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The oxidation induced by antimyeloperoxidase antibodies triggers fibrosis in microscopic polyangiitis

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ABSTRACT: Lung fibrosis is considered a severe manifestation of microscopic polyangiitis (MPA). Antimyeloperoxidase (anti-MPO) antibodies in MPA patients' sera can activate MPO and lead to the production of reactive oxygen species (ROS). While high levels of ROS are cytotoxic, low levels can induce fibroblast proliferation. Therefore, we hypothesised that the oxidative stress induced by anti-MPO antibodies could contribute to lung fibrosis.

24 MPA patients (45 sera) were enrolled in the study, including nine patients (22 sera) with lung fibrosis. Serum advanced oxidation protein products (AOPP), MPO-induced hypochlorous acid (HOCl) and serum-induced fibroblast proliferation were assayed.

AOPP levels, MPO-induced HOCl production and serum-induced fibroblast proliferation were higher in patients than in healthy controls ($p<0.0001$, $p=0.0001$ and $p=0.0005$, respectively). Increased HOCl production was associated with active disease ($p=0.002$). Serum AOPP levels and serum-induced fibroblast proliferation were higher in patients with active MPA and lung fibrosis ($p<0.0001$). A significant linear relationship between fibroblast proliferation, AOPP levels and HOCl production was observed only in patients with lung fibrosis.

Oxidative stress, in particular the production of HOCl through the interaction of MPO with anti-MPO antibodies, could trigger the fibrotic process observed in MPA.

KEYWORDS: Anti-neutrophil cytoplasm antibodies, fibrosis, hypochlorous acid, myeloperoxidase, vasculitis

Microscopic polyangiitis (MPA) is a necrotising vasculitis affecting small-sized vessels. While cutaneous, gastrointestinal, musculoskeletal and neurological manifestations can be observed in MPA, the most typical manifestations include rapidly progressive glomerulonephritis and pulmonary involvement. Classical pulmonary involvement consists of alveolar haemorrhage secondary to pulmonary capillaritis [1, 2]. Pulmonary fibrosis is also a potentially severe manifestation of MPA, but mild pulmonary fibrosis is significantly associated with an increased rate of mortality [2–4]. Although pulmonary fibrosis in MPA might result from iterative episodes of alveolar haemorrhage, half of patients with pulmonary fibrosis have no history of haemoptysis and pulmonary fibrosis can be the initial manifestation of the disease, sometimes several years prior to the diagnosis of MPA [2, 5].

MPA is associated with a variety of circulating autoantibodies, in particular anti-neutrophil cytoplasm antibodies (ANCAs), which can be detected in 75–80% of cases. In MPA, ANCAs exhibit mainly a perinuclear fluorescent pattern and are directed to myeloperoxidase (MPO). Although anti-MPO antibodies (Abs) are associated with pulmonary fibrosis [2, 5], their role in this pathophysiological process has been poorly understood so far.

Anti-MPO Abs play a key role in endothelial damage *in vitro* and the development of vasculitis *in vivo* [6–8]. They can trigger an oxidative burst in neutrophils *in vitro* [6] and cause damage to endothelial cells through MPO activation and hypochlorous acid (HOCl) production [8]. Altogether, these findings argue for the cytotoxic effects of anti-MPO Abs through the generation of reactive oxygen species (ROS) in MPA. ROS are already known to trigger fibroblast proliferation

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and the development of fibrosis, as observed in other pathological conditions, such as idiopathic pulmonary fibrosis, systemic sclerosis (SSc) and malignancies [9–11]. Therefore, we hypothesised that the oxidative stress induced by anti-MPO Abs could contribute to the development of fibrosis in MPA patients. We compared the serum levels of anti-MPO Abs with markers of oxidative stress and cellular proliferation, and correlated these parameters with the presence or the absence of lung fibrosis.

PATIENTS AND METHODS

Patients and serum sampling

24 patients (45 sera) with MPA (nine males and 15 females; mean±SD age 59.3±12.5 yrs, ranges 25–77 yrs) were included in the study (table 1). Nine out of the 24 patients (22 out of the 45 sera) had lung fibrosis. All patients had anti-MPO Ab-associated vasculitis meeting the definition of the Chapel Hill Nomenclature [12] for the diagnosis of MPA. Lung fibrosis was defined by the presence of the following findings on high-resolution computed tomography (CT) scans of the chest: honeycombing in a peripheral distribution and/or marked traction bronchiectasias. In the MPA patients with CT scan abnormalities consistent with lung fibrosis, pulmonary function tests were performed using spirometry and plethysmography (table 2).

21 serum samples were obtained at the time of flare, including seven sera from patients with lung fibrosis. 24 sera were also obtained during clinical remission, including 15 sera from patients with lung fibrosis. Thus, 22 sera from nine MPA patients with lung fibrosis and 23 sera from 15 MPA patients with no lung fibrosis were analysed. 12 sera from 12 patients with active anti-proteinase 3 (anti-PR3) Ab-positive granulomatosis with polyangiitis (GPA) (Wegener's) (anti-PR3-GPA) and five sera from five patients with active anti-MPO Ab-positive GPA (MPO-GPA) served as controls. All cases of GPA were biopsy-proven. In addition, 40 sera from 40 healthy donors served as controls. All donors gave their written informed consent. All sera were prospectively collected during follow-up between April 1995 and February 2010 in the National Referral Center of Necrotising Vasculitides (Paris, France).

Disease activity was assessed using the Birmingham Vasculitis Activity Score (BVAS) [13]. Mean±SD BVAS score at diagnosis of MPA was 22.7±3.9 in patients with lung fibrosis and 20.0±5.2 in those without lung fibrosis ($p=0.1935$). BVAS score was 22.6±8.9 and 23.2±5.2 in patients with PR3-GPA and in those with MPO-GPA, respectively. Active disease corresponded to a BVAS >3, whereas inactive disease corresponded to a BVAS <3. At the time of sampling, MPA patients with lung fibrosis received low doses of prednisone

