

# S100A8, S100A9 and S100A12 Expression in myeloid cells from whole blood from patients with common lung diseases

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## Abstract

**Introduction or Hypothesis:** The myeloid-associated calgranulins, S100A8, S100A9 and S100A12 are associated with inflammatory lung diseases and S100A9 is proposed as a possible prognostic marker in non-small cell lung cancer (NSCLC). We proposed that expression patterns of the calgranulins may discriminate between healthy persons and those with lung disease and/or cancer.

**Methods:** We compared S100A8, S100A9 and S100A12 expression in CD14<sup>+</sup>CD11b<sup>+</sup> monocytes and CD14<sup>-</sup>CD11b<sup>+</sup> granulocytes in whole blood from patients with lung diseases (NSCLC, chronic obstructive pulmonary disease (COPD)), with their expression in the corresponding controls (smokers and non-smokers) using flow cytometry. S100A8, S100A9 and S100A12 levels serum were measured by enzyme-linked immunosorbent assays (ELISAs); nitrate/nitrite (NO<sub>x</sub>) concentrations in serum and exhaled breath condensate (EBC) were quantitated using a modified fluorometric assay based on the Griess method.

**Results:** S100A9 was more abundant than S100A8 or S100A12 in granulocytes, but S100A9 levels in monocytes and granulocytes were not significantly different. S100A8 levels in monocytes were significantly lower than in granulocytes in all groups except in patients with NSCLC. We identified a subset of granulocytes, differentiated by high/low expression of CD11b. Except for S100A12, there was significantly more S100 expression in CD11-high populations; CD11b-low granulocytes from smokers or patients with lung cancer contained significantly less S100A12 than CD11b-high cells. Serum S100A8, S100A9 and S100A12 concentrations were similar in all groups, but serum from patients with NSCLC contained significantly higher NO<sub>x</sub> levels than samples from non-smokers ( $9.8 \pm 1.5$  vs  $6.9 \pm 0.78$   $\mu$ M respectively,  $p=0.0019$ ) while no differences were found in EBC.

**Conclusions:** Calgranulin levels in granulocytes and monocytes from patients with lung diseases did not discriminate between groups although uncharacteristically, S100A8 levels in granulocytes and monocytes from patients with NSCLC were similar. Serum NO<sub>x</sub> was increased in NSCLC and may be associated with MDSC, but roles for the S100 proteins in terms of pathogenesis, or distinguishing between the various pulmonary diseases need further assessment.

## Introduction

The S100 proteins known as calgranulins, S100A8, S100A9 and S100A12, are constitutively expressed in neutrophils and monocytes, and are generally associated with inflammatory processes [1,2]. In lung diseases, S100A8/S100A9 have been suggested as a potential surrogate marker of disease severity in human tuberculosis and proposed to mediate chronic airway inflammation and remodelling in asthma [3,4]. There are relatively few studies investigating levels of the individual proteins, although S100A8 is elevated in sputum from groups of patients with bronchiectasis and cystic fibrosis [5]. S100A12 is also significantly higher than S100A8/S100A9 in lung lavage fluid from patients with the acute respiratory distress syndrome (ARDS) [6]. Sputum from patients with eosinophilic asthma contains elevated S100A12 concentrations and S100A12 is possibly a marker of sepsis-related lung injury, and a prognostic marker of idiopathic pulmonary fibrosis [7-10]. In lung-associated cancers, S100A8 is elevated in lavage fluid from patients with adenocarcinoma and expression appears related to cancer progression [11]. Murine models implicate S100A8 in the establishment of a pre-metastatic niche in the lung while S100A9 over-expression in lesions from patients with non-small cell lung cancer (NSCLC) is associated with a poor prognosis [12,13]. On the other

