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The oxidation induced by anti-MPO antibodies triggers fibrosis in microscopic polyangiitis

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Abstract

Question of the study: Lung fibrosis is considered as a severe manifestation of microscopic polyangiitis (MPA). Antimyeloperoxidase (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 hypothesized that the oxidative stress induced by anti-MPO antibodies could contribute to lung fibrosis.

Methods: Twenty-one MPA patients (42 sera) were enrolled in the study, including six patients (19 sera) with lung fibrosis. Serum advanced oxidation protein products (AOPP), MPO-induced hypochlorous acid (HOCl), and serum-induced fibroblast proliferation were assayed.

Results: 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 ($P<0.0001$). Significant linear relationship between fibroblast proliferation, AOPP levels and HOCl production was observed only in patients with lung fibrosis.

Answer to the question. The 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.

Microscopic polyangiitis (MPA) is a necrotizing 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 patients with pulmonary fibrosis have no history of hemoptysis 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 (ANCA) that can be detected in 75-80% of cases. In MPA, ANCA 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 damages *in vitro* and in the development of vasculitis *in vivo* [6-8]. They can trigger an oxidative burst in neutrophils *in vitro* [6] and cause damages 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 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 have hypothesized that the oxidative stress induced by anti-MPO Abs could contribute to the development of fibrosis in MPA patients. We have

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

Twenty-four patients (45 sera) with MPA (9 males and 15 females; mean age: 59.3 years ±12.5, ranges: 25-77) were included in the study (Table 1). Nine of the 24 patients (22 of the 45 sera) had lung fibrosis. All patients had anti-MPO Abs-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 CT scan 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 by spirometry and plethysmography (Table 2).

Twenty-one serum samples were obtained at the time of flare, including 7 sera from patients with lung fibrosis. Twenty-four sera were also obtained during clinical remission, including 15 sera from patients with lung fibrosis. Thus, 22 sera from 9 MPA patients with lung fibrosis and 23 sera from 15 MPA patients with no lung fibrosis were analyzed. Twelve sera from 12 patients with active anti-proteinase 3 (PR3) Ab-positive Wegener's granulomatosis (PR3-WG), 5 sera from 5 patients with active anti-MPO Ab-positive Wegener's granulomatosis (MPO-WG) served as controls. All WG were biopsy proven. In addition, 40 sera from 40 healthy donors served as controls. All of them 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 Necrotizing Vasculitides.

Disease activity was assessed by the Birmingham Vasculitis Activity Score (BVAS) [13]. Mean 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). Mean BVAS score was 22.6±8.9 and 23.2±5.2 in patients with PR3-WG and in those with MPO-WG respectively. Active disease corresponded to a BVAS higher than 3, whereas inactive disease corresponded to a BVAS

lower than 3. At the time of sampling, MPA patients with lung fibrosis received low doses of prednisone (less than 10mg/a day) in 20 cases, azathioprine in 4 cases and no treatment in 2 cases. At the time of sampling, MPA patients without lung fibrosis received low doses prednisone (less than 10mg/a day) in 10 cases, intermediate dose of prednisone (between 10 and 30 mg/a day) in 4 cases, azathioprine in 2 cases, intravenous cyclophosphamide in 1 case and no treatment in 9 cases". The 12 PR3-WG patients and the 5 MPO-WG patients respectively received low doses of prednisone (less than 10mg/a day) in 9 and 4 cases, azathioprine in 5 and 2 cases, methotrexate in 4 and 0 cases and no treatment in 4 and 1 cases

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 were expressed in arbitrary units (AU) /ml. Concentrations <20 AU/ml were considered negative. Anti-PR3 AAbs were measured using the Varelisa PR3, (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) was diluted at 2µg/ml in PBS and coated onto 96-well plates (Black Optiplate, Packard, Warrenville, Illinois, USA). After three washes with PBS,100µl of each serum diluted 1:10 were deposited into wells and incubated for 60 minutes at room temperature. 36µM luminol and 400µM H_2O_2 diluted in PBS were added to start the reactionHOCl production was measured by chemiluminescence using a luminometer (Fusion, Packard, Warrenville, Illinois, USA) at 37°C. HOCl production was expressed in

AU. Notably, our technical approach enables to eliminate MPA sera from the wells by several washing. Thus, MPA sera are not incubated with H2O2.

Assay of serum myeloperoxidase. Serum concentrations of myeloperoxidase were determined by sandwich ELISA as recommended by the manufacturer (Sigma) in all sera from patients and healthy controls. The threshold of sensitivity of the assay was >1.5ng/ml.

Quantification of serum ceruloplamin. Serum concentrations of ceruloplamin were measured by immuno-nephelometry as described by the manufacturer (BNII, Dade-Behring, Paris La Défense, France). Normal values were between 0.17 and 0.70mg/l.

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

Fibroblast proliferation assay. The fibroblast proliferation assay was performed as previously described [10]. Briefly, NIH 3T3 fibroblasts (ATCC n°CRL-1658; $4x10^3$ cells per well) were seeded in 96-well plates (Costar, Cambridge, MA) and incubated with 50µl of MPA or control serum diluted in 150µl of culture medium RPMI-1640 (Invitrogen, Carlsbad, CA, USA) without fetal calf serum at 37°C in 5% CO_2 for 48h. Cell proliferation was

determined by pulsing the cells with [^{3}H]thymidine (1µCi/well) during the last 16h of culture. Results were expressed as absolute numbers of counts per minute.

Measurement of total antioxidant capacity of the serum

The antioxidant capacity of sera was determined by measuring the formation of the radical cation ABTS 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 were expressed as means ± SD of mM Trolox equivalents.

Fibroblast proliferation in the presence of HOCl or AOPP. NIH 3T3 fibroblasts ($4x10^3$ per well) were seeded in 96-well plates (Costar) and incubated with various amounts of HOCl (concentration ranging from $1.5x10^{-13}$ to $2.5x10^{-9}$ M) for 48h. Cell proliferation was determined by thymidine incorporation . Results were expressed as absolute numbers of counts per min (cpm). BSA was oxidized with 1mM HOCl for 1h at room temperature. Proteins were then dialyzed overnight against PBS and tested for AOPP content. NIH 3T3 fibroblasts ($4x10^3$ per well) were seeded in 96-well plates (Costar) and incubated with 50µl of a dilution of the oxidized or non-oxidized protein preparations and 150µl of culture medium without fetal calf serum at 37°C in 5% CO_2 for 48 h. Cell proliferation was determined by thymidine incorporation. Results were expressed as absolute numbers of counts per min (cpm).

Statistical analysis. Data were expressed as means ±S.D. Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test for unpaired data or regression analysis according to the Spearman's rank correlation test. A P value <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 (75,00 I.U. [ranges: 1-227] vs 2,000 I.U. [ranges: 0-7], $p<0.0001$; 201 A.U. [ranges: 133-425] vs 155 A.U. [ranges: 136-170], $p<0.0001$; 243 [ranges: 10-952] vs 130 [ranges: 0-260] μmol/l of chloramine-T equivalents, $p=0.0001$ and 23174 [ranges: 2000-144749] vs 9516 [ranges: 303-31745] cpm, $p=0.0005$, respectively) (Fig 1). The MPO-WG patients without lung fibrosis exhibited similar HOCl production by serum-activated MPO, serum AOPP levels, but lower serum-induced proliferation of fibroblasts than MPA patients (median=23174 cpm [ranges: 2000-144749] vs 9021cpm [ranges: 4490-23362]; $p<0.001$). Results in MPA, MPO-WG and PR3-WG patients are indicated in Additional Table 1.