TABLE 1 General characteristics of micrscopic polyangiitis (MPA) patients

Patient	Sex	Age at diagnosis yrs	Lung fibrosis	Organs affected	Histological confirmation	Anti-MPO Abs	BVAS at diagnosis
1	M	77	Yes	GS, K, L, PN	Not available	Present	24
2	M	70	Yes	GS, K, L, Mu, PN	Muscle	Present	29
3	M	69	Yes	E, ENT, GS, K, L, S	Kidney	Present	28
4	F	33	Yes	GS, K, L, S	Skin	Present	21
5	F	57	Yes	E, ENT, K, L, Mu	Kidney	Present	20
6	M	62	Yes	GS, K, L	Kidney	Present	18
7	M	70	Yes	GS, K, L,	Kidney	Present	18
8	F	57	Yes	GS, K, L, PN	Kidney	Present	23
9	F	60	Yes	GS, K, L, Mu, S	Skin	Present	24
10	F	68	No	GS, Mu, PN, S	Nerve	Present	14
11	M	45	No	GS, K, L	Not available	Present	20
12	F	73	No	ENT, GS, PN	Muscle, temporal artery	Present	13
13	M	47	No	ENT, GS, K, L	Kidney	Present	22
14	M	53	No	GS, H, K, L, PN	Kidney	Present	33
15	F	70	No	K	Kidney	Present	12
16	F	25	No	GS, K, L, Mu, S	Kidney, skin	Present	23
17	M	60	No	B, E, ENT, GS, PN	Muscle	Present	17
18	F	52	No	B, E, ENT, GS, Mu, PN, S	Muscle	Present	20
19	F	56	No	GS, L, K, S	Kidney	Present	22
20	F	60	No	GS, K, PN	Muscle, kidney	Present	23
21	F	67	No	GS, ENT, Mu, PN, S	Skin	Present	20
22	F	70	No	GS, K, Mu, S	Kidney	Present	17
23	F	56	No	B, ENT, GS, PN	Muscle	Present	21
24	F	66	No	GS, H, K, L	Kidney	Present	24

Organs affected by MPA at diagnosis of the disease and histological confirmation for each MPA patient from the study. MPO: myeloperoxidase; Ab: antibody; BVAS: Birmingham Vasculitis Activity Score; M: male; F: female; GS: general symptoms; K: kidney involvement; L: lung; PN: peripheral neuropathy; Mu: muscle; E: eye involvement; ENT: ear, nose and throat involvement; S: skin; H: heart; B: bowel.

TABLE 2 Pulmonary function tests in micrscopic polyangiitis patients with lung fibrosis

Parameter	Mean±SD
VC % pred	93.0±11.2
FEV_1 % pred	88.2±23.0
FVC % pred	93.6±10.3
FEV_1/VC %	71.9±12.0
TLC % pred	86.0±9.9
RV % pred	80.4±9.4
RV/TLC %	38.2±6.2
$D_{L,CO}$ % pred	58.9±9.0

Spirometry and plethysmography were carried out and the parameters were recorded. VC: vital capacity; % pred: % predicted; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; TLC: total lung capacity; RV: residual volume; $D_{L,CO}$: diffusing capacity of the lung for carbon monoxide.

(<10 mg·day^{-1}) in 20 cases, azathioprine in four cases and no treatment in two cases. At the time of sampling, MPA patients without lung fibrosis received low doses of prednisone (<10 mg·day^{-1}) in 10 cases, intermediate doses of prednisone (10–30 mg·day^{-1}) in four cases, azathioprine in two cases, intravenous cyclophosphamide in one case and no treatment in nine cases. The 12 PR3-GPA patients and the five MPO-GPA patients received low doses of prednisone (<10 mg·day^{-1}) in nine and four cases, azathioprine in five and two cases, methotrexate in four and no cases, and no treatment in four and one cases, respectively.

ANCA, anti-MPO Ab and anti-PR3 Ab assays

All sera were screened for ANCA by indirect immunofluorescence using ethanol-fixed normal fresh neutrophils [14]. Anti-MPO Abs were determined by ELISA, as recommended by the manufacturer (Bio Advance, Emerainville, France). Results are expressed in AU·mL^{-1}. Concentrations <20 AU·mL^{-1} were considered negative. Anti-PR3 Abs were measured using the Varelisa PR3 kit (Phadia, Montigny-le-Bretonneux, France).

In vitro generation of HOCl by MPO in the presence of MPA sera

The quantification of the production of HOCl by MPO in the presence of MPA sera was performed as previously described [8].

Purified MPO (Calbiochem, San Diego, CA, USA) was diluted to 2 μg·mL^{-1} in PBS and used to coat 96-well plates (black Optiplate; Packard, Warrenville, IL, USA). After three washes with PBS, 100 μL of each serum diluted 1:10 was deposited into wells and incubated for 60 min at room temperature. 36 μM luminol and 400 μM H_2O_2 diluted in PBS were added to start the reaction. HOCl production was measured by chemiluminescence using a luminometer (Fusion; Packard) at 37°C. HOCl production was expressed in AU. Notably, our technical approach enabled the elimination of MPA sera from the wells by repeated washing. Thus, MPA sera were not incubated with H_2O_2.

Assay of serum MPO

Serum concentrations of MPO were determined by sandwich ELISA, as recommended by the manufacturer (Sigma, St Louis, MO, USA), in all sera from patients and healthy controls. The threshold of sensitivity of the assay was >1.5 ng·mL^{-1}.

Quantification of serum ceruloplamin

Serum concentrations of ceruloplamin were measured by immunonephelometry as described by the manufacturer (BNII; Dade-Behring, Paris, France). Normal values were between 0.17 and 0.70 mg·L^{-1}.

Assay of advanced oxidation protein products in sera

Advanced oxidation protein products (AOPP) were assayed by spectrophotometry as previously described [15]. In test wells, 200 μL serum diluted 1:20 in PBS was distributed onto a 96-well plate and 20 μL acetic acid was added. Next, 10 μL 1.16 M potassium iodide was added. In standard wells, 10 μL 1.16 M potassium iodide was added to 200 μL chloramine-T solution followed by 20 μL acetic acid. Calibration used chloramine-T within the range of 0–100 μmol·L^{-1}. The absorbance was immediately read at 340 nm on a microplate reader (Fusion). AOPP concentrations was expressed as μmol·L^{-1} chloramine-T equivalents.

Fibroblast proliferation assay

The fibroblast proliferation assay was performed as previously described [10]. Briefly, NIH 3T3 fibroblasts (American Type Culture Collection strain number CRL-1658; 4×10^3 cells·well^{-1}) were seeded into 96-well plates (Costar, Cambridge, MA, USA) and incubated with 50 μL MPA or control serum diluted in 150 μL RPMI-1640 culture medium (Invitrogen, Carlsbad, CA, USA) without fetal calf serum at 37°C in 5% CO_2 for 48 h. Cell proliferation was determined by pulsing the cells with [^{3}H]thymidine (1 μCi·well^{-1}) during the final 16 h of culture. Results are expressed as absolute numbers of counts per minute (cpm).

Measurement of total antioxidant capacity of the serum

The antioxidant capacity of sera was determined by measuring the formation of the radical cation 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) using the Antioxidant Assay Kit (Cayman Chemical-Interchim, Montluçon, France) based on the photometric method previously described by MILLER and RICE-EVANS [16]. Samples were measured in triplicate and results are expressed as mean±SD in mM Trolox equivalents.

