC examination was 59.6±14 years. Fifteen patients (15%) were current smokers or had quit smoking within the last 10 years, with a mean tobacco exposure of 23.7±16.5 pack/years. Median time since onset of RP was 12.9 years (IQR 8.1–21.7). Sixty-eight patients (70%) had limited cutaneous SSc, 17 (18%) had diffuse cutaneous SSc, and 12 (12%) had the sine scleroderma subset. The most frequent organ involvement in our cohort was interstitial lung disease (ILD), present in 41 cases (42%), and 30 patients (31%) had a history of digital ulcers (DU). Other manifestations were less common, with PAH, heart involvement, and scleroderma renal crisis observed respectively in 3%, 3%, and 5% of the population. At inclusion in the study, median modified Rodnan skin score (mRSS) was 2 (IQR 0-6). Antinuclear antibodies (ANA) were positive in 97% of patients and the most frequent SSc-specific autoantibodies were anti-centromere antibodies (ACA), present in 49 (50%) patients, and anti-topoisomerase I antibodies (ATA), found in 17 (17%).

Baseline characteristics of the patients who replied to the questionnaire were compared to those of 267 female patients included in the CCISS cohort, who did not reply to the questionnaire. All analysed patients fulfilled the 2013 ACR/EULAR classification criteria (3). No significant difference in age, median time since onset of RP and of first non-RP SSc-related symptom, was observed. Moreover, at time of inclusion in the CCISS cohort, prevalence of ATA and ACA, and of limited and diffuse disease subsets, were comparable between responders and non-responders.

CEEE and reproductive history

Patients who underwent hysterectomy before the reported age of menopause were excluded due to the impossibility to reliably determine transition to menopause. Patients who underwent bilateral oophorectomy in childbearing age were included and the age at surgery was considered as the age of menopause. For CEEE calculation, few missing values had to be handled. Five patients did not remember the age at menopause, and 3 the age at menarche. We considered them to be, respectively, 47.4 and 13.7 years, which were the average ages of natural menopause and of menarche in our cohort. For 3 out of 144 born children, the duration of breastfeeding was unknown and we counted 5 months for each, which was the average breastfeeding length in our cohort. For post-menopausal patients who could not remind the average duration of menstrual cycles, we assumed a mean of 28 days. CEEE was thus calculated till the time of the scored NVC for women in childbearing age and in peri-menopause, and till menopause for post-menopausal patients. In our

Table I. Demographic, clinical, reproductive, and capillaroscopic characteristics of patients included in the study.

Patients' characteristics	n=97
Demographic	
Age (years), mean (±SD)	59.6 (14)
BMI, mean (±SD)	24.2 (4.8)
Smokers within last 10 years, n (%)	15 (15)
Pack/years (n=15), mean (±SD)	23.7 (16.5)
Clinical and laboratory	
SSc type	
lcSSc, n (%)	68 (70)
dcSSc, n (%)	17 (18*)
ssSSc, n (%)	12 (12)
Disease duration, median (IQR)	12.9 (8.1-21.7)
mRSS, median (IQR)	2 (0-6)
ILD, n (%)	41 (42)
PAH, n (%)	3 (3)
DU, n (%)	30 (31)
Heart, n (%)	3 (3)
SRC, n (%)	5 (5)
Antibodies	
ANA, n (%)	94 (97)
ACA, n (%)	49 (50)
ATA, n (%)	17 (17)
anti-RNA-pol III, n (%)	2 (2)
anti-Pm/Scl, n (%)	7 (7)
anti-RNP, n (%)	5 (5)
Reproductive history	
Post-menopausal, n (%)	81 (83)
OCP use, n (%)	87 (90)
OCP years (n=87), mean (±SD)	13.9 (9)
Years of CEEE, mean (±SD)	9 (5.5)
Nailfold videocapillaroscopy	
Non-scleroderma pattern, n (%)	20 (21)
Early scleroderma pattern, n (%)	11 (11)
Active scleroderma pattern, n (%)	31 (32)
Late scleroderma pattern, n (%)	35 (36)

SD: standard deviation; IQR; interquartile range; BMI: body mass index; SSc: systemic sclerosis; lcSSc: limited cutaneous SSc; dcSSc: diffuse cutaneous SSc; ssSSc: SSc sine scleroderma (disease duration is considered as time since onset of Raynaud's phenomenon); mRSS: modified Rodnan skin score; ILD: interstitial lung disease; PAH: pulmonary arterial hypertension; DU: digital ulcers; SRC: scleroderma renal crisis; ANA: anti-nuclear antibodies; ACA: anti-centromere antibodies; ATA: anti-topoisomerase I antibodies; RNA-pol III: anti-RNA polymerase III; OCP: oral contraceptive pill; CEEE: cumulative endogenous estrogen exposure.
*17.5% rounded to 18% to avoid rounding error.

cohort, 83% of patients were in postmenopause and 90% had a history of oral contraceptives use, on average for 13.9±9 years. Mean CEEE in our population was 9±5.5 years (Table I).