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 of the BVAS value was 18 [ranges: 6-27] in patients with active MPA and 1 [ranges: 0-2] in patients with remittent MPA.

The anti-MPO Ab levels were similar in MPA patients with active disease than in those with remittent disease (median= 75 I.U. [ranges: 20-227] *vs* 77 I.U. [ranges: 5-200], ns) (Fig 2.A). *In vitro,* the HOCl production by serum-activated MPO was significantly higher in patients with active disease than in those with remittent disease (median= 228 A.U. [172-425] *vs* 189 A.U. [ranges: 133-241], $p<0.01$) (Fig 2.B). The medians of serum AOPP levels and serum-induced fibroblast proliferation were not significantly different in patients with active

or remittent disease (median= 241 µmol/l of chloramine-T equivalents [ranges: 37-952] vs 246 µmol/l of chloramine-T equivalents [ranges: 10-674], ns and 15089 cpm [ranges: 2687-144749] vs 23769 cpm [ranges: 2000-110355], ns respectively) (Fig 2.C and 2.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 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 I.U. [ranges : 20-200] vs 75 I.U. [ranges : 20-227], p=0.93 and 224 A.U. [ranges : 167- 425] vs 201 A.U. [ranges : 133-280], p=0.58, respectively), but were significantly higher in patients with pulmonary fibrosis than in healthy controls (median= 2 I.U. [ranges : 1-7], p<0.0001 and 155 A.U. [ranges : 136-174], p<0.0001, respectively) (Fig 3.A and 3.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).

The mean serum AOPP levels were higher in patients with pulmonary fibrosis than in patients without fibrosis (median= 389 µmol/l of chloramine-T equivalents [ranges : 10-952] vs 210 µmol/l of chloramine-T equivalents [ranges: 10-797]), but the difference did not reach significance (p=0.11) (Fig 3.C). 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 (median= 130 µmol/l of chloramine-T equivalents [ranges: 0-260] , 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 than in healthy controls (45434 cpm [2000-144749] vs 9366 cpm [9366-28533], p<0.0001 and 45434 cpm [2000-144749] vs 9516 cpm [303- 31745], cpm, p<0.0001 respectively), but there was no difference between patients with no fibrosis and healthy controls (p=0.58) (Fig 3.D). When including only one serum per patient, serum-induced fibroblast proliferation remained significantly higher in patients with pulmonary fibrosis compared to patients with no fibrosis (77974 cpm [60,565-132,941] vs 9444 cpm [2687-28075], 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 (0,4885 mM Trolox equivalents [ranges: 0,4203-0,6792] vs 0,6843 mM Trolox equivalents [ranges : 0,4223-0,8989] and 0,7077 mM Trolox equivalents [0,5281-0,8920], respectively; p<0.0001 in both cases) but was not statistically different between patients with no lung fibrosis and healthy controls (Fig 3.E).

Serum levels of MPO and of ceruloplasmin were not different between MPA patients with and without lung fibrosis (4.5 ±3.4ng/ml vs 3.5 ±5.2ng/ml p=0.2098 and 0.34 ±0.06g/l vs 0.37 ±0.07g/l p=0.3536, respectively) or healthy subjects (4.5 ±3.4ng/ml vs 5.2 ±3.5ng/ml p=0.6628 and 0.34 ±0.06g/l vs 0.36±0.07g/l, 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 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 5.A and 5.B). An increase in AOPP levels was observed in patients with active disease and lung fibrosis compared to 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 (477,4 µmol/l of chloramine-T equivalents [ranges: 148-952] vs 200,9 µmol/l of chloramine-T equivalents [ranges: 37-797], p<0.05 and 91948 cpm [ranges: 42171-144749] vs 9,444 cpm [ranges: 2,687- 28,075], p<0.001, respectively) (Fig 5.C and 5.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 to patients with remittent MPA without fibrosis significance (192,3 A.U. [ranges: 167-231] vs 177,0 A.U. [ranges: 133-240], $p<0.05$). 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 $1.5x10^{-13}$ to $1.5x10^{-10}$ 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 6.A). Low concentrations of AOPP generated by oxidation of BSA with HOCl (25µg/ml) significantly increased the rate of fibroblast proliferation by 8.5% ($P<0.05$), while higher concentrations of HOCL-oxidized BSA induced a significant decrease of 17% in the rate of fibroblast proliferation versus non-oxidized BSA ($P<0.0001$) (Fig 6.B).

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* is higher in MPA than in healthy subjects. Serum AOPP levels, a marker of protein oxidation [15], are also higher in MPA patients than in healthy controls. The amount of HOCl produced following the activation of MPO by MPA sera *in vitro* is correlated with the activity of the disease. These results are in agreement with recent studies that have highlighted the role of the 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. found higher levels of antibodies to HOCl–low-density lipoproteins 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 [17]. 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 is 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 MAP 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-mercapto-ethanol totally abrogates the fibrotic process [19].

In active MPA, HOCl production is increased both in patients with lung fibrosis and in those with no lung fibrosis. However, in patients with lung fibrosis, serum AOPP levels are increased and probably contribute to the *in vitro* serum-induced proliferation of fibroblasts and lung fibrosis as already observed in systemic sclerosis [10]. Although a causal relationship cannot 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-AOPP contribute to lung fibrosis is strengthened by our observation that HOCl-AOPP increase fibroblast proliferation. Finally, patients with lung fibrosis exhibit 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 are similar between patients with lung fibrosis and patients with no lung fibrosis suggests that the way HOCl oxidize proteins is different between individuals. Thus, despite similar levels of HOCl production, patients with lung fibrosis differ from those with no lung fibrosis by the following steps in the fibrotic process (ie, HOCl-AOPP generation and fibroblast proliferation), because of 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 systemic sclerosis, 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 intra tracheal instillation of bleomycin. Bleomycin induces lung fibrosis through a ROS-mediated mechanism that is abrogated by the anti-oxidant molecule N-Acetyl-Cysteine. 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 HOCl-AOPP increase fibroblast proliferation, whereas high doses of HOCl and AOPP are highly cytotoxic, is in line 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 the 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.

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Table 1. General characteristics of MPA patients. Organs affected by MPA at diagnosis of the disease and histological confirmation for each MPA patient from the study. Anti-MPO Abs: anti-myeloperoxidase antibodies; B: bowel; E: eye involvement; ENT: ear, nose, throat involvement; F: female; GS: general symptoms; H: heart; .J: joint involvement; K: kidney involvement; L: lung; M: male; Mu: muscle; PN: peripheral neuropathy; S: skin

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, M, 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, M	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, M, S	skin	present	24
# 10	F / 68	no	GS,M, 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, M, 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, M, 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, M, PN, S	skin	present	20
# 22	F / 70	no	GS, K, M, 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

Table 2. Pulmonary function tests in MPA patients with lung fibrosis. Spirometry and plethysmography were carried out and the following parameters were recorded: VC : vital capacity ; FEV1 : forced expiratory volume in 1 second ; FVC : forced vital capacity ; FEV1/FVC : FEV1 to FVC ratio ; TLC : total lung capacity ; RV : residual volume ; RV/TLC : RV to TLC ratio ; DLCO : diffusing capacity of the lung for carbon monoxide. These data are given as percentages of predicted values (pred) ± standard deviations (SD).