Fibroblast proliferation in the presence of HOCl or AOPP

NIH 3T3 fibroblasts (4×10^3 cells·well^{-1}) were seeded into 96-well plates (Costar) and incubated with various amounts of HOCl (concentration ranging from 1.5×10^{-13} to 2.5×10^{-9} M) for 48 h. Cell proliferation was determined by thymidine incorporation. Results were expressed as absolute numbers of cpm. Bovine serum albumin (BSA) was oxidised with 1 mM HOCl for 1 h at room temperature. Proteins were then dialysed overnight against PBS and tested for AOPP content. NIH 3T3 fibroblasts (4×10^3 cells·well^{-1}) were seeded into 96-well plates (Costar) and incubated with 50 μL of a dilution of the oxidised or nonoxidised protein preparations and 150 μL culture medium without fetal calf serum at 37°C in 5% CO_2 for 48 h. Cell proliferation was determined by thymidine incorporation. Results are expressed as absolute numbers of cpm.

Statistical analysis

Data are presented as mean±SD. Statistical analysis was performed using the nonparametric Mann–Whitney U-test for unpaired data or regression analysis according to Spearman's rank correlation test. A p-value of <0.05 was considered significant. Statistical analysis was performed twice: a first time including the sera and a second time using only one serum sample per patient. The sera corresponding to the most active stage were chosen and for the comparison between active and remittent stages, we also used the sera corresponding to the longest remission period.

RESULTS

Anti-MPO Ab levels, oxidative stress markers and in vitro *fibroblast proliferation in MPA patients compared to controls*

The median of anti-MPO Ab levels, HOCl production by serum-activated MPO, serum AOPP levels and serum-induced proliferation of fibroblasts were significantly higher in MPA patients than in healthy controls (median 75 (range 1–227) *versus* 2 (0–7) IU ($p<0.0001$), 201 (133–425) *versus* 155 (136–170) AU ($p<0.0001$), 243 (10–952) *versus* 130 (0–260) $\mu mol \cdot L^{-1}$ of chloramine-T equivalents ($p=0.0001$) and 23,174 (2,000–144,749) *versus* 9,516 (303–31,745) cpm ($p=0.0005$), respectively; fig. 1). The MPO-GPA patients without lung fibrosis exhibited similar HOCl production by serum-activated MPO and serum AOPP levels, but lower serum-induced proliferation of fibroblasts than MPA patients (23,174 (2,000–144,749) *versus* 9,021 (4,490–23,362) cpm; $p<0.001$). Results in MPA, MPO-GPA and PR3-GPA patients are presented in table 1 of the online supplementary material.

Anti-MPO Ab levels, oxidative stress markers and in vitro *fibroblast proliferation in active or remittent MPA*

At the time of serum sampling, the median BVAS was 18 (range 6–27) in patients with active MPA and 1 (0–2) in patients with remittent MPA.

The anti-MPO Ab levels in MPA patients with active disease were similar to those in patients with remittent disease (median 75 (range 20–227) *versus* 77 (5–200) IU; nonsignificant; fig. 2a). *In vitro*, the HOCl production by serum-activated MPO was significantly higher in patients with active disease than in those with remittent disease (228 (172–425) *versus* 189 (133–241) AU; $p<0.01$; fig. 2b). The medians of serum AOPP levels and serum-induced fibroblast proliferation were not significantly different

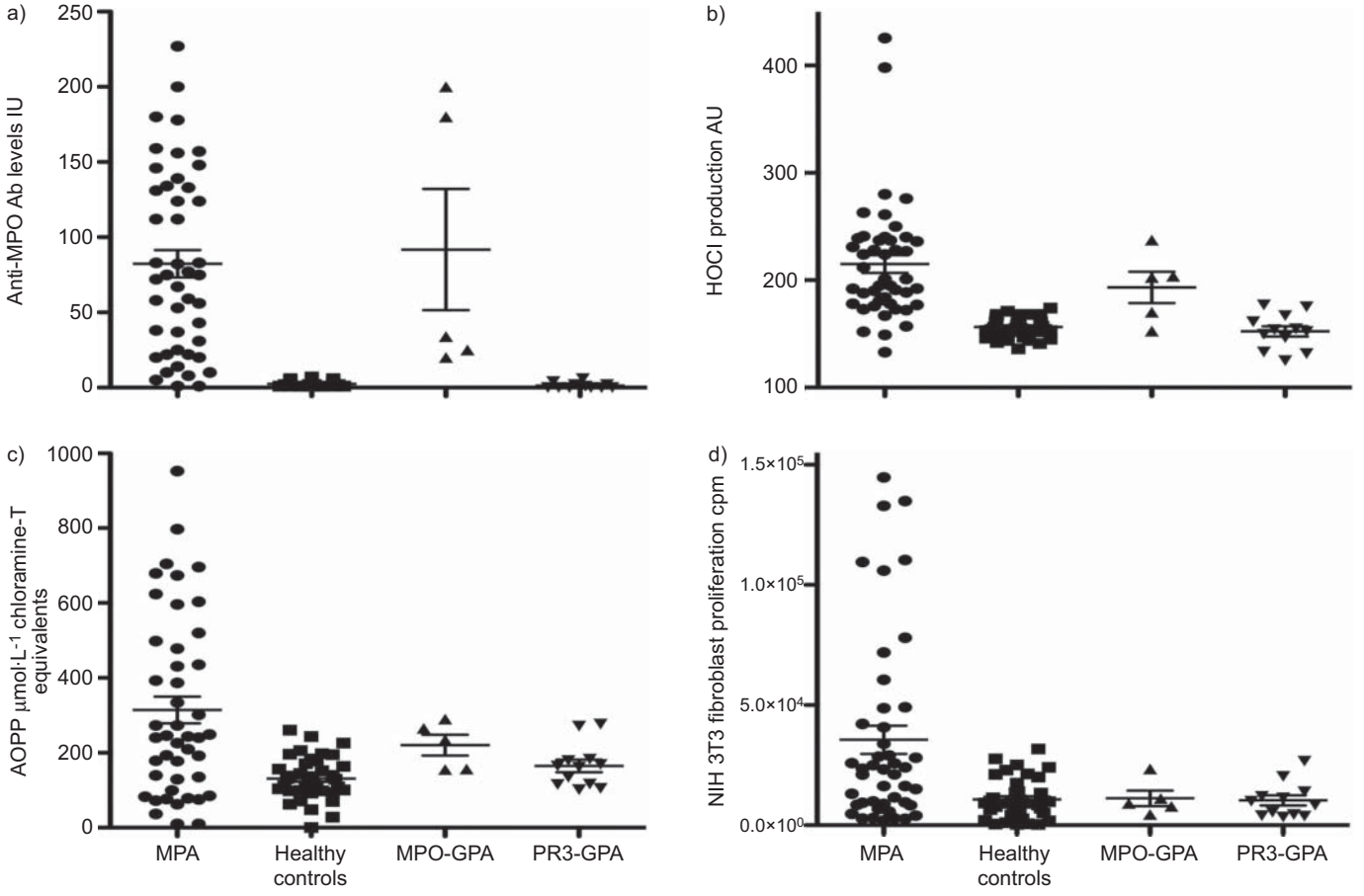

FIGURE 1. Antimyeloperoxidase (anti-MPO) antibody (Ab) levels, oxidative stress markers and *in vitro* fibroblast proliferation in microscopic polyangiitis (MPA) patients compared to healthy controls. Mean a) anti-MPO Ab levels, b) hypochlorous acid (HOCl) production by serum-activated MPO, c) serum advanced oxidation protein products (AOPP) levels and d) serum-induced proliferation of NIH 3T3 fibroblasts were assayed in 45 sera from 24 patients with MPA, in 40 sera from healthy controls, five sera from five patients with active anti-MPO Ab-positive granulomatosis with polyangiitis (anti-MPO-GPA), and in 12 sera from 12 patients with active anti-proteinase 3 Ab-positive GPA (anti-PR3-GPA), as described in the Materials and Methods section. Solid lines represent means and whiskers represent standard deviations. cpm: counts per minute.

