High DNA Oxidative Damage in Systemic Sclerosis

JEROME AVOUAC, DIDIER BORDERIE, OVANESSE GARABED EKINDJIAN, ANDRE KAHAN, and YANNICK ALLANORE

ABSTRACT. ***Objective.*** Several lines of evidence suggest that the generation of reactive oxygen species (ROS) is of major importance in the pathogenesis of SSc. Protein and lipid damage have previously been demonstrated, but scarce data are available on oxidative damage to DNA. In patients with SSc, we evaluated levels of 8-hydroxy-2'-deoxyguanosine (8-oxodG), the main validated biomarker of endogenous oxidative damage to DNA, compared to levels of F2-isoprostane, a product of free radical-mediated peroxidation of arachidonic acid.

Methods. Urinary levels of 8-oxodG and 8-isoprostaglandin-$F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) were determined by competitive ELISA method in consecutive SSc patients and controls matched for age and sex.

Results. We included 80 unrelated SSc patients (72 women, mean age 56 ± 11 yrs) and 39 controls (33 women, mean age 64 ± 8 yrs). Urinary levels of 8-oxodG/creat and 8-iso-$PGF_{2\alpha}$/creat in SSc patients were found to be higher than in controls (6.5 ng/mg vs 3.7 ng/mg, $p = 0.0001$; and 11.4 ng/mg vs 4.2 ng/mg, $p < 0.0001$). In multivariate analysis, 8-oxodG levels were associated with the presence of pulmonary fibrosis on computerized tomography scan, decreased forced vital capacity, and decreased DLCO/alveolar volume. In patients with the diffuse cutaneous subset, a modified Rodnan skin score > 14 was independently associated with 8-oxodG levels. In SSc, 8-oxodG and 8-iso-$PGF_{2\alpha}$ values were correlated ($r = 0.32$; $p = 0.005$).

Conclusion. Our study confirmed marked oxidative stress in SSc. We also found increased values of 8-oxodG in SSc patients and a relevant association with a fibrotic phenotype. The predictive value of this marker and its potential influence on fibrotic disturbances remain to be determined. (J Rheumatol First Release Sept 15 2010; doi:10.3899/jrheum.100398)

Key Indexing Terms:
SYSTEMIC SCLEROSIS — OXIDATIVE STRESS
OXIDATIVE DNA DAMAGE — LIPID PEROXIDATION

Systemic sclerosis (SSc) is a connective tissue disease characterized by early generalized microangiopathy, including vasospastic tendencies, that culminates in systemic fibrosis. Several lines of evidence suggest that generation of free radicals may be important in SSc[1]. Free radicals damage proteins, lipids, DNA, collagens, and the immune system. They are mainly generated by ischemia/reperfusion injuries or during the inflammatory process[2]. Moreover, monocytes from patients with SSc can produce large amounts of the superoxide anion[3,4], and fibroblasts produce reactive oxygen species (ROS)[5]. In addition, some tissue antigens are susceptible to fragmentation in an oxidative microenvironment[6]. An increase in susceptibility to oxidation may be related to an increase in oxidative stress or a decrease in antioxidants[7]. In SSc, markers of lipid peroxidation, including plasma malondialdehyde[8], thiobarbituric acid-reactive substances[9], antibodies against oxidized low-density lipoproteins[10], and bioactive F2-isoprostane (F2-isoPs) concentrations are elevated[11,12]. Among these markers, F2-isoPs, a reliable marker of lipid oxidant injury *in vivo*, has been well studied and was shown to correlate with severity of both microangiopathy and lung involvement[13,14]. This large amount of data on lipid peroxidation contrasts with the lack of evidence of oxidative DNA damage in SSc. 8-Oxodeoxyguanosine (8-oxodG) is the most abundant DNA lesion caused by ROS. It is highly mutagenic, resulting in GC to TA transversions. After cleavage from DNA as a result of DNA repair, 8-oxodG is excreted in urine. Another significant source of extracellular 8-oxodG may be oxidation of the nucleotide pool[15]. Urinary 8-oxodG levels are therefore considered as a general biomarker of oxidative stress. Methods used for 8-oxodG detection include high-performance liquid chromatography, tandem mass spectroscopy, and a recently developed competitive ELISA[16].

The aim of our study was to evaluate DNA oxidative damage in SSc. We measured urinary concentrations of 8-oxodG and urinary levels of 8-isoprostaglandin-$F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in a large group of SSc patients as compared with healthy controls.

From the Service de Rhumatologie A and Service de Biochimie A, Hôpital Cochin, Université Paris Descartes, AP-HP, Paris, France.

J. Avouac, MD, PhD, Service de Rhumatologie; D. Borderie, MD, PhD; O.G. Ekindjian, MD, PhD, Service de Biochimie; A. Kahan, MD, PhD; Y. Allanore, MD, PhD, Service de Rhumatologie.