hand, low expression of S100A8 and S100A12 in tissue sections from patients with oropharyngeal squamous cell carcinoma was associated with decreased overall survival [14]. We recently identified novel anti-inflammatory properties of S100A8. It induces interleukin-10 (IL-10) in airway lining epithelial cells and, like glucocorticosteroids, completely suppressed leukocyte recruitment in acute lung injury (ALI) [15]. Moreover, S100A8 is a potent scavenger of NO, ROS and hypochlorous acid (HOCl) and these properties contribute to its anti-inflammatory effects in ALI, and acute asthma in mice [16-18]. These seemingly paradoxical properties of S100A8 may be dependent on the cell types affected within the microenvironment, its concentration and its anti-oxidant properties that promote post-translational modifications that may act as switches that regulate function [16,19].

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Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immunoregulatory myeloid cells that moderate ongoing inflammation and consist of myeloid progenitors, macrophage precursors, granulocytes, and dendritic cells (DC) and can be found at tumour sites [20]. Human MDSC express the common myeloid markers CD33 and CD11b, but not markers of more mature myeloid or lymphoid cells [21]. MDSC dampen innate and adaptive immunity by suppressing natural killer (NK) cell cytotoxicity and CD4<sup>+</sup> Th1 helper T cell or CD8<sup>+</sup> cytotoxic T lymphocyte activation, partially by depleting L-arginine and cysteine and increasing toxic nitric oxide (NO) levels via regulation of inducible nitric oxide synthase (iNOS), arginase 1 (ARG1) and generation of reactive oxygen species (ROS) [22-24].

S100A9 overexpression promoted MDSC accumulation in peripheral blood and inhibited macrophage and dendritic cell differentiation [25]. Although reportedly associated with immunological abnormalities in cancer, MDSC also modulate immunosuppression in chronic inflammatory conditions, including asthma-related airway inflammation [26]. Interestingly, passive transfer of tumour-derived MDSC restored the Type-2 helper T (Th2) cell balance to normal in murine asthma and suppressed recruitment of inflammatory cells into lungs of antigen-challenged animals, prolonged corneal allograft survival and reduced graft-versus-host disease, indicating important roles for these cells in normal homeostasis [27-29].

S100A9 is proposed as a marker of MDSC in the circulation of patients with certain cancers including NSCLC, where over-expression in monocytes is associated with a poor prognosis and reduced responses to chemotherapy [21,30]. Feng et al. found that peripheral blood mononuclear cells (PBMC) from these patients expressed a higher frequency of S100A9-positive cells, intracellular levels were elevated, and S100A8 was also increased compared to normal donors. S100A9 levels also correlated with the suppressive capacity of CD11b<sup>+</sup>CD14<sup>+</sup> MDSCs in NSCLC and reduced responses to chemotherapy [31]. Supporting these observations, immunohistochemistry indicated increased S100A8 and S100A9 expression in NSCLC tissue compared to adjacent and peripheral lung tissue [32]. More recently, following the discovery of monocytic CD14<sup>+</sup>IL-4Rα<sup>+</sup> and CD11b<sup>+</sup>CD14<sup>+</sup>HLA-DR<sup>low/neg</sup> MDSCs in the circulation of patients with colon cancer and melanoma respectively, a population of monocytic CD11b<sup>+</sup>CD14<sup>+</sup>CD15<sup>low/neg</sup>CD33<sup>+</sup>S100A9<sup>+</sup> cells were linked to NSCLC. S100A8, S100A9 and S100A12 were found specifically in CD14<sup>+</sup> HLA-DR<sup>low</sup> MDSC, and increased numbers of CD14<sup>+</sup> cells containing S100A9 were reported in blood from patients with colon cancer [21,31,33-35].