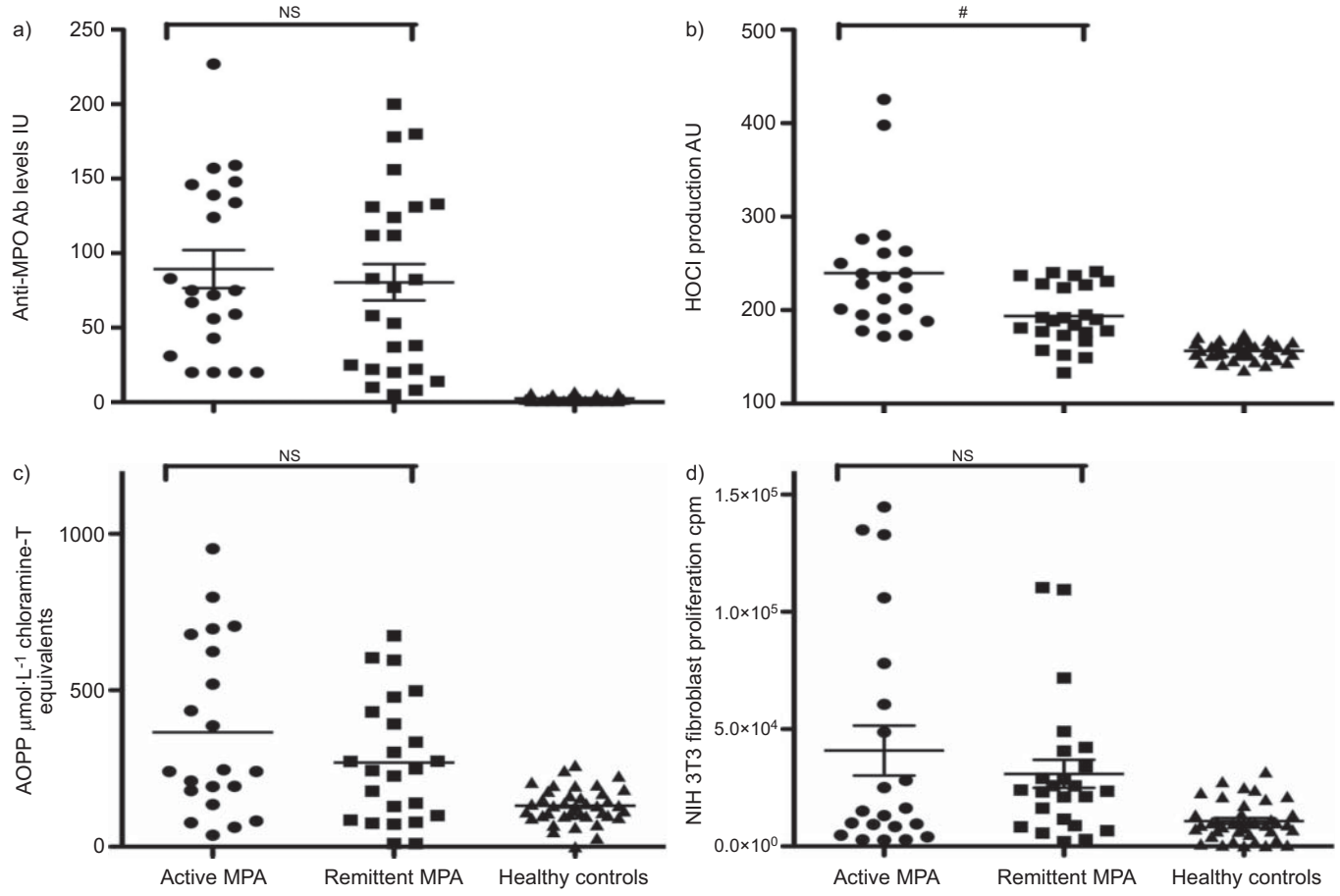

FIGURE 2. Antimyeloperoxidase (anti-MPO) antibody (Ab) levels, oxidative stress markers and *in vitro* fibroblast proliferation in active and remittent microscopic polyangiitis (MPA). Mean a) anti-MPO Ab levels, b) hypochlorous acid (HOCl) production by serum-activated MPO, c) serum advanced oxidation protein products (AOPP) levels and d) serum-induced proliferation of NIH 3T3 fibroblasts were assayed in 21 sera from 16 MPA patients with active disease and in 24 sera from 14 patients with remittent MPA. Active disease was defined as Birmingham Vasculitis Activity Score score >3. Solid lines represent means and whiskers represent standard deviations. cpm: counts per minute. NS: nonsignificant; #: $p<0.0001$.

in patients with active or remittent disease (241 (37–952) *versus* 246 (10–674) µmol·L^{-1} chloramine-T equivalents (nonsignificant) and 15,089 (2,687–144,749) *versus* 23,769 (2,000–110,355) cpm (nonsignificant), respectively; fig. 2c and d). In all cases, the mean values of these markers were higher in MPA patients with active or remittent disease than in healthy controls.

Anti-MPO Ab levels, oxidative stress markers and in vitro *fibroblast proliferation in MPA associated or not associated with pulmonary fibrosis*

The anti-MPO Ab levels and HOCl production by serum-activated MPO were not significantly different between patients with and without pulmonary fibrosis (median 72 (range 20–200) *versus* 75 (20–227) IU ($p=0.93$) and 224 (167–425) *versus* 201 (133–280) AU ($p=0.58$), respectively), but were significantly higher in patients with pulmonary fibrosis than in healthy controls (2 (1–7) IU ($p<0.0001$) and 155 (136–174) IU ($p<0.0001$), respectively) (fig. 3a and b). The mean anti-MPO Ab levels and HOCl production by serum-activated MPO were significantly higher in patients without pulmonary fibrosis than in healthy controls ($p<0.0001$ and $p<0.0001$, respectively).

Serum AOPP levels were higher in patients with pulmonary fibrosis than in patients without fibrosis (median 389 (range 10–952) *versus* 210 (10–797) µmol·L^{-1} chloramine-T equivalents), but the difference did not reach significance ($p=0.11$) (fig. 3c). However, the mean serum AOPP levels were significantly higher in patients with pulmonary fibrosis and, to a lesser extent, in patients without pulmonary fibrosis than in healthy controls (130 (0–260) µmol·L^{-1} chloramine-T equivalents; $p<0.0001$ and $p<0.05$, respectively).