Address correspondence to Dr. Y. Allanore, Service de Rhumatologie A, Hôpital Cochin, Université Paris Descartes, 27 rue du Faubourg Saint-Jacques, 75014 Paris, France. E-mail: yannick.allanore@cch.aphp.fr

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MATERIALS AND METHODS

Inclusion criteria. We included consecutive patients with SSc who had been hospitalized in the Rheumatology Department for systematic followup. All patients were classified as having limited or diffused cutaneous SSc according to LeRoy's criteria[17]. The local ethics committee approved the study, and all subjects gave written informed consent. Three months of stable current treatment were necessary for inclusion, and prednisone use at a dose of less than 10 mg/day was authorized. Vasodilators, including calcium channel blockers and angiotensin-converting enzyme inhibitors, had to be withdrawn at least 3 days before inclusion (corresponding to > 5 times the drug half-life), as they were shown to decrease oxidative stress[1]. The control group consisted of 39 healthy nonsmokers, matched for age and sex.

Exclusion criteria. We excluded patients who could not stop vasodilator therapy, as well as those who were pregnant or current smokers, or who had diabetes, current treatment for dyslipidemia, or severe disease (e.g., cardiac, hepatic failure, or gangrene).

Clinical assessment. Detailed information was collected in all participating patients, including age, sex, cutaneous SSc subset as defined by LeRoy, *et al*[17], disease duration (date of first non-Raynaud's symptom), skin involvement according to the modified Rodnan skin score (mRSS)[18], and history of digital ulceration.

Laboratory assessments. Laboratory studies were obtained at baseline, on the morning of hospital admission. They included complete blood cell count, Westergren erythrocyte sedimentation rate (ESR, considered elevated above 28 mm/h), C-reactive protein level (CRP, considered elevated above 10 mg/l), serum and urinary creatinine concentration, and tests for antinuclear antibodies and anticentromere antibodies (both detected in immunofluorescence on Hep2 cells) and antitopoisomerase I antibodies (counter immunoelectrophoresis). von Willebrand factor antigen concentration was determined by ELISA (VIDAS von Willebrand, BioMerieux, Marcy l'Etoile, France).

Pulmonary and cardiac assessment. Pulmonary involvement was assessed at baseline by chest radiography, computed tomography (CT), and measurements of forced vital capacity (FVC) and the carbon monoxide diffusion capacity/alveolar volume (DLCO/AV) ratio. Suspicion of pulmonary hypertension was considered at baseline by Doppler echocardiography and defined by systolic pulmonary arterial pressure (sPAP) > 40 mm Hg. Confirmed pulmonary arterial hypertension (PAH) was defined as a resting mean PAP ≥ 25 mm Hg with a pulmonary capillary wedge pressure of ≤ 15 mm Hg measured at right heart catheterization.

Composite scores. Disease activity was assessed according to the preliminary composite index proposed by the European Scleroderma Study Group, and disease severity was assessed according to the Medsger severity score[19,20].

Sample acquisition. All the patients underwent urine collection at the same time as physical examination and laboratory investigations. Urinary excretion of 8-oxodG and isoprostane was evaluated from overnight urine collection. The timing and total volume were recorded, and two 50-ml aliquots were stored at –80°C until extraction.

8-oxodG competitive ELISA. Urine levels of 8-oxodG were measured using a competitive ELISA (Bioxytech® 8-OxodG-EIA, Oxis International, Beverly Hills, CA, USA). Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer. The analytical range was 0.125 to 200 ng/ml. Inter and intraassay coefficients of variation were 2.1% and 4.5%, respectively. Urinary 8-oxodG concentration was expressed as ng 8-oxodG/mg creatinine (creat).

8-iso-$PGF_{2\alpha}$ competitive ELISA. Urine levels of 8-iso-$PGF_{2\alpha}$ were measured using a competitive ELISA (Cayman Chemical, Ann Arbor, MI, USA). Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer. The EIA typically displays an IC50 (50% B/B_0) of about 10 pg/ml and a detection limit (80% B/B_0) of about 2.7 pg/ml. In our assay, analytical range extended from 0.8 to 500 pg/ml. Interassay and intraassay coefficients of variation were 9.5% and 10.7%, respectively. Urinary 8-iso-$PGF_{2\alpha}$ concentration was expressed as ng 8-iso-$PGF_{2\alpha}$/mg creat.

Statistical analysis. All data are presented as median (range) for continuous variables and numbers and percentages for categorical variables, unless stated otherwise. Data were analyzed with the Mann-Whitney (unpaired data) test. Spearman's rank correlation test was used to assess the relation between quantitative variables. A multiple linear regression analysis was also performed for all variables identified with $p \leq 0.10$ univariately. P values < 0.05 were considered significant.