Parameters	**Mean (± SD)**
VC (% of pred)	93.0 (± 11.2)
FEV1 (% of pred)	88.2 (± 23.0)
FVC (% of pred)	93.6 (±10.3)
FEV1/VC (%)	71.9 (±12.0)
TLC (% of pred)	86.0 (±9.9)
RV (% of pred)	80.4 (±9.4)
RV/TLC (%)	38.2 (± 6.2)
DLCO (% of pred)	58.9 (± 9.0)

FIGURE LEGENDS

Figure 1. Anti-MPO Ab levels, oxidative stress markers and *in vitro* fibroblast proliferation in MPA patients compared to healthy controls. Mean anti-MPO Ab levels (A), HOCl production by serum-activated MPO (B), serum AOPP levels (C) and serum-induced proliferation of NIH 3T3 fibroblasts (D) were assayed in 45 sera from 24 patients with MPA, in 40 sera from healthy controls, in 5 sera from 5 patients with active anti-MPO Ab-positive Wegener's granulomatosis (MPO-WG), and in 12 sera from 12 patients with active anti-proteinase 3 (PR3) Ab-positive Wegener's granulomatosis (PR3-WG) as described in the materials and methods section. Data are expressed as means ±S.D. * *P* <0. 05; ** *P* < *0. 01; *** P < 0. 001; ****P < 0. 0001.*

Figure 2. Anti-MPO Ab levels, oxidative stress markers and *in vitro* fibroblast proliferation in active and remittent MPA. Mean anti-MPO Ab levels (A), HOCl production by serum-activated MPO (B), serum AOPP levels (C) and serum-induced proliferation of NIH 3T3 fibroblasts (D) 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 BVAS score >3. Data are expressed as means ±S.D. * *P* <0. 05; ** *P < 0. 01; *** P < 0. 001; ****P < 0. 0001.*

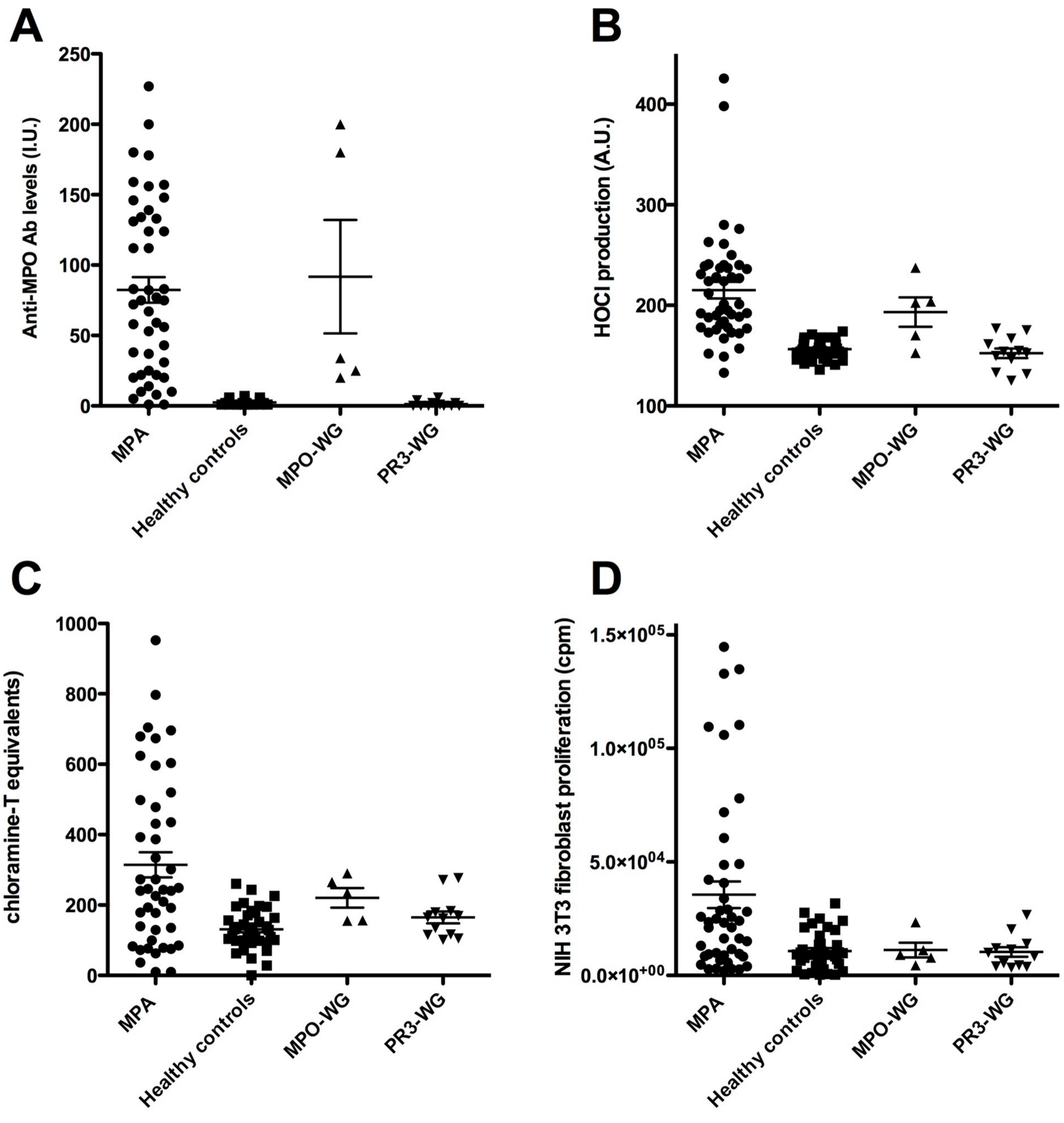
Figure 3. Anti-MPO Ab levels, oxidative stress markers and *in vitro* fibroblast proliferation in MPA associated or not with lung fibrosis. Mean anti-MPO Ab levels (A), HOCl production by serum-activated MPO (B), serum AOPP levels (C) and serum-induced proliferation of NIH 3T3 fibroblasts (D) were assayed in 22 sera from 9 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 CT scan associated with alteration of