Serum-induced fibroblast proliferation was significantly higher in patients with pulmonary fibrosis than in patients with no fibrosis and healthy controls (median 45,434 (range 2,000–144,749) *versus* 9,366 (9,366–28,533) ($p<0.0001$) and 9,516 (303–31,745) cpm ($p<0.0001$), respectively), but there was no difference between patients with no fibrosis and healthy controls ($p=0.58$) (fig. 3d). When including only one serum per patient, serum-induced fibroblast proliferation remained significantly higher in patients with pulmonary fibrosis compared with patients with no fibrosis (77,974 (60,565–132,941) *versus* 9,444 (2,687–28,075) cpm; $p=0,0098$).

The total antioxidant activity was lower in MPA patients with lung fibrosis than in patients with no lung fibrosis and healthy controls (median 0.4885 (range 0.4203–0.6792) *versus* 0.6843 (0.4223–0.8989) and 0.7077 (0.5281–0.8920) mM Trolox equivalents, respectively;

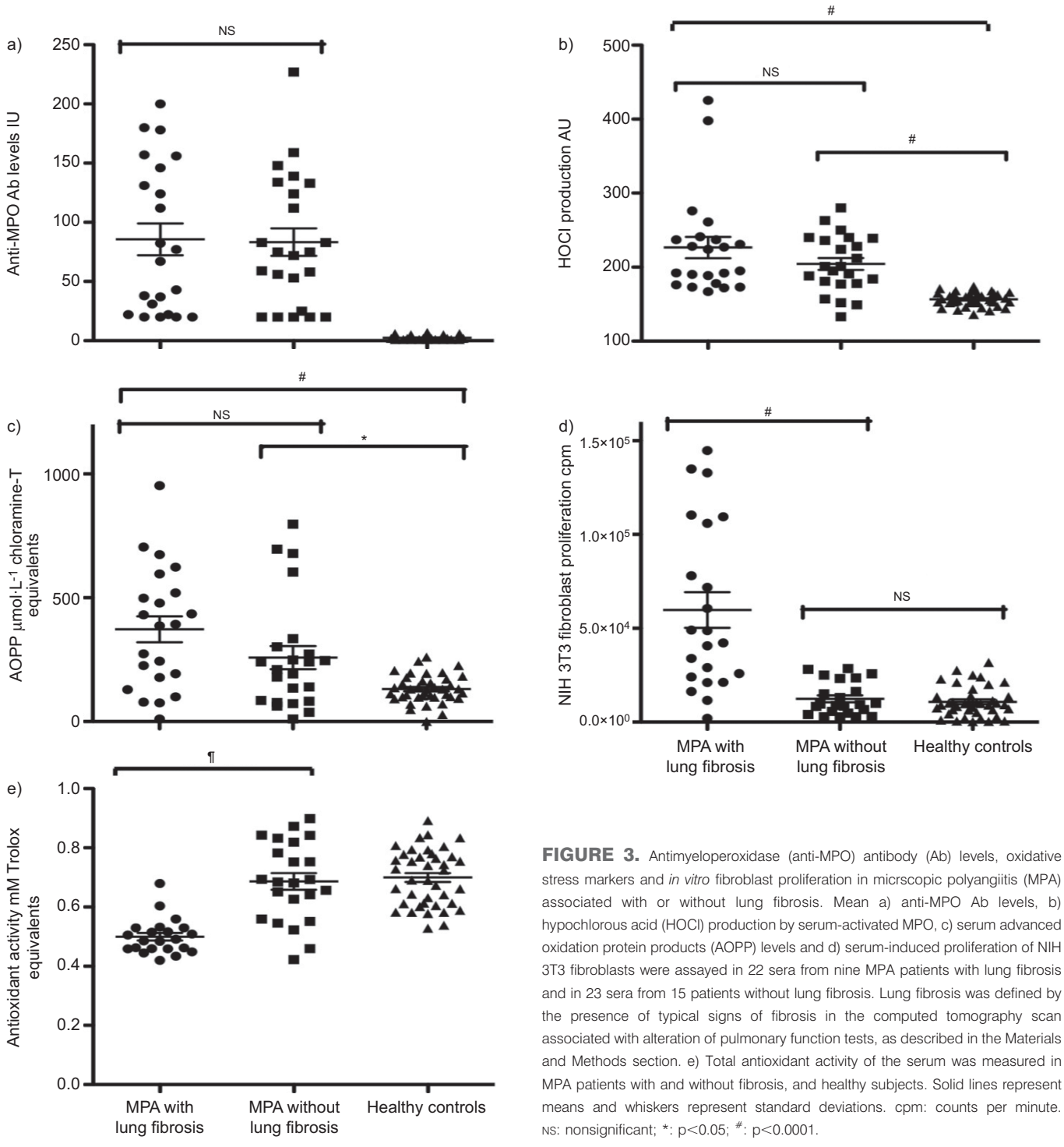

FIGURE 3. Antimyeloperoxidase (anti-MPO) antibody (Ab) levels, oxidative stress markers and *in vitro* fibroblast proliferation in micrscopic polyangiitis (MPA) associated with or without lung fibrosis. Mean a) anti-MPO Ab levels, b) hypochlorous acid (HOCl) production by serum-activated MPO, c) serum advanced oxidation protein products (AOPP) levels and d) serum-induced proliferation of NIH 3T3 fibroblasts were assayed in 22 sera from nine MPA patients with lung fibrosis and in 23 sera from 15 patients without lung fibrosis. Lung fibrosis was defined by the presence of typical signs of fibrosis in the computed tomography scan associated with alteration of pulmonary function tests, as described in the Materials and Methods section. e) Total antioxidant activity of the serum was measured in MPA patients with and without fibrosis, and healthy subjects. Solid lines represent means and whiskers represent standard deviations. cpm: counts per minute. NS: nonsignificant; *: $p<0.05$; #: $p<0.0001$.

$p<0.0001$ in both cases), but was not statistically different between patients with no lung fibrosis and healthy controls (fig. 3e).

Serum levels of MPO and ceruloplasmin were not different between MPA patients with and without lung fibrosis (mean±SD 4.5±3.4 *versus* 3.5±5.2 ng·mL^{-1} ($p=0.2098$) and 0.34±0.06 *versus* 0.37±0.07 g·L^{-1} ($p=0.3536$), respectively) or healthy subjects (4.5±3.4 *versus* 5.2±3.5 ng·mL^{-1} ($p=0.6628$) and 0.34±0.06 *versus* 0.36±0.07 g·L^{-1} ($p=0.4330$), respectively).

Correlations between anti-MPO Ab levels, HOCl production, serum AOPP levels and proliferation of NIH 3T3 fibroblasts in MPA with or without lung fibrosis

No significant correlation was found between anti-MPO Ab levels, HOCl production, serum AOPP levels and proliferation of fibroblasts in MPA patients with no pulmonary fibrosis (data not shown).