RESULTS

Study population. We included 80 SSc patients, of whom 72 were women (90%), with a mean age of 56 ± 11 years and a mean disease duration of 7 ± 6 years (disease duration was < 5 yrs in 44 patients). All patients had Raynaud's phenomenon, 39 had the diffuse cutaneous subset and 41 the limited subset (Table 1). No included patient was treated with cyclophosphamide. The control group consisted of 39 patients, of whom 33 were women (85%), with a mean age of 64 ± 8 years.

Levels of 8-oxodG and 8-iso-$PGF_{2\alpha}$ in SSc patients compared to controls. Urine levels of 8-oxodG/creat were found to be higher in SSc patients than in controls (median 6.5 ng/mg, range 0.06–55.38 ng/mg vs median 3.7 ng/mg, range 0.10–10.41 ng/mg; p = 0.0001; Figure 1A). As expected, urine levels of 8-iso-$PGF_{2\alpha}$/creat were also significantly higher in SSc patients (median 11.4 ng/mg, range 2.7–56.9 ng/mg vs 4.2 ng/mg, range 0.7–9.2 ng/mg; p < 0.0001; Figure 1B). In SSc patients, 8-oxodG/creat and 8-iso-$PGF_{2\alpha}$/creat values were correlated (r = 0.32; p = 0.005; Figure 2).

Relationship between oxidative stress and age/gender in SSc patients and controls. Urinary levels of 8-oxodG/creat and 8-iso-$PGF_{2\alpha}$/creat positively correlated with age in the control population (r = 0.35; p = 0.02 and r = 0.41; p = 0.03, respectively). Conversely, no significant correlation with age was found in the SSc population (r = 0.14; p = 0.4 and r = 0.11; p = 0.5, respectively). No association between oxidative stress and gender was found in SSc patients and controls.

Relationship between 8-oxodG levels and clinical features (Table 2). There was no difference between patients with the diffuse or limited cutaneous subset for the urinary values of 8-oxodG/creat (median 7.09 ng/mg, range 0.06–55.38 ng/mg vs median 2.74 ng/mg, range 0.10–45.71 ng/mg; p = 0.3).

In univariate analysis, SSc patients with disease duration less than 5 years exhibited higher values of 8-oxodG/creat versus patients with longer disease duration (median 8.83 ng/mg, range 0.16–43.25 ng/mg vs median 1.73 ng/mg, range 0.06–55.38 ng/mg; p = 0.03). The likelihood of pulmonary involvement, with pulmonary fibrosis on CT scan (p = 0.01), decreased FVC < 75% predicted (p = 0.002), and decreased DLCO/AV < 5% predicted (p = 0.003) was significantly higher in patients with higher levels of

Table 1. Clinical and biological characteristics of 80 patients with systemic sclerosis (SSc).

Characteristic	SSc Patients, n = 80	Diffuse Cutaneous Subset, n = 39	Limited Cutaneous Subset, n = 41
Disease duration, yrs, mean ± SD	6.7 ± 6.0	5.4 ± 5.2	7.9 ± 6.4
Disease duration ≤ 5 yrs, n (%)	44 (55)	25 (64)	19 (46)
Modified Rodnan skin score, mean ± SD	8.8 ± 6.5	13.6 ± 6.2	4.5 ± 2.8
Modified Rodnan skin score > 14, n (%)	16 (20)	16 (41)	0 (0)
Digital ulcers, n (%)	24 (30)	13 (33)	11 (27)
sPAP > 40 mm Hg, n (%)	8 (10)	6 (15)	2 (5)
PAH on right heart catheterization	6 (8)	4 (10)	2 (5)
Pulmonary fibrosis on CT scan, n (%)	40 (50)	34 (87)	6 (15)
Erythrocyte sedimentation rate > 28 mm/h, n (%)	17 (21)	11 (28)	6 (15)
CRP > 10 mg/l, n (%)	14 (17.5)	10 (26)	4 (10)
von Willebrand antigen concentration, %, mean ± SD	157 ± 47	163 ± 68	149 ± 33
von Willebrand antigen concentration > 200%, n (%)	15 (19)	9 (23)	6 (15)
Positive antinuclear antibodies (> 1/160), n (%)	70 (87.5)	33 (85)	37 (90)
Positive anti-topoisomerase I antibodies, n (%)	25 (31)	25 (64)	0 (0)
Positive anticentromere antibodies, n (%)	21 (26)	0 (0)	21 (51)
Decreased FVC < 75% normal, n (%)	23 (29)	18 (46)	5 (12)
Decreased DLCO/VA < 75% normal, n (%)	33 (41)	20 (51)	13 (32)
Active disease (Valentini activity score ≥ 3), n (%)	35 (44)	29 (69)	6 (15)
Severe disease (Medsger severity score ≥ 3), n (%)	17 (21)	11 (28)	6 (15)
Treatment with calcium channel blockers, n (%)	80 (100)	39 (100)	41 (100)
Treatment with angiotensin-converting enzyme inhibitors, n (%)	34 (42)	22 (56)	12 (29)
Treatment with low-dose corticosteroids, n (%)	30 (38)	19 (49)	11 (27)

sPAP: systolic pulmonary artery pressure; PAH: pulmonary arterial hypertension; CRP: C-reactive protein; FVC: forced vital capacity; DLCO/VA: decrease in carbon monoxide diffusion capacity divided by alveolar volume.