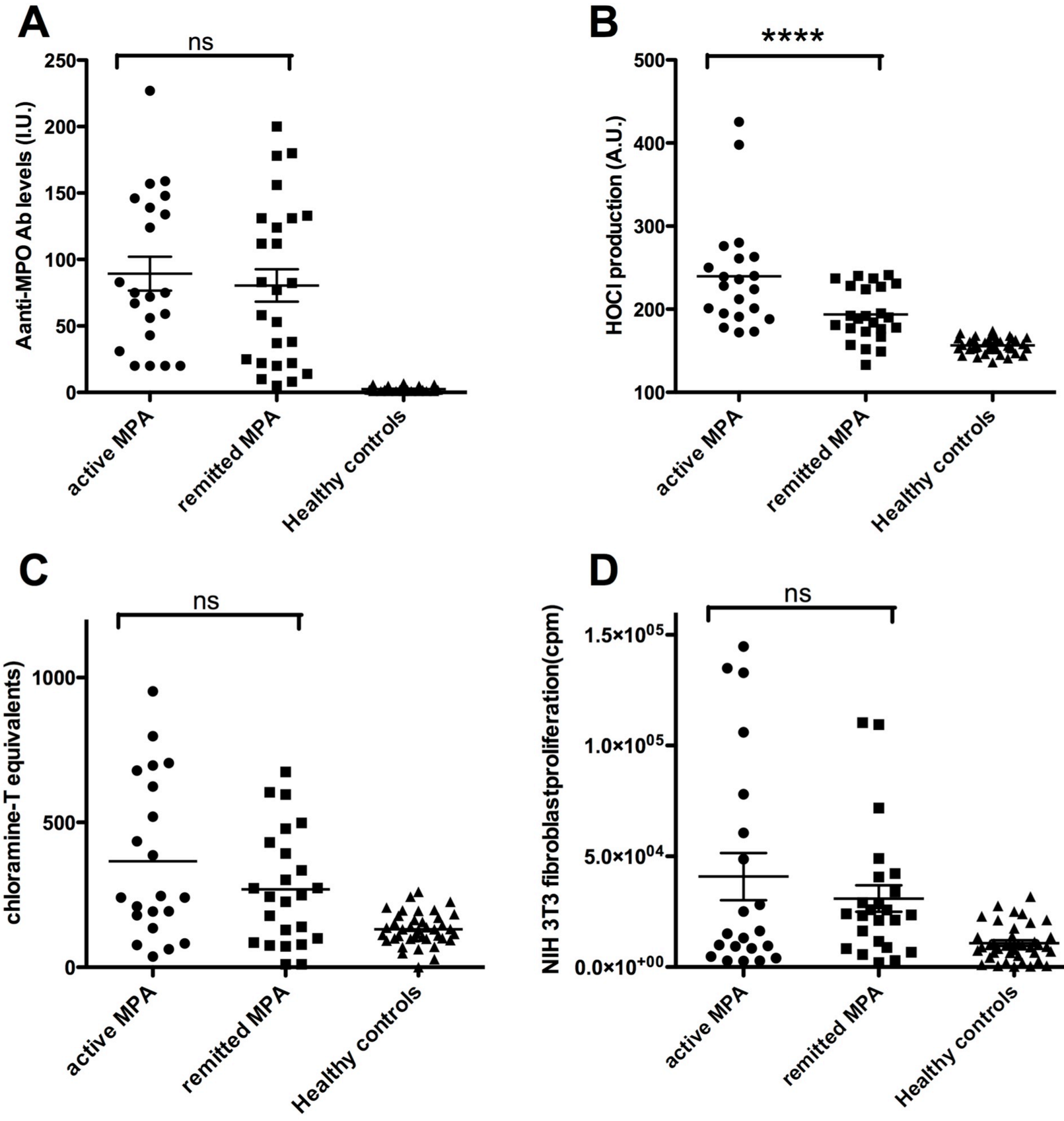
pulmonary function tests as described in the materials and methods section. (E) Total anti-oxidant activity of the serum was measured in MPA patients with and without fibrosis and healthy subjects. Data are expressed as means ±S.D. * *P* <0. 05; ** *P < 0. 01;* *** *P < 0. 001;* ****P < 0. 0001.*

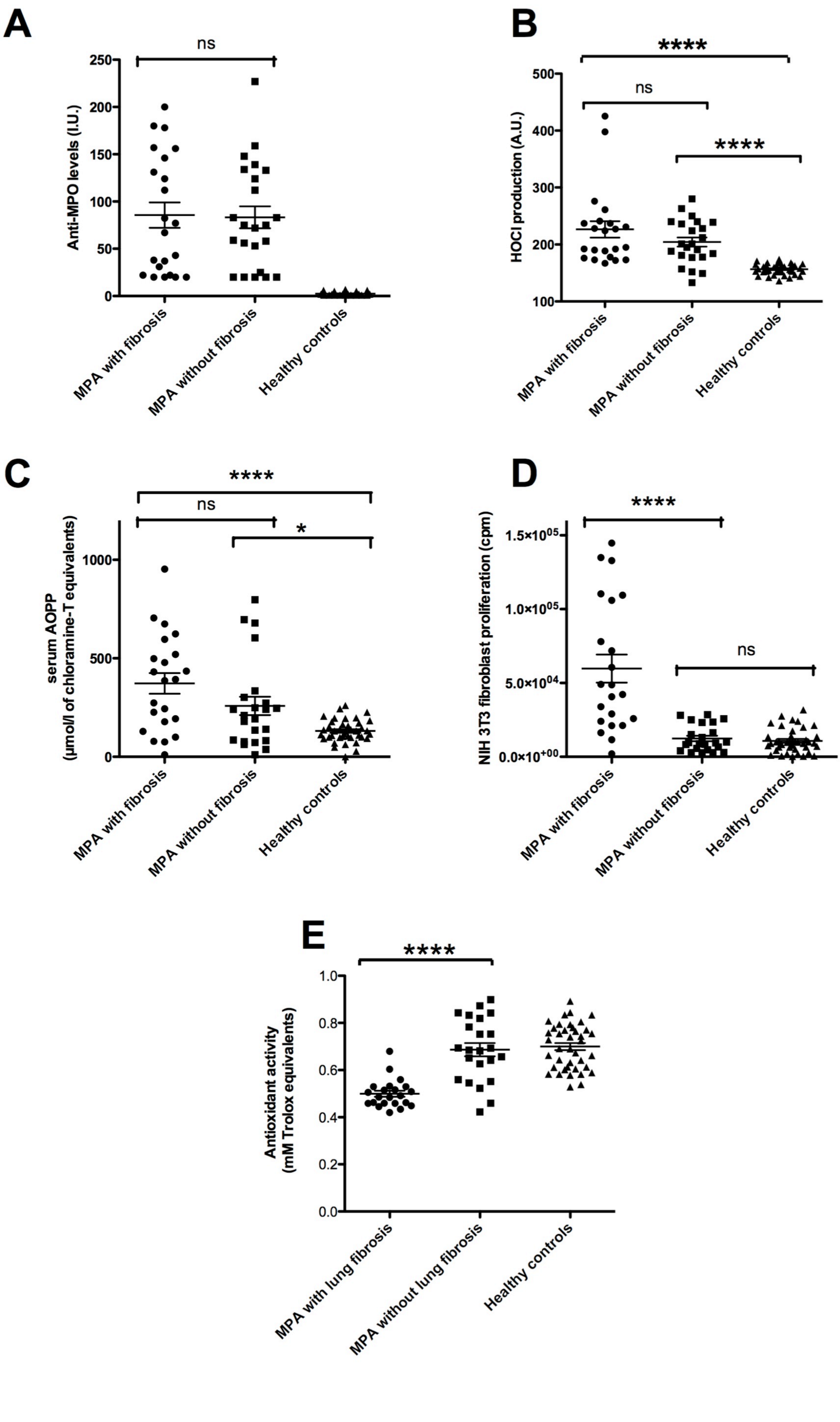
Figure 4. Correlations between anti-MPO Ab levels, HOCl production, serum AOPP levels and *in vitro* proliferation of NIH 3T3 fibroblasts in MPA associated or not with lung fibrosis. Regression analysis according to the Spearman test for paired data was used to analyze the correlations between anti-MPO Ab levels, HOCl production, serum AOPP levels and *in vitro* proliferation of NIH 3T3 fibroblasts in MPA patients with and without fibrosis.