Microvascular damage

Capillaroscopic examinations used for scoring were performed within the last 2 years in 87% of the patients and scleroderma pattern was present in 79% of cases. Of these, 11 (11%) had an "early" pattern, 31 (32%) an "active" pattern, and 35 (36%) were classified as "late" pattern. Twenty patients (21%) did not show a scleroderma pattern. Each parameter of microvascular damage was evaluated in all 97 patients and semiquantitative scores were derived as described (Table II). In the different domains of microangiopathy, the most represented scores were 2 for density reduction (49%), 1 for dilated (67%) and giant capillaries (59%) and 0 for ramifications (47%).

Association of CEEE with each parameter of microvascular damage

Ordinal logistic regression did not show a statistically significant contribution of CEEE to microvascular impairment (Table III). In particular, after adjusting for age and disease duration since onset of RP, CEEE was not associated with the semiquantitative scores of capillary loss (OR 1.05, 95% CI 0.97–1.14), dilated capillaries (OR 1.05, 95% CI 0.96–1.14), giants (OR 1.03, 95% CI 0.95–1.12) and ramified capillaries (OR 0.99, 95% CI 0.92–1.07). We also repeated the analysis computing CEEE length obtained before subtraction of post-ovulatory phases in the equation. No difference was observed.

Association of CEEE with NVC parameters and clinical characteristics

No effect of CEEE on NVC patterns was found using binary logistic regression (Table IV) after adjusting for time since onset of RP, age, disease subset, and autoantibody positivity. SSc pattern *vs*. non-SSc pattern (OR 0.99, 95% CI 0.89–1.1), or late SSc pattern *versus* non-late pattern (OR 0.96, 95% CI 0.88–1.05) were used as dependent variables. Logistic regression (Table IV) failed to show an association of CEEE with the presence of ILD (OR 0.98, 95% CI 0.88–1.08) but a non-significant trend of association between CEEE and DU (OR 1.09, 95% CI 0.99-1.19) was observed. In the 30 patients with DU, mean CEEE was 10.3±5.8 years, compared to 8.5±5.3 years in the 67 patients without DU (p=0.145). Predictive value of CEEE on other clinical characteristics was not analysed in consideration of their low prevalence in our cohort, as shown in Table I.

Table II. Distribution of microangiopathy scores in the cohort.

	Score			
	0	1	2	3
Microangiopathy parameter				
Density loss, n (%)	7 (7)	33 (34)	47 (49)	10 (10)
Dilated capillaries, n (%)	7 (7)	65 (67)	25 (26)	0
Giant capillaries, n (%)	32 (33)	57 (59)	8 (8)	0
Ramified capillaries, n (%)	45 (47)	37 (38)	12 (12)	3 (3)

Score 0: no abnormalities/reduction; score 1: <33% abnormalities/reduction; score 2: 33-66% abnormalities/reduction; score 3: >66% abnormalities/reduction.

Table III. Association between CEEE and each parameter of microvascular damage assessed through semiquantitative scores.

	Density loss OR (95% CI)	Dilated capillaries OR (95% CI)	Giant capillaries OR (95% CI)	Ramified capillaries OR (95% CI)
CEEE	1.05 (0.97-1.14)	1.05 (0.96-1.14)	1.03 (0.95-1.12)	0.99 (0.92-1.07)

Multivariate analysis was adjusted for age and SSc duration since onset of Raynaud's phenomenon. CEEE: cumulative endogenous estrogen exposure; OR: odds ratio; CI: confidence interval.

Table IV. Association of CEEE in multivariate analysis, with clinical characteristics and NVC pattern.

	ILD OR (95% CI)	DU OR (95% CI)	SSc pattern* OR (95% CI)	late SSc pattern** OR (95% CI)
CEEE	0.98 (0.88-1.08)	1.09 (0.99-1.19)	0.99 (0.89-1.1)	0.96 (0.88-1.05)

Multivariate analysis for ILD and DU was adjusted for disease subset, autoantibodies, SSc duration since onset of Raynaud's phenomenon. Multivariate analysis for SSc pattern and late SSc pattern was adjusted for disease subset, autoantibodies, age, SSc duration since onset of Raynaud's phenomenon. CEEE: cumulative endogenous estrogen exposure; OR: odds ratio; CI: confidence interval; ILD: interstitial lung disease; DU: digital ulcers; SSc: systemic sclerosis.
*vs. non-SSc pattern.
**vs. non-late patterns.