In contrast, in patients with pulmonary fibrosis, a correlation was observed between anti-MPO Ab levels and

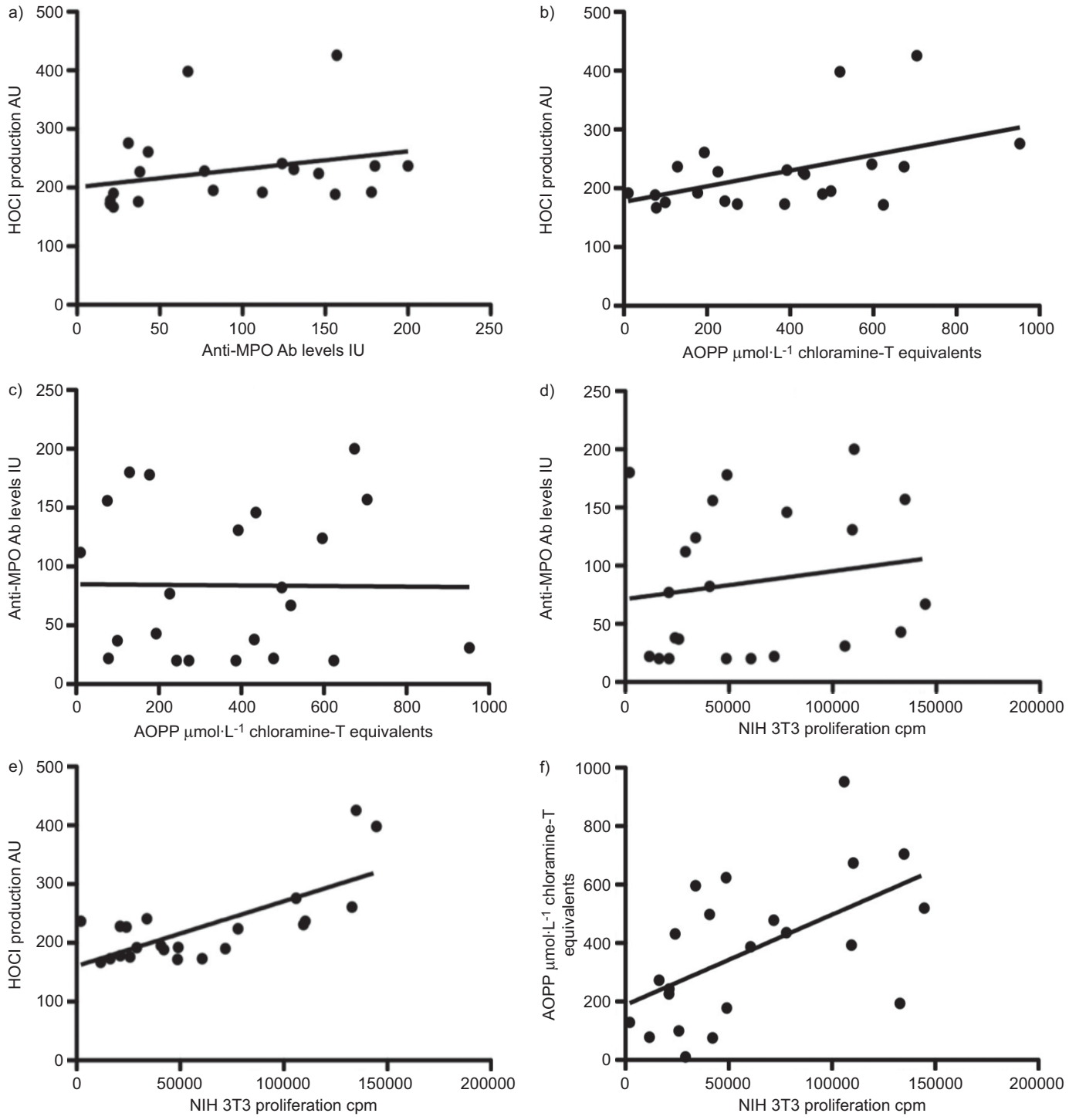

FIGURE 4. Correlations between antimyeloperoxidase (anti-MPO) antibody (Ab) levels, hypochlorous acid (HOCl) production, serum advanced oxidation protein products (AOPP) levels and *in vitro* proliferation of NIH 3T3 fibroblasts in microscopic polyangiitis associated with or without lung fibrosis. Regression analysis according to the Spearman test for paired data was used to analyse the correlations between a) anti-neutrophil cytoplasm antibody (ANCA) and HOCl ($r=0.56$; $p<0.01$), b) AOPP and HOCl ($r=0.48$; $p<0.05$), c) AOPP and ANCA (nonsignificant), d) NIH 3T3 proliferation and ANCA (nonsignificant), e) NIH 3T3 proliferation and HOCl ($r=0.72$; $p<0.001$), and f) NIH 3T3 proliferation and AOPP ($r=0.56$; $p<0.01$). cpm: counts per minute.

HOCl production ($r=0.56$; $p<0.01$), as previously described [8]. We also found a significant correlation between HOCl production by MPO and serum AOPP levels in those patients ($r=0.48$; $p<0.05$) (fig. 4). However, no correlation was found between serum AOPP levels and anti-MPO Ab levels.

In MPA patients with lung fibrosis, no correlation was observed between the *in vitro* proliferation of fibroblasts and anti-MPO Ab levels (r=0.16; p=0.45) (fig. 4). A strong correlation was observed between the proliferation of fibroblasts and the production of HOCl (r=0.72; p<0.001) or the levels of serum AOPP (r=0.56; p<0.01).

No correlations were observed between creatinine levels and production of ROS or proliferation of fibroblasts. In addition, there were no differences in the phenotypes of patients and no differences in renal involvement between MPA patients with or without fibrosis.

Relationship between disease activity and lung fibrosis

At the time of active disease, no significant difference was observed in terms of anti-MPO Ab levels and HOCl production between patients with or without lung fibrosis (fig. 5a and b). An increase in AOPP levels was observed in patients with active disease and lung fibrosis, compared with patients with active disease and no lung fibrosis. At the time of active disease, both serum AOPP levels and serum-induced fibroblast proliferation were significantly higher in patients with lung fibrosis than in patients with no lung fibrosis (median 477.4 (range 148–952) *versus* 200.9 (37–797) μmol·L^{-1} chloramine-T equivalents (p<0.05) and 91,948 (42,171–144,749) *versus* 9,444 (2,687–28,075) cpm (p<0.001), respectively) (fig. 5c and d).

At the time of remission, no significant difference in anti-MPO Ab levels and in serum AOPP levels was observed between patients with lung fibrosis and patients with no fibrosis. A significant increase in HOCl production was also observed in patients with remittent disease and lung fibrosis compared with patients with remittent MPA without fibrosis significance (mean 192.3 (range 167–231) *versus* 177.0 (133–240) AU; p<0.05). In patients with remittent disease, serum-induced fibroblast proliferation was higher in patients with lung fibrosis than in patients with no lung fibrosis (p<0.01).