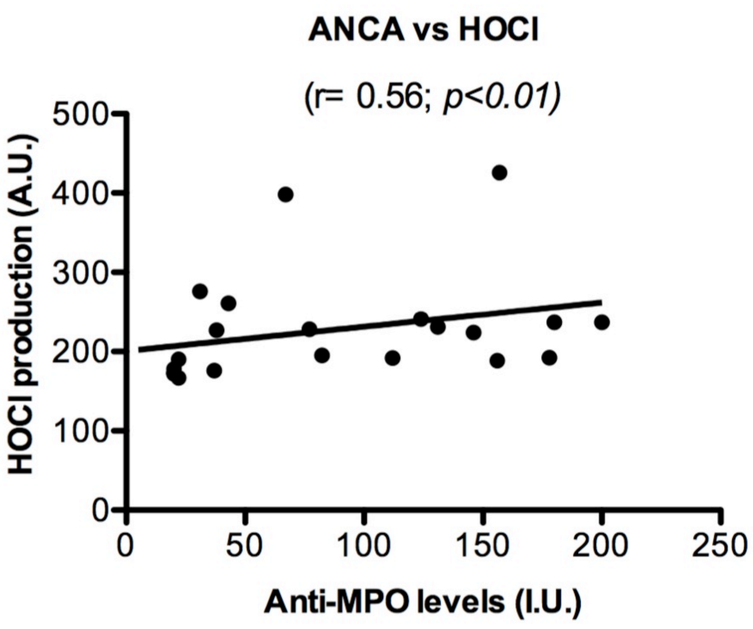
Figure 5. Relationship between MPA activity and lung fibrosis. Mean anti-MPO Ab levels (A), HOCl production by serum-activated MPO (B), serum AOPP levels (C) and serum-induced proliferation of NIH 3T3 fibroblasts (D) were assayed in 7 sera from 4 patients with active MPA and lung fibrosis, in 14 sera from 12 patients with active MPA with no lung fibrosis, in 15 sera from 6 patients with remittent MPA and lung fibrosis, in 9 sera from 8 patients with remittent MPA with no lung fibrosis. Data are expressed as means ±S.D. * *P* <0. 05; ** *P < 0. 01;* *** *P < 0. 001;* ****P < 0. 0001.*

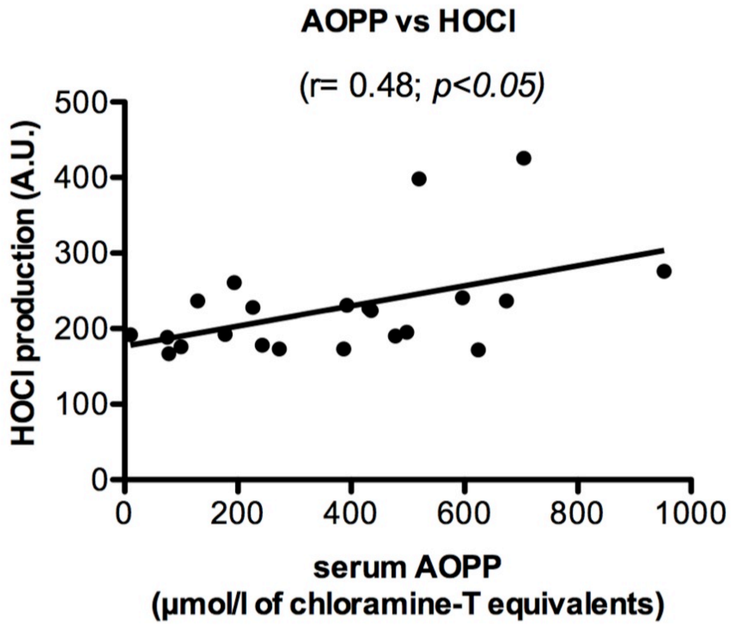
Figure 6. HOCl and AOPP modulate fibroblast proliferation *in vitro*. NIH 3T3 fibroblasts were incubated with $1.5x10^{-13}$ to $1.5x10^{-10}$ M HOCl or with AOPP (generated by oxidation of BSA with HOCl as described in the materials and methods section) for 48 hours. Fibroblast proliferation was determined by thymidine incorporation. Experiments were carried out in triplicates. Results are expressed as means ±S.D. * *P* <0. 05; ** *P < 0. 01;* *** *P < 0. 001;* ****P < 0. 0001.*

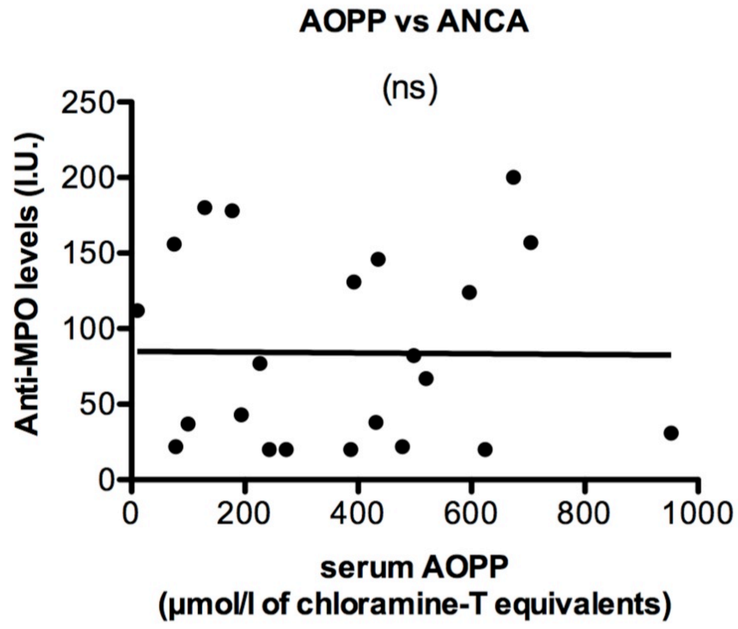

A
Anti-MPO Ab levels (I.U.)
250
200
150
100
50
0
MPA
Healthy controls
MPO-WG
PR3-WG
B
HOCl production (A.U.)
400
300
200
100
MPA
Healthy controls
MPO-WG
PR3-WG
C
AOPP (µmol/l of chloramine-T equivalents)
1000
800
600
400
200
0
MPA
Healthy controls
MPO-WG
PR3-WG
D
NIH 3T3 fibroblast proliferation (cpm)
1.5×10^05
1.0×10^05
5.0×10^04
0.0×10^+00
MPA
Healthy controls
MPO-WG
PR3-WG