Discussion

This is the first study investigating the influence of CEEE in patients with SSc, with particular interest in the contribution to the degree of microvascular damage.

As evidenced by a recent review (19), NVC may help in the prediction of SSc outcomes, as microangiopathic abnormalities are associated with disease manifestations, in particular calcinosis, DU, and PAH. However, to what extent the observed microangiopathy can be contributed by comorbidities, or by environmental or endogenous factors, not directly related to SSc, has not been thoroughly investigated. Smoking, diabetes mellitus, repeated microtrauma and chemical exposure (20) have all been proposed to affect microangiopathy, while little is known about hormones. The interest in sex hormones in SSc is supported by the striking sex disparity in prevalence and by the knowledge, derived from large cohorts, that men and women have different clinical and serological charac-

teristics (21, 22). Besides that, clear evidence about a direct association between estrogens and SSc onset or progression has never been demonstrated and our results do not show an effect of CEEE on the severity of microvascular impairment.

Being microangiopathy our primary outcome, we decided to use not only NVC patterns, but also semiquantitative scores based on the calculation adopted to derive MES (18), to see if CEEE was associated with any particular aspect of microvascular damage. In the primary analysis, no correlation with scores based on density reduction, on percentage of dilated or giant capillaries, and on proportion of ramifications, could be found in our population. Then, in the second analysis, no association could be found between CEEE and presence/absence of SSc pattern, or presence/absence of late SSc pattern. Finally, CEEE was not associated with presence of ILD and DU, also after correction for autoantibody positivity.

Lifetime estrogen exposure has been investigated in non-rheumatic diseases, particularly in neurology, where estrogens have been advocated as a neuroprotective factor (17, 23), and in cardiology, where CEEE has been studied as a possible predictor of cardiovascular mortality (24). In rheumatology, though, CEEE has never been investigated, and for this reason we decided to assess whether it is associated with different aspects of SSc or not.

We included 97 patients in the study, which we think are representative of our CCISS cohort, but the sample size was too small to investigate other organ manifestations, as heart involvement or PAH, due to the low prevalence in our population. However, the major limitation of this study was the risk of recall bias, especially in consideration of the high prevalence of women in postmenopausal age. We tried to reduce this risk structuring the questionnaire in a clear and comprehensive way, but recall bias is unavoidable in the estimation of CEEE. Considering that the most difficult item to remember in our questionnaire was the average length of menstrual cycles, we also considered the hypothesis that a recall bias could have influenced our results, and we repeated the analysis taking it out from CEEE equation, but no difference was observed. Furthermore, given the small effect of CEEE on DU, we also compared mean CEEE between patients with and without DU, but again no significant difference was found. However, we cannot exclude that our study was underpowered to identify a minor contribution of CEEE to the risk of DU, with possible false negative results conditioned by limited sample size. Thus, replication of the study in other, preferably larger, cohorts, would be valuable in determining whether our observation was spurious or was indicative of a trend not reaching significance due to problems inherent statistical power.

Few published studies have investigated a possible effect of estrogens on the different aspects of SSc. In a Canadian retrospective cohort (25), higher mean mRSS was observed in childbearing-age patients compared to women in the hypoestrogenic state of menopause. A potential effect of estrogens has also been outlined in two other retrospective studies, showing that the prevalence of PAH increases after transition to menopause (26) and that post-menopausal patients treated with HRT have significantly lower risk of being diagnosed with PAH, compared to women who did not receive HRT (12). In none of these studies, however, was NVC assessed as an outcome, neither was the presence of DU as expression of small-vessel vasculopathy, and whether estrogen modulation influences peripheral microcirculation has not been determined.

In conclusion, our findings failed to demonstrate a statistically significant association between CEEE and the degree of microangiopathy, nor with clinical characteristics observed in patients with SSc. However, our results do not exclude a contribution of sex hormones to the pathogenesis of SSc, especially considering that CEEE estimation is only one of the possible approaches to investigate estrogens in SSc. We believe that longitudinal studies on sex hormone levels could clarify their role in disease progression. Further basic and applied research is needed to elucidate the interaction of estrogens and other sex hormones in SSc, but also to characterise their effects on target tissues.

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