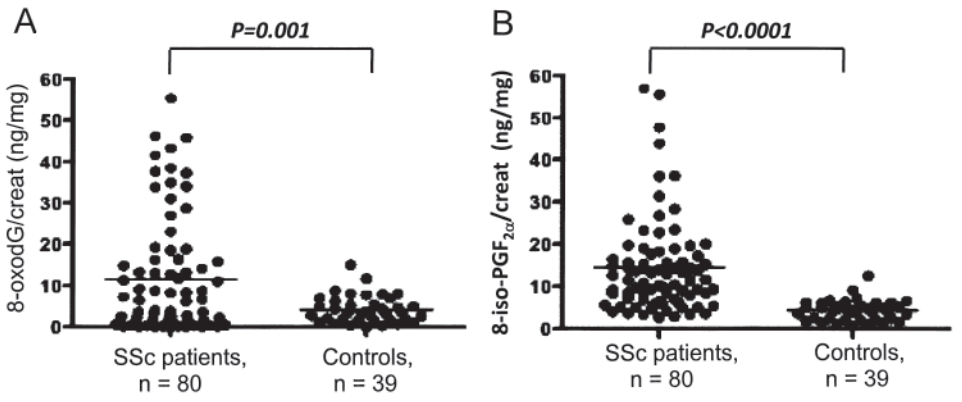

Figure 1. Levels of 8-oxodG and 8-iso-$PGF_{2\alpha}$ in patients with SSc versus controls.

8-oxodG/creat (Table 2). In multiple linear regression analysis, pulmonary fibrosis on CT scan (p = 0.03), FVC < 75% predicted (p = 0.04), and DLCO/AV < 75% predicted (p = 0.02) were independent factors associated with increased urine 8-oxodG/creat levels.

Among patients with the diffuse cutaneous disease subset, in univariate analysis, patients with higher 8-oxodG/creat levels were more likely to have shorter disease duration (median 8.49 ng/mg, range 0.16–43.25 ng/mg vs median 1.73 ng/mg, range 0.06–55.38 ng/mg; p = 0.04), mRSS > 14 (p = 0.02), decreased FVC (< 75% predicted; p = 0.04), and active disease according to the Valentini index (p = 0.01). In multivariate analysis, only mRSS > 14 (p = 0.04) was independently associated with higher 8-oxodG/creat levels.

Patients with the limited cutaneous disease subset and higher 8-oxodG/creat levels also exhibited decreased FVC, < 75% predicted (p = 0.02). However, FVC was not independently associated with 8-oxodG levels in multivariate analysis performed on variables with p < 0.1 univariately.

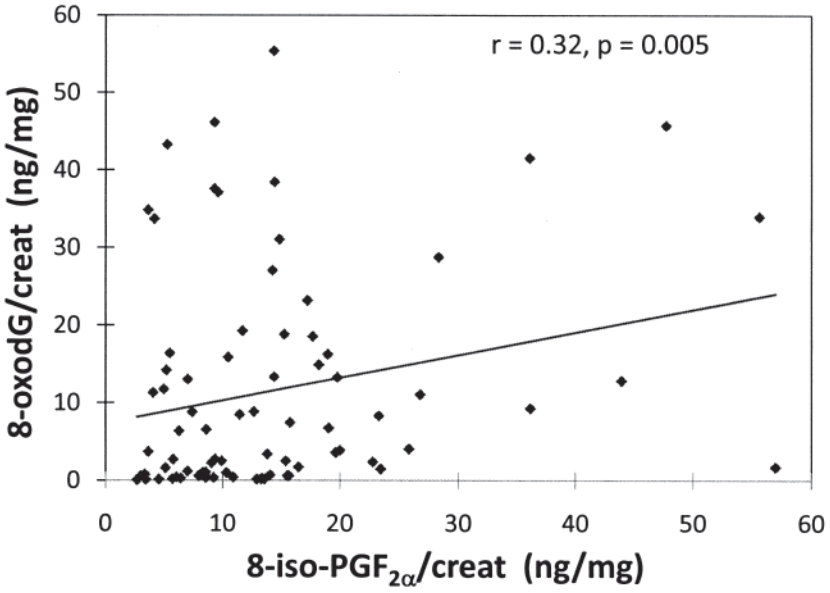

Figure 2. Correlation between urinary levels of 8-oxodG and 8-iso-$PGF_{2\alpha}$ in patients with SSc (log transformation).