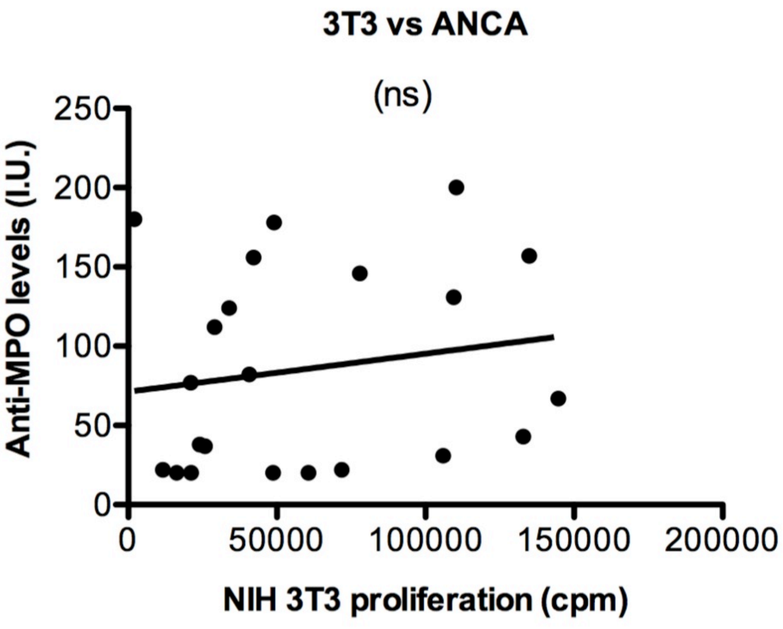

A
ns
Aanti-MPO Ab levels (I.U.)
250
200
150
100
50
0
active MPA
remitted MPA
Healthy controls
B
****
HOCl production (A.U.)
500
400
300
200
100
active MPA
remitted MPA
Healthy controls
C
ns
AOPP (µmol/l of chloramine-T equivalents)
1000
500
0
active MPA
remitted MPA
Healthy controls
D
ns
NIH 3T3 fibroblastproliferation(cpm)
1.5×10^05
1.0×10^05
5.0×10^04
0.0×10^+00
active MPA
remitted MPA
Healthy controls

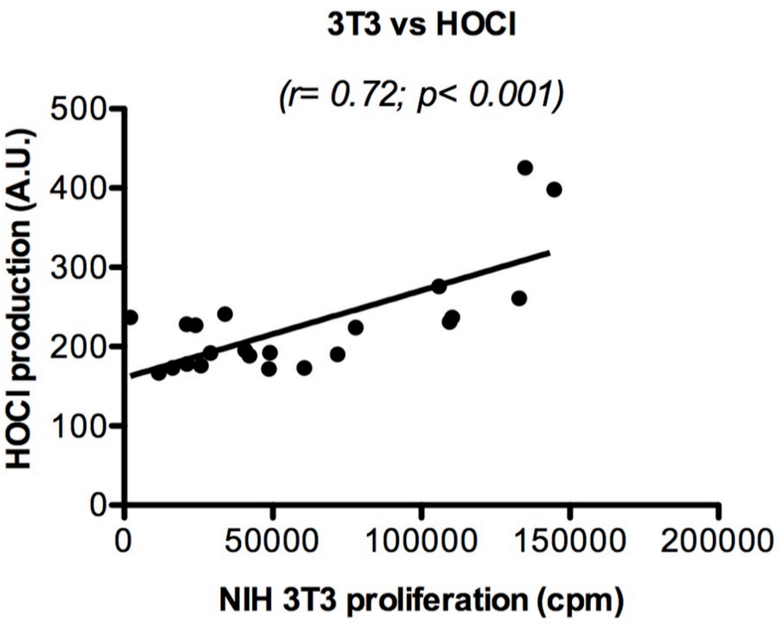

A
ns
Anti-MPO levels (I.U.)
250
200
150
100
50
0
MPA with fibrosis
MPA without fibrosis
Healthy controls
B
****
ns
****
HOCl production (A.U.)
500
400
300
200
100
MPA with fibrosis
MPA without fibrosis
Healthy controls
C
****
ns
*
serum AOPP
(µmol/l of chloramine-T equivalents)
1000
500
0
MPA with fibrosis
MPA without fibrosis
Healthy controls
D
****
NIH 3T3 fibroblast proliferation (cpm)
1.5×10^05
1.0×10^05
5.0×10^04
0.0×10^+00
ns
MPA with fibrosis
MPA without fibrosis
Healthy controls
E
****
Antioxidant activity
(mM Trolox equivalents)
1.0
0.8
0.6
0.4
0.2
0.0
MPA with lung fibrosis
MPA without lung fibrosis
Healthy controls

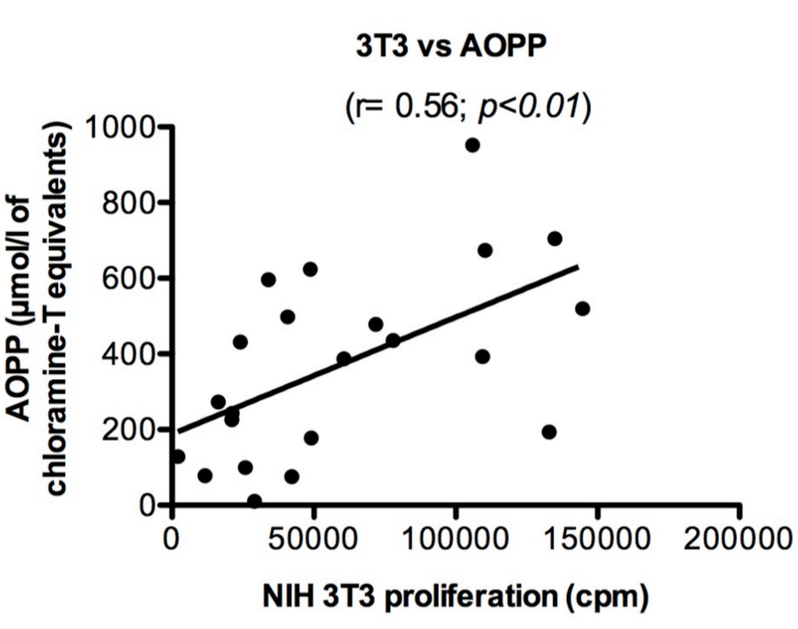

ANCA vs HOCl
(r= 0.56; p<0.01)
HOCl production (A.U.)
0
100
200
300
400
500
0
50
100
150
200
250
Anti-MPO levels (I.U.)