HOCl and AOPP modulate fibroblast proliferation

Low concentrations of HOCl (from 1.5×10^{-13} to 1.5×10^{-10} M) induced a significant increase in fibroblast proliferation (p<0.05), whereas higher concentrations of HOCl induced a decrease in fibroblast proliferation and cell death (p<0.05) (fig. 6a). Low concentrations of AOPP generated by oxidation

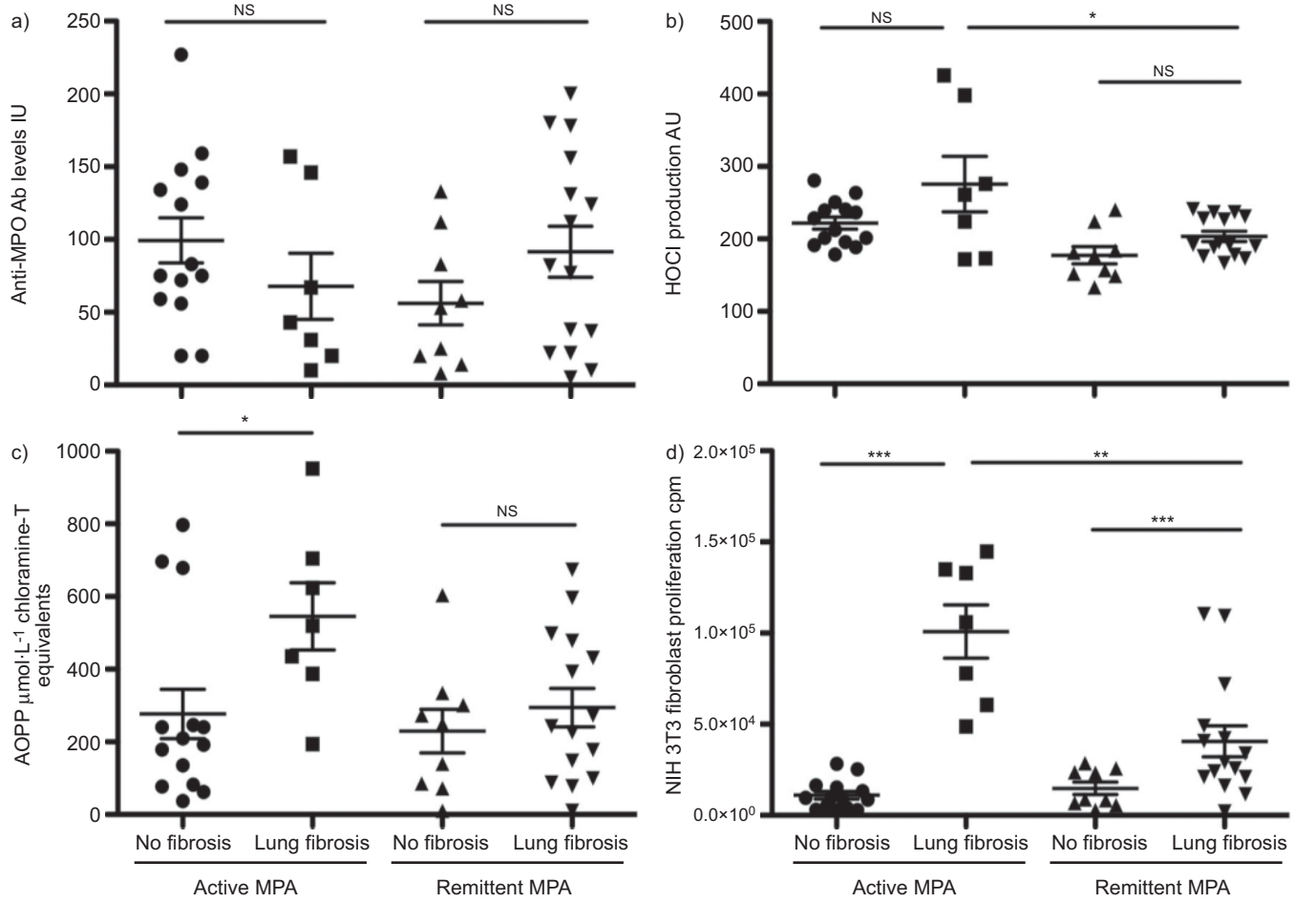

FIGURE 5. Relationship between miscroscopic angiitis (MPA) activity and lung fibrosis. Mean a) antimyeolperoxidase (anti-MPO) antibody (Ab) levels, b) hypochlorous acid (HOCl) production by serum-activated MPO, c) serum advanced oxidation protein products (AOPP) levels and d) serum-induced proliferation of NIH 3T3 fibroblasts were assayed in seven sera from four patients with active MPA and lung fibrosis, in 14 sera from 12 patients with active MPA without lung fibrosis, in 15 sera from six patients with remittent MPA and lung fibrosis, and in nine sera from eight patients with remittent MPA without lung fibrosis. Solid lines represent means and whiskers represent standard deviations. cpm: counts per minute. NS: nonsignificant; *: p<0.05; **: p<0.01; ***: p<0.001.

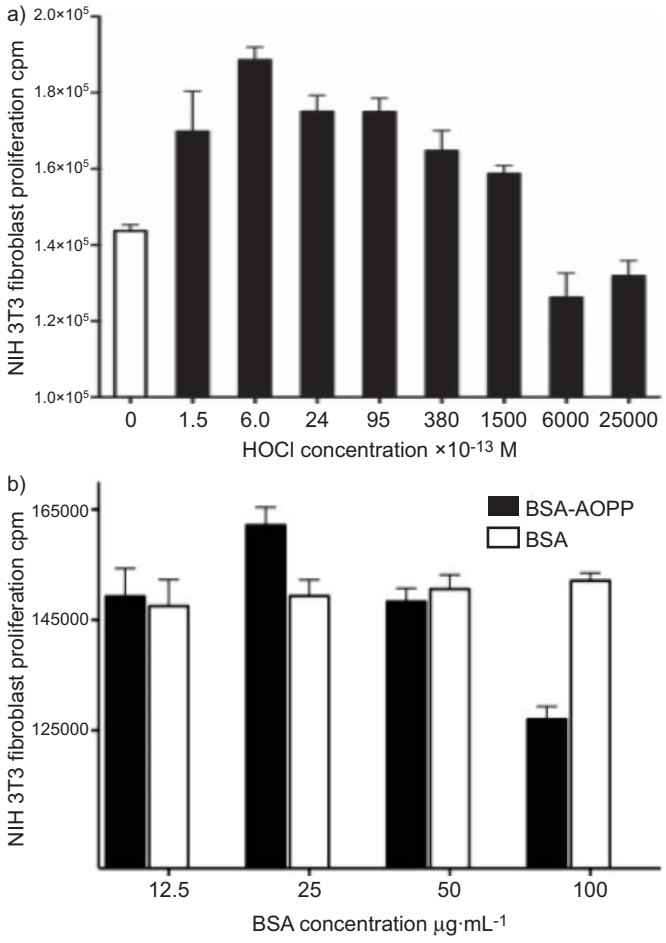

FIGURE 6. Hypochlorous acid (HOCl) and advanced oxidation protein products (AOPP) modulate fibroblast proliferation *in vitro*. NIH 3T3 fibroblasts were incubated with 1.5×10^{-13}–1.5×10^{-10} M HOCl or AOPP (generated by oxidation of bovine serum albumin (BSA) with HOCl, as described in the Materials and Methods section) for 48 h. Fibroblast proliferation was determined by thymidine incorporation. Experiments were carried out in triplicates. Solid lines represent means and whiskers represent standard deviations. cpm: counts per minute.