Relationship between 8-iso-$PGF_{2\alpha}$ levels and clinical features (Table 3). Patients with the diffuse cutaneous disease subtype had significantly higher values of 8-iso-$PGF_{2\alpha}$/creat than patients with the limited cutaneous subset (median 14.34 ng/mg, range 2.68–56.96 ng/mg vs median 9.31, range 3.41–55.58 ng/mg). Consistently, the likelihood of positive anticentromere antibodies was significantly lower in patients with increased 8-iso-$PGF_{2\alpha}$/creat levels ($p = 0.03$). As for 8-oxodG, patients with higher 8-iso-$PGF_{2\alpha}$ levels were more likely to experience pulmonary involvement, characterized by presence of pulmonary fibrosis on CT scan ($p = 0.02$), decreased FVC (< 75% predicted; $p = 0.002$), and decreased DLCO/AV (< 75% predicted; $p = 0.04$). The likelihood of elevated sPAP on echocardiography (> 40 mm Hg; $p = 0.03$) and increased CRP levels (> 10 mg/l; $p = 0.02$) was significantly higher in patients with increased levels of 8-iso-$PGF_{2\alpha}$/creat. In multiple regression analysis, decreased FVC < 75% predicted ($p = 0.04$) was the only independent factor associated with increased 8-iso-$PGF_{2\alpha}$/creat levels.

Among patients with the diffuse cutaneous subset, in univariate analysis, patients with elevated 8-iso-$PGF_{2\alpha}$/creat levels were more likely to have PAH ($p = 0.04$), decreased DLCO/AV (< 75% predicted; $p = 0.03$), and severe disease according to the Medsger index (score > 2; $p = 0.003$). In multivariate analysis, we did not identify any factor independently associated with elevated 8-iso-$PGF_{2\alpha}$ levels.

Patients with the limited cutaneous disease subset had no factor associated with levels of 8-iso-$PGF_{2\alpha}$/creat.

DISCUSSION

Oxygen free radical damage has been suggested to play an important role in SSc, and this is supported by considerable evidence showing increased lipid peroxidation in this disease[13,14,21,22]. This contrasts with scarce data on the existence of oxidative DNA damage[23]. Our results confirm the marked trend towards oxidative stress in SSc and represent the first report of the existence of high oxidative DNA damage in this disease. Indeed, we found levels of urinary 8-oxodG, the most abundant DNA lesion caused by ROS, that were significantly higher in SSc patients than in the control group. Moreover, 8-oxodG levels were positively correlated with lipid peroxidation, assessed by urinary 8-iso-$PGF_{2\alpha}$ levels. Our results also showed a strong association in multivariate analysis between urinary 8-oxodG levels and a more fibrotic phenotype, at least for lung fibrosis.

The presence of high levels of urinary 8-oxodG that correlated with 8-iso-$PGF_{2\alpha}$ is a first confirmation that SSc is associated with marked oxidative stress. Whether these events are involved in the pathogenesis of the disease or are a consequence of tissue injury cannot be elucidated from our results. However, since free radicals stimulate both fibroblast proliferation and vasospasm, a causal link between free radical generation and SSc pathogenesis is strongly suspected. Unlike the association observed in healthy controls, urinary 8-oxodG and 8-iso-$PGF_{2\alpha}$ levels were independent of the age of patients with SSc, indicating that the increase was a reflection of oxidative damage associated with the disease rather than a nonspecific aspect of aging.

Table 2. Associations between urinary 8-oxodG levels and clinical features.