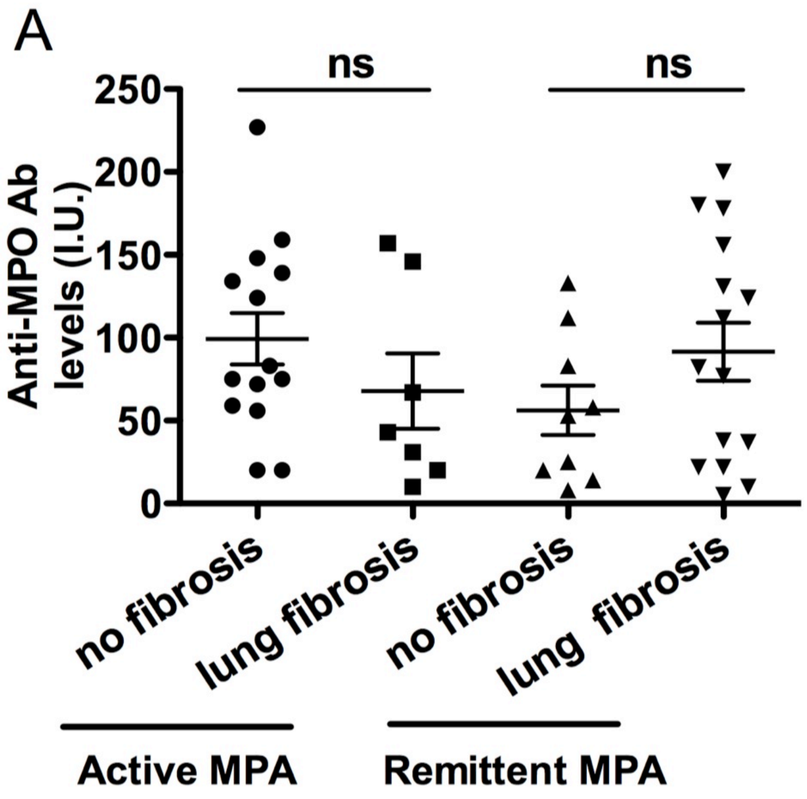

AOPP vs HOCl
(r= 0.48; p<0.05)
HOCl production (A.U.)
0
100
200
300
400
500
0
200
400
600
800
1000
serum AOPP
(µmol/l of chloramine-T equivalents)

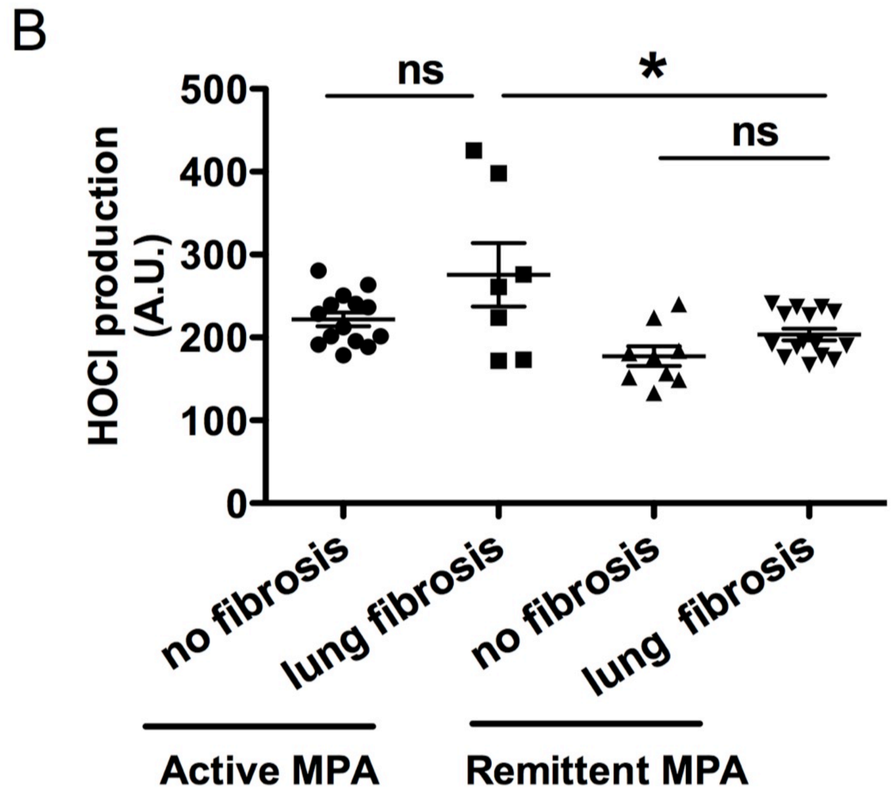

AOPP vs ANCA
(ns)
Anti-MPO levels (I.U.)
0
50
100
150
200
250
0
200
400
600
800
1000
serum AOPP
(µmol/l of chloramine-T equivalents)


3T3 vs ANCA
(ns)
Anti-MPO levels (I.U.)
0
50
100
150
200
250
0
50000
100000
150000
200000
NIH 3T3 proliferation (cpm)


3T3 vs HOCl
(r= 0.72; p< 0.001)
HOCl production (A.U.)
0
100
200
300
400
500
0
50000
100000
150000
200000
NIH 3T3 proliferation (cpm)


3T3 vs AOPP
(r= 0.56; p<0.01)
AOPP (µmol/l of chloramine-T equivalents)
0
200
400
600
800
1000
0
50000
100000
150000
200000
NIH 3T3 proliferation (cpm)


A
ns
ns
250
200
150
100
50
0
Anti-MPO Ab levels (I.U.)
no fibrosis
lung fibrosis
no fibrosis
lung fibrosis
Active MPA
Remittent MPA