of BSA with HOCl (25 $\mu g \cdot mL^{-1}$) significantly increased the rate of fibroblast proliferation by 8.5% ($p<0.05$), whereas higher concentrations of HOCl-oxidised BSA induced a significant decrease of 17% in the rate of fibroblast proliferation *versus* nonoxidised BSA ($p<0.0001$) (fig. 6b).

DISCUSSION

We have recently shown the correlation between serum anti-MPO Ab levels and HOCl production in MPA [8], and the profibrotic role of sera rich in AOPP in scleroderma [10]. We have now observed a similar phenomenon in MPA and shown the role of the oxidative stress in the development of lung fibrosis in this condition.

In a first step, we found that the production of HOCl by serum-activated MPO *in vitro* was higher in MPA than in healthy subjects. Serum AOPP levels, a marker of protein oxidation [15], were also higher in MPA patients than in healthy controls. The amount of HOCl produced following the activation of MPO by MPA sera *in vitro* was correlated with the activity of the disease. These results are in agreement with recent studies that have highlighted the role of oxidative stress (especially MPO-mediated) in the pathogenesis of MPA [8, 17, 18]. We have shown in a previous report that anti-MPO Abs can activate MPO and enhance the production of HOCl leading to endothelial cell damage [8]. In addition, SLOT *et al.* [17] found higher levels of Abs to HOCl–low-density lipoproteins (LDLs) in patients with vasculitis and anti-MPO Abs, than in patients with anti-PR3 Abs, suggesting that enhanced MPO-mediated LDL oxidation occurs in patients with vasculitis and anti-MPO Abs. Furthermore, mercuric chloride-induced vasculitis can be inhibited by antioxidant molecules [18].

In a second step, we observed that the *in vitro* serum-induced proliferation of fibroblasts was higher in MPA patients than in healthy subjects. This phenomenon probably results from the pro-proliferative properties of ROS [10, 11] and not from the direct action of anti-MPO Abs that, under certain circumstances, can increase fibroblast proliferation through the activation of the mitogen-activated protein kinase pathway [11]. Indeed, we recently described the pivotal role of ROS and AOPP in the pathogenesis of SSc, a connective tissue disorder associated with skin and lung fibrosis [10]. In SSc, the sera from patients with lung fibrosis contain high levels of AOPP that trigger fibroblast proliferation more than sera from patients with no lung fibrosis. Moreover, neutralisation of AOPP with the reducing agent β-mercaptoethanol totally abrogates the fibrotic process [19].

In active MPA, HOCl production was increased both in patients with lung fibrosis and in those with no lung fibrosis. However, in patients with lung fibrosis, serum AOPP levels were increased and probably contributed to the *in vitro* serum-induced proliferation of fibroblasts and lung fibrosis, as already observed in SSc [10]. Although a causal relationship could not be established, the strong and significant correlations between HOCl concentrations, AOPP levels and fibroblast proliferation suggest that HOCl resulting from MPO activation by anti-MPO Abs induces the formation of AOPP that trigger fibroblast proliferation and lead to lung fibrosis. Our hypothesis that HOCl and AOPP contribute to lung fibrosis is strengthened by our observation that HOCl and AOPP increase fibroblast proliferation. Finally, patients with lung fibrosis exhibited a lower serum antioxidant activity than other MPA patients with no lung fibrosis, suggesting that additional factors and molecules may be involved in the modulation of the fibrosis triggered by HOCl. The fact that the levels of HOCl production were similar between patients with lung fibrosis and patients with no lung fibrosis suggests that the way HOCl oxidise proteins is different between individuals. Thus, despite similar levels of HOCl production, patients with lung fibrosis differ from those with no lung fibrosis in the subsequent steps in the fibrotic process (*i.e.* HOCl and AOPP generation, and fibroblast proliferation), due to decreased antioxidant properties in the serum. Importantly, our results are specific to anti-MPO-associated MPA since in another pathological autoimmune condition with lung fibrosis such as SSc, the sera from patients do not induce an increase in the production of HOCl compared to sera from healthy subjects (data not shown).

Our results were similar when considering all the sera from the study and when considering only one serum sample per

patient. Despite this limitation related to the use of translational values, our results argue for the pro-fibrotic role of ROS.

The role of ROS in the development of lung fibrosis has already been reported in a large number of pathological conditions, such as bronchopulmonary dysplasia in pre-term infants [20], adult respiratory distress syndrome [21, 22], sarcoidosis [23], idiopathic pulmonary fibrosis [24], and in the animal models of silicosis and asbestos-induced pulmonary fibrosis [25, 26]. The most relevant model demonstrating the role of ROS in lung fibrosis is certainly the murine model of lung fibrosis induced by intratracheal instillation of bleomycin. Bleomycin induces lung fibrosis through a ROS-mediated mechanism that is abrogated by the antioxidant molecule *N*-acetylcysteine. However, the type of ROS involved in this process and their mechanism of action remain unclear [11].

Indeed, while the overproduction of ROS is often associated with cellular apoptosis or necrosis [27, 28], the exposure to low levels of ROS can increase the growth of many types of mammalian cells. In addition, decreasing the basal level of ROS with scavengers of ROS suppresses normal cell proliferation in human and rodent fibroblasts, demonstrating the link between intracellular ROS concentration and the rate of fibroblast proliferation [29–31]. Thus, our observation that low concentrations of HOCl and of its oxidative products AOPP increase fibroblast proliferation, whereas high doses of HOCl and AOPP are highly cytotoxic, is consistent with the previous reports.

Although its role is not directly demonstrated in the present study, our results suggest that HOCl produced by the interaction of MPO and anti-MPO Abs can, either by itself or following oxidation of proteins, induce fibroblast proliferation and initiate the fibrotic process in some individuals. In addition, serum AOPP levels and serum-induced fibroblast proliferation *in vitro* could be prospectively evaluated as biological markers of lung fibrosis activity in patients with MPA.

STATEMENT OF INTEREST

A statement of interest for D. Montani can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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