Feature	SSc Patients: All, n = 80 8-OxodG/Creatinine, ng/mg, median (range)	p	SSc Patients: Diffuse Cutaneous Subset, n = 39 8-OxodG/Creatinine, ng/mg, median (range)	p	SSc Patients: Limited Cutaneous Subset, n = 41 8-OxodG/Creatinine, ng/mg median (range)	p
Skin						
Modified Rodnan skin score						
< 14	4.80 (0.06–31.06)	0.1†	**1.39 (0.06–31.06)**	**0.02**†	NA*	NA
> 14	10.30 (0.19–41.54)		**10.30 (0.19–41.54)**			
Vascular involvement						
Digital ulcers	3.88 (0.13–55.38)	0.6	8.49 (0.34–55.38)	0.08†	3.19 (0.13–33.96)	0.2
Present	6.45 (0.06–46.13)		1.73 (0.06–46.13)		7.89 (0.10–45.71)	
Absent						
Systolic pulmonary artery pressure						
> 40 mm Hg	1.90 (0.34–14.18)	0.3	1.90 (0.54–14.18)	0.7	NA**	NA
≤ 40 mm Hg	6.54 (0.06–55.38)		3.40 (0.06–55.38)			
Pulmonary arterial hypertension						
Present	5.18 (0.34–14.18)	0.5	6.40 (0.54–14.18)	0.5	NA**	NA
Absent	5.22 (0.06–55.38)		2.72 (0.06–55.38)			
Laboratory measures						
Erythrocyte sedimentation rate						
≥ 28 mm	6.54 (0.13–55.38)	0.7	3.69 (0.54–55.38)	0.2	7.91 (0.13–27.06)	0.7
< 28 mm	4.08 (0.06–45.71)		2.55 (0.06–43.25)		7.89 (0.10–45.71)	
C-reactive protein						
≥ 10 mg/l	1.73 (0.06–55.38)	0.2	1.19 (0.06–55.38)	0.5	2.26 (0.34–9.29)	0.3
< 10 mg/l	7.44 (0.1–45.71)		6.09 (0.16–43.25)		7.44 (0.10–45.71)	
von Willebrand factor antigen						
≥ 200%	9.96 (1.05–34.84)	0.2	19.96 (1.05–34.84)	0.3	8.71 (2.26–16.24)	0.7
< 200%	3.61 (0.13–46.13)		1.73 (0.19–46.13)		4.08 (0.42–28.78)	
Antinuclear antibodies						
Present	3.98 (0.10–55.38)	0.9	2.72 (0.16–55.38)	0.5	6.64 (0.1–45.71)	0.7
Absent	9.37 (0.06–31.06)		13.59 (0.06–31.06)		4.22 (0.42–27.06)	
Antitopoisomerase antibodies						
Present	7.09 (0.16–55.38)	0.2	6.12 (0.16–55.38)	0.5	NA***	NA
Absent	2.53 (0.06–45.71)		2.59 (0.06–43.25)			
Anticentromere antibodies						
Present	3.54 (0.13–45.71)	0.3	NA****	NA	4.43 (0.13–45.71)	0.2
Absent	8.80 (0.06–55.38)				11.06 (0.1–37.58)	
Lung involvement						
Pulmonary fibrosis on CT scan						
Present	**8.33 (0.06–55.38)**	**0.01**†	8.85 (0.06–55.38)	0.08†	7.89 (0.20–37.58)	0.2
Absent	**2.39 (0.1–45.71)**		2.62 (0.19–41.54)		3.39 (0.10–45.71)	
Forced vital capacity						
< 75%	**8.85 (0.16–55.38)**	**0.002**†	**8.82 (0.16–55.38)**	**0.04**†	**9.29 (0.10–32.29)**	**0.02**†
≥ 75%	**1.73 (0.10–45.71)**		**1.73 (0.19–43.25)**		**0.34 (0.10–45.71)**	
DLCO/AV						
< 75%	**11.31 (0.16–55.38)**	**0.003**†	10.57 (0.16–55.38)	0.07†	14.90 (0.34–37.12)	0.1†
≥ 75%	**3.06 (0.10–38.45)**		2.39 (0.19–16.38)		6.65 (0.1–38.45)	
Composite indexes						
Active disease (Valentini)						
Yes (≥ 3)	8.67 (0.16–55.38)	0.5	**8.85 (0.16–55.38)**	**0.01**†	4.95 (0.2–37.12)	0.6
No (< 3)	4.08 (0.06–45.71)		**0.92 (0.06–15.86)**		6.32 (0.10–45.71)	
Severe disease (Medsger)						
Yes (> 2)	2.5 (0.06–43.25)	0.3	0.80 (0.06–16.38)	0.06†	10.18 (0.34–28.78)	0.9
No (≤ 2)	6.45 (0.10–55.38)		3.54 (0.16–55.38)		6.64 (0.10–45.71)	

† Variables included in multiple linear regression analysis. Comparison was not possible as: * no patient with the limited cutaneous subset had mRSS > 14; ** only 2 patients had sPAP > 40 or PAH and the limited cutaneous subtype. *** no patient with limited cutaneous SSc had antitopoisomerase-1 antibodies; **** only one patient had diffuse cutaneous SSc and anticentromere antibodies. NA: not applicable. Significant values appear in bold type.

Table 3. Associations between urinary 8-iso-$PGF_{2\alpha}$ levels and clinical features.