B
ns
*
ns
500
400
300
200
100
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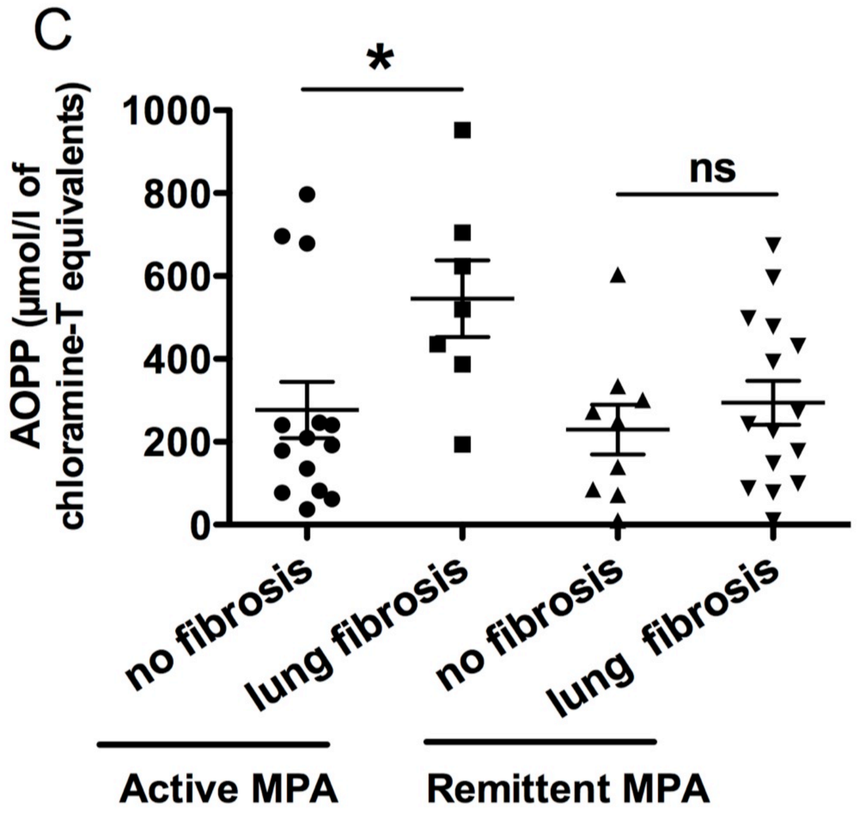
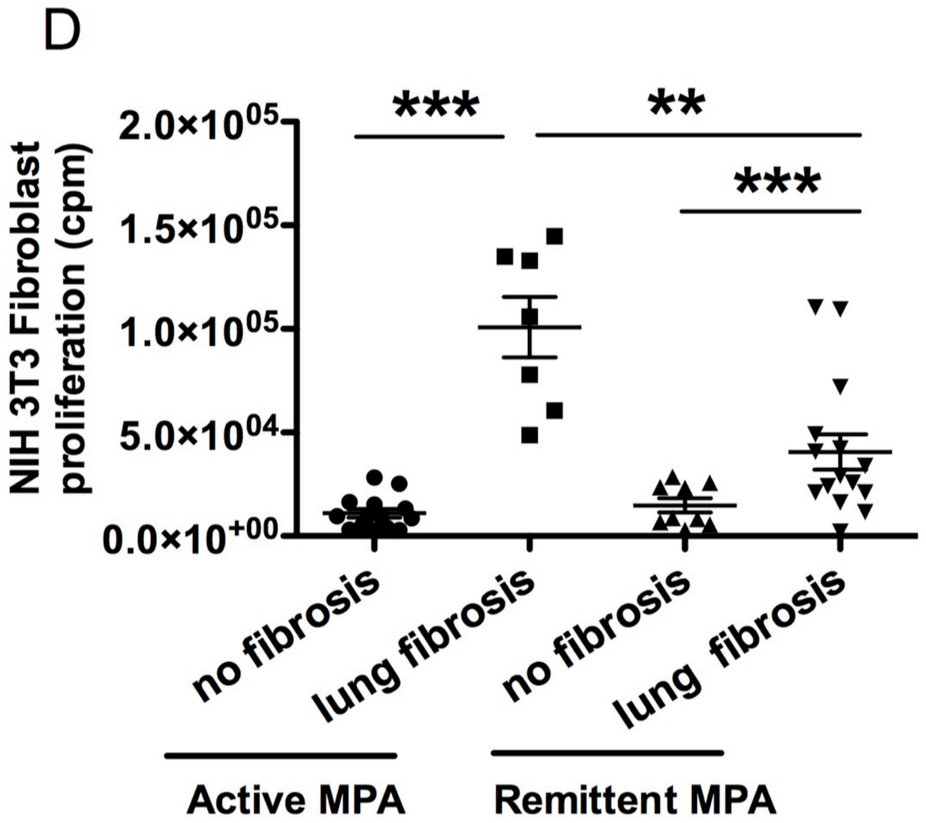
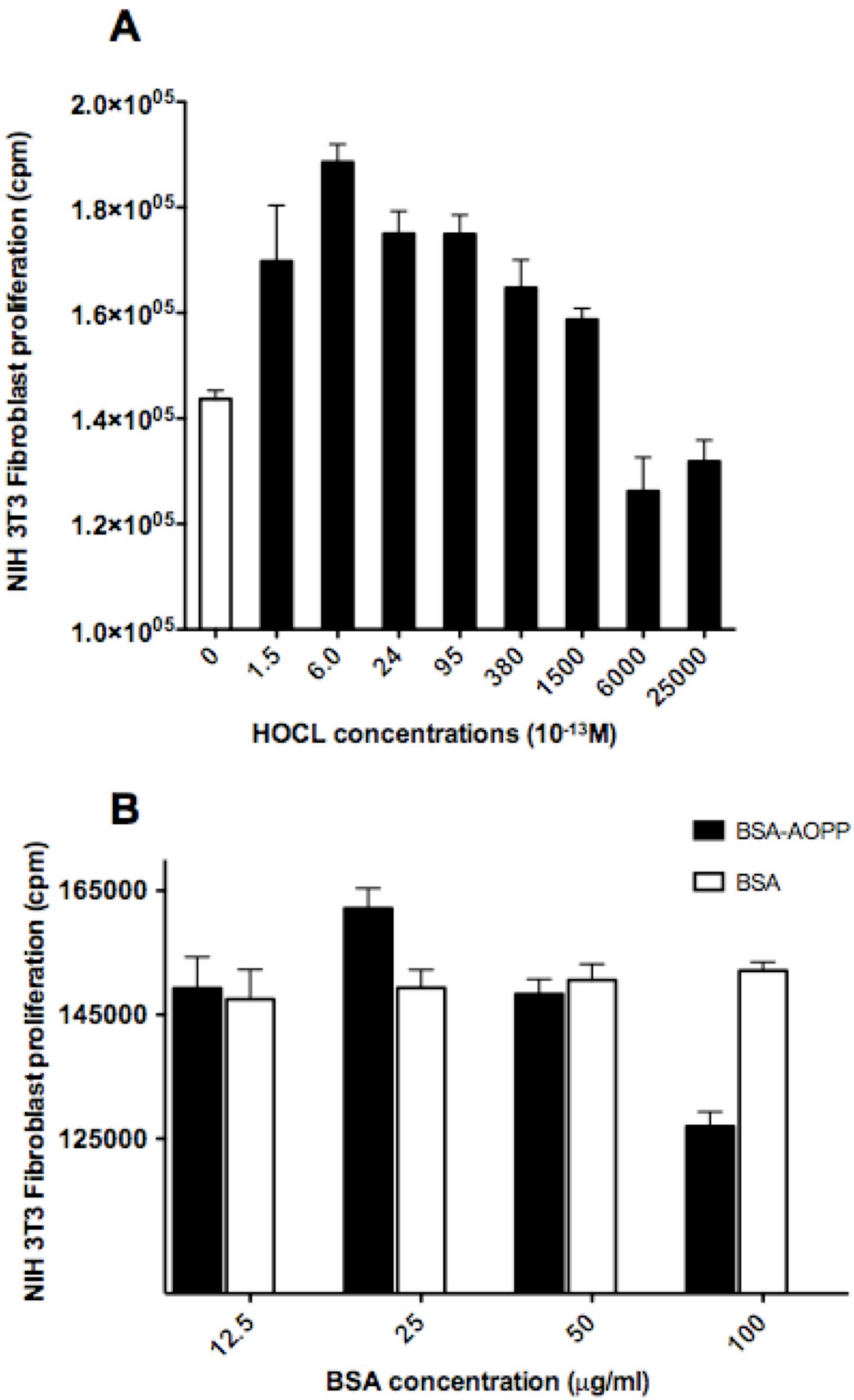
HOCl production (A.U.)
no fibrosis
lung fibrosis
no fibrosis
lung fibrosis
Active MPA
Remittent MPA


C
*
ns
1000
800
600
400
200
0
AOPP (µmol/l of chloramine-T equivalents)
no fibrosis
lung fibrosis
no fibrosis
lung fibrosis
Active MPA
Remittent MPA


D
***
**
***
2.0×10^05
1.5×10^05
1.0×10^05
5.0×10^04
0.0×10^+00
NIH 3T3 Fibroblast proliferation (cpm)
no fibrosis
lung fibrosis
no fibrosis
lung fibrosis
Active MPA
Remittent MPA


A
2.0×10^05
1.8×10^05
1.6×10^05
1.4×10^05
1.2×10^05
1.0×10^05
NIH 3T3 Fibroblast proliferation (cpm)
0
1.5
6.0
24
95
380
1500
6000
25000
HOCL concentrations (10^-13M)
B
BSA-AOPP
BSA
165000
145000
125000
NIH 3T3 Fibroblast proliferation (cpm)
12.5
25
50
100
BSA concentration (μg/ml)