Feature	SSc Patients: All, n = 80 8-OxodG/Creatinine, ng/mg, median (range)	p	SSc Patients: Diffuse Cutaneous Subset, n = 39 8-OxodG/Creatinine, ng/mg, median (range)	p	SSc Patients: Limited Cutaneous Subset, n = 41 8-OxodG/Creatinine, ng/mg median (range)	p
Skin						
Modified Rodnan skin score						
< 14	9.89 (3.62–43.93)	0.5	9.89 (3.62–43.93)	0.6	NA*	NA
> 14	12.30 (2.68–56.96)		9.92 (2.68–56.96)			
Vascular involvement						
Digital ulcers						
Present	12.91 (2.68–56.96)	0.6	10.45 (2.68–56.96)	0.2	14.25 (3.41–25.83)	0.2
Absent	9.90 (2.96–55.58)		9.22 (2.96–36.06)		16.80 (4.54–55.58)	
Systolic pulmonary artery pressure						
> 40 mm Hg	**13.10 (2.68–56.96)**	**0.03**†	**12.06 (2.68–56.96)**	**0.04**†	NA**	NA
≤ 40 mm Hg	**6.85 (2.96–36.14)**		**5.18 (2.96–9.34)**			
Pulmonary arterial hypertension						
Present	12.79 (2.68–56.96)	0.1†	**10.95 (2.68–56.96)**	**0.04**†	NA**	NA
Absent	6.85 (2.96–36.14)		**5.06 (2.96–8.53)**			
Laboratory measures						
Erythrocyte sedimentation rate						
≥ 28 mm	12.79 (2.68–56.96)	0.4	9.89 (2.68–56.96)	0.9	15.46 (3.41–55.58)	0.5
< 28 mm	9.29 (2.96–43.93)		9.29 (2.96–43.93)		11.42 (4.54–36.14)	
C-reactive protein						
≥ 10 mg/l	**13.82 (3.34–56.96)**	**0.02**†	11.44 (3.34–56.96)	0.2	15.25 (3.41–55.58)	0.8
< 10 mg/l	**8.79 (2.68–36.14)**		8.53 (2.68–15.39)		9.04 (8.54–36.14)	
von Willebrand factor antigen						
≥ 200%	13.39 (3.34–56.96)	0.4	11.87 (3.34–56.96)	0.6	15.25 (4.54–28.31)	0.9
< 200%	9.22 (3.62–26.76)		9.22 (3.62–14.85)		14.00 (6.25–26.76)	
Antinuclear antibodies						
Present	12.18 (2.96–56.96)	0.5	9.31 (2.96–56.96)	0.4	14.84 (3.41–55.58)	0.8
Absent	10.68 (2.68–15.77)		7.24 (2.68–14.85)		12.58 (10.32–15.77)	
Antitopoisomerase antibodies						
Present	13.45 (2.68–55.58)	0.06†	11.56 (2.68–36.06)	0.7	NA***	NA
Absent	8.76 (2.96–56.96)		9.25 (2.96–56.96)			
Anticentromere antibodies						
Present	**9.90 (2.68–56.96)**	**0.03**†	NA****	NA	9.90 (3.41–19.05)	0.06
Absent	**16.94 (4.15–55.58)**				18.20 (4.15–55.58)	
Lung involvement						
Pulmonary fibrosis on CT scan						
Present	**15.25 (3.41–55.58)**	**0.02**†	13.29 (5.47–36.06)	0.3	15.72 (3.41–55.58)	0.1
Absent	**9.29 (2.68–56.96)**		9.22 (2.68–56.96)		10.10 (6.25–14.25)	
Forced vital capacity						
< 75%	**13.61 (3.34 –55.58)**	**0.002**†	10.45 (3.34–43.93)	0.6	15.67 (3.41–55.58)	0.05
≥ 75%	**8.53 (2.96–56.96)**		8.53 (2.96–56.96)		8.54 (6.25–13.60)	
DLCO/AV						
< 75%	**14.07 (3.41–55.58)**	**0.04**†	**13.61 (5.47–36.06)**	**0.03**†	14.43 (3.41–55.58)	0.8
≥ 75%	**9.29 (2.96–56.96)**		**6.99 (2.96–56.96)**		15.25 (4.15–36.14)	
Composite indexes						
Active disease (Valentini)						
Yes (≥ 3)	12.49 (2.68–56.96)	0.5	9.34 (2.68–56.96)	0.5	14.43 (3.41–55.58)	0.8
No (< 3)	11.44 (2.96–43.93)		8.31 (2.96–43.93)		13.91 (8.54–36.14)	
Severe disease (Medsger)						
Yes (> 2)	13.39 (3.34–56.96)	0.08†	**13.29 (3.34–56.96)**	**0.003**†	16.76 (9.90–36.14)	0.4
No (≤ 2)	8.53 (2.68–36.14)		**5.69 (2.68–12.67)**		13.93 (3.41–55.58)	

† Variables included in multiple linear regression analysis. Comparison was not possible as: * no patient with the limited cutaneous subset had mRSS > 14; ** only 2 patients had sPAP > 40 or PAH and the limited cutaneous subtype; *** no patient with limited cutaneous SSc had antitopoisomerase-1 antibodies; **** only one patient had the diffuse cutaneous SSc and anticentromere antibodies. NA: not applicable. Significant values appear in bold type.

Recent data have shown that selective oxidation of DNA topoisomerase-I induced skin and lung fibrosis in mice, which support that DNA oxidative damage may dictate the subset of SSc[24]. In the present study, levels of 8-oxodG were higher in patients with the diffuse cutaneous disease subset, but this result did not reach statistical significance. Thus, DNA damage measurement cannot be used to adequately discriminate between these 2 subsets of SSc, as suggested[12,13]. Further studies are also needed to assess the influence of oxidative stress on the perturbation of the immune system in SSc, especially on the production of autoantibodies.

We found a clear association in multivariate analysis between oxidative stress and interstitial lung involvement. This was particularly true for 8-oxodG. Previous studies have failed to find a correlation between lipid peroxidation, assessed by urinary 8-iso-$PGF_{2\alpha}$ levels, and pulmonary fibrosis[12,13]. These studies were performed on a small sample and detected lung involvement only on chest radiographs, which may have accounted for the different results. However, it is noteworthy that one of these studies showed that 8-iso-$PGF_{2\alpha}$ levels correlated with DLCO and with a more severe lung involvement, as assessed by the lung severity assessment score[13].

Although oxidative damage is associated with microvascular dysfunction[25] and is known to contribute to abnormalities of vascular remodeling and angiogenesis[26], we failed to find an association between oxidative stress and clinical or biological markers of SSc-related vasculopathy. This may be partly explained by the low number of patients in our study with digital ulcers, increased sPAP, or increased von Willebrand factor. Further studies with adequate sample size are thus required to explore this association.

The occurrence of DNA damage in autoimmune diseases, especially in SSc, has not been assessed precisely. One study showed increased 8-oxodG and increased susceptibility to cytotoxic killing by hydrogen peroxide in lymphocytes from patients with different autoimmune diseases[23]. Another recent study found a strong expression of 8-oxodG around synovial tissue in patients with rheumatoid arthritis[27,28,29]. In SSc, no data are available, but indirect evidence may suggest a contribution of DNA damage for development of fibrosis. First, higher urinary 8-oxodG levels were more frequently found in a subgroup of patients with a more fibrotic phenotype, and who were characterized by higher mRSS and presence of symptomatic interstitial lung involvement. Second, a close relationship between oxidative DNA damages and epigenetic modifications has been highlighted, especially in some models of cancer[30]. In SSc, recent evidence has emerged on the role of epigenetic alterations in the promotion of fibrosis, but further studies in SSc are needed to assess if these epigenetic modifications leading to fibrosis are related to increased DNA damage induced by oxidative stress[31]. Oxidative damage to DNA is important in mutagenesis and carcinogenesis[32,33]. Experimental studies *in vitro* and in animals show that malignant cells contain high levels of oxidized DNA lesion. In humans, elevated 8-oxodG levels have been detected in various tumors, especially in breast, prostate, bladder, and small-cell lung cancer[16,34,35,36]. Thus, urinary 8-oxodG is considered a useful indicator of cancer risk, even though there is no direct evidence linking oxidative DNA modification to cancer.

In SSc, the link with cancer seems not overwhelming, but recent evidence suggests a modest increase in risk, with standardized incidence ratios ranging from 0.75 to 2.73[37]. The incidence of malignancy ranges from 3.6% to 10.7%, with a weighted average of 6.3% across the largest studies reported[37]. Lung and breast cancer are the most frequently reported types of malignancy. No unifying mechanism has established a direct link between SSc and cancer risk. Although some risk factors have been identified, no predictor of cancer risk in SSc is available[37,38]. Thus, the predictive value of 8-oxodG for the risk of cancer in SSc patients should be determined, regarding increased risk of cancer and high levels of 8-oxodG in SSc patients.

Limitations of our study that merit consideration include its observational design; moreover, any pathogenic link emerging from this type of study should be considered cautiously. Our sample size was too limited to adequately assess disease phenotype associations in specific subsets of patients, especially those with peripheral or pulmonary vasculopathy. The range of 8-oxodG values was broad in both SSc patients and controls, which may reflect their successive recruitment, and thus the absence of selection criteria. The values of 8-iso-$PGF_{2\alpha}$ reported here were higher than those previously published. The method of measurement may account for such discrepancies, as we used a competitive ELISA whereas others used chromatography[12,13]. It is noteworthy that the levels found in the control group were within the manufacturer's determined threshold; and the fold increase observed in the patient groups was close to that of a previous report[13]. It was not possible to consider nailfold videocapillaroscopy in our study because, in a significant number of cases, this examination was not performed at the same time as the measurements of urine concentrations of 8-oxodG and 8-iso-$PGF_{2\alpha}$. It was also not possible to assess the relationship between 8-oxodG and cancer in our population.

Our results confirm the striking association between SSc and oxidative stress, targeting lipids and nucleic acids. Our study is the first to report high oxidative DNA damage in SSc, reflected by increased urinary 8-oxodG levels. As this marker is considered a useful indicator of cancer risk, further investigations are warranted to assess the predictive value of 8-oxodG as a cancer predictor in SSc, a disease characterized by increased frequency of cancer.

Increased levels of this 8-oxodG were more likely to be found in patients with a high degree of skin fibrotic extension

and with interstitial lung involvement, supporting the potential contribution of oxidative DNA damage in fibrosis. Functional analyses are now needed to confirm this hypothesis.

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