Here we assessed S100A8, S100A9 and S100A12 levels in CD11b<sup>+</sup>CD14<sup>+</sup> monocytes and CD11b<sup>+</sup>CD14<sup>+</sup> granulocytes in whole blood and serum from patients with NSCLC, COPD, and smokers and non-smokers without these diseases to test the hypotheses that the calgranulins would differ between diseases and cell type. Intensity of S100A9 expression was higher than that of S100A8 and S100A12. S100A8 expression in granulocytes was higher than in monocytes, whereas S100A9 and S100A12 levels were generally similar. However, in contrast to the other groups, granulocytes and monocytes from patients with NSCLC contained similar levels of S100A8 whereas serum levels of S100A8, S100A9 and S100A12 did not differ between clinical groups. We quantitated nitrate/nitrite (NOx) in serum and exhaled breath condensate (EBC) as NO can promote neoplastic growth through DNA damage, by modifying protein structures during oxidative stress, and MDSC reduce T cell activation via NO-related pathways [39,40]. Serum NOx concentrations were significantly higher in the NSCLC group compared to non-smokers.

## Materials and methods

### Subjects

Ethics approval was granted by the South Eastern Sydney Area Health Service Research Ethics Committee (SESAHS HREC12/119); all participants completed informed, written consent. The study comprised four clinical groups: subjects with newly-diagnosed untreated NSCLC, stable COPD, current smokers and non-smokers (Table 1). NSCLC was diagnosed by cytology or histopathology; COPD was diagnosed clinically together with appropriate response to bronchodilators using spirometry [41]. Smokers included subjects who regularly smoked one or more cigarettes per day [42]. Demographic and medical details were collected by questionnaire and participants with other respiratory conditions, current or recent infections, or pregnancy, excluded.

### Flow cytometry

**Whole Blood Leukocyte Staining:** Expression of intracellular S100A8, S100A9 and S100A12 was measured using a FACSCalibur cytometer (BD Biosciences, California, USA). Reactivities of rabbit anti-S100A8, anti-S100A9 and S100A12 immunoglobulins G (IgGs) prepared in-house are described by McCormick et al., and Goyette et al. [43,44]. Anti-S100A8 and anti-S100A12 IgGs did not cross-react with other calgranulins; anti-S100A9 reacted with the S100A8/S100A9 complex but not with S100A8.

Fresh venous blood was collected in heparin-coated Vacutainer tubes (BD Biosciences Pharmingen, California, USA) and 100 µl incubated in 2ml NH4Cl-containing lysis solution for 10 minutes at room temperature (RT) in the dark. Samples were centrifuged at 300x g for 5 minutes at RT then lysed erythrocytes in supernates removed. Cells were washed thrice with 2 ml 1% bovine serum albumin (BSA, Sigma-Aldrich, NSW, Australia) in phosphate-buffered saline (PBS, called wash buffer), resuspended in 200 µl wash buffer, fixed by addition of 50 µl 10% paraformaldehyde (PFA) in PBS and incubated at 4°C overnight. After washing twice with 10ml of wash buffer, cells were resuspended to a final volume of 200 µl then kept on ice for 5 minutes. After adding 1.8ml 100% methanol kept at -80°C, cells were immediately transferred to -20°C for 30 minutes. After three washes, cells resuspended in 100 µl wash buffer were stained with 10µl phycoerythrin-conjugated anti-CD14 (clone number M5E2, BD Biosciences Pharmingen, California, USA), 1 µg fluorescein isothiocyanate-conjugated mouse anti-human CD11b (clone number M1/70, BioLegend, California, USA), 1 µg of either biotin-conjugated anti-human S100A8, S100A9 or S100A12 IgG or biotin-conjugated rabbit IgG control (Molecular Innovations, Michigan, USA) and incubated for 20 minutes at RT in the dark in a final volume of 117 µl. After two washes, 5 µl of allophycocyanin-conjugated streptavidin (BD Biosciences Pharmingen, California, USA) were added and cells incubated for 20 minutes at RT in the dark, washed twice, then fixed with 300 µl 1% PFA in PBS and kept at 4°C until analysis. All staining procedures were performed within two hours of blood collection to minimise signal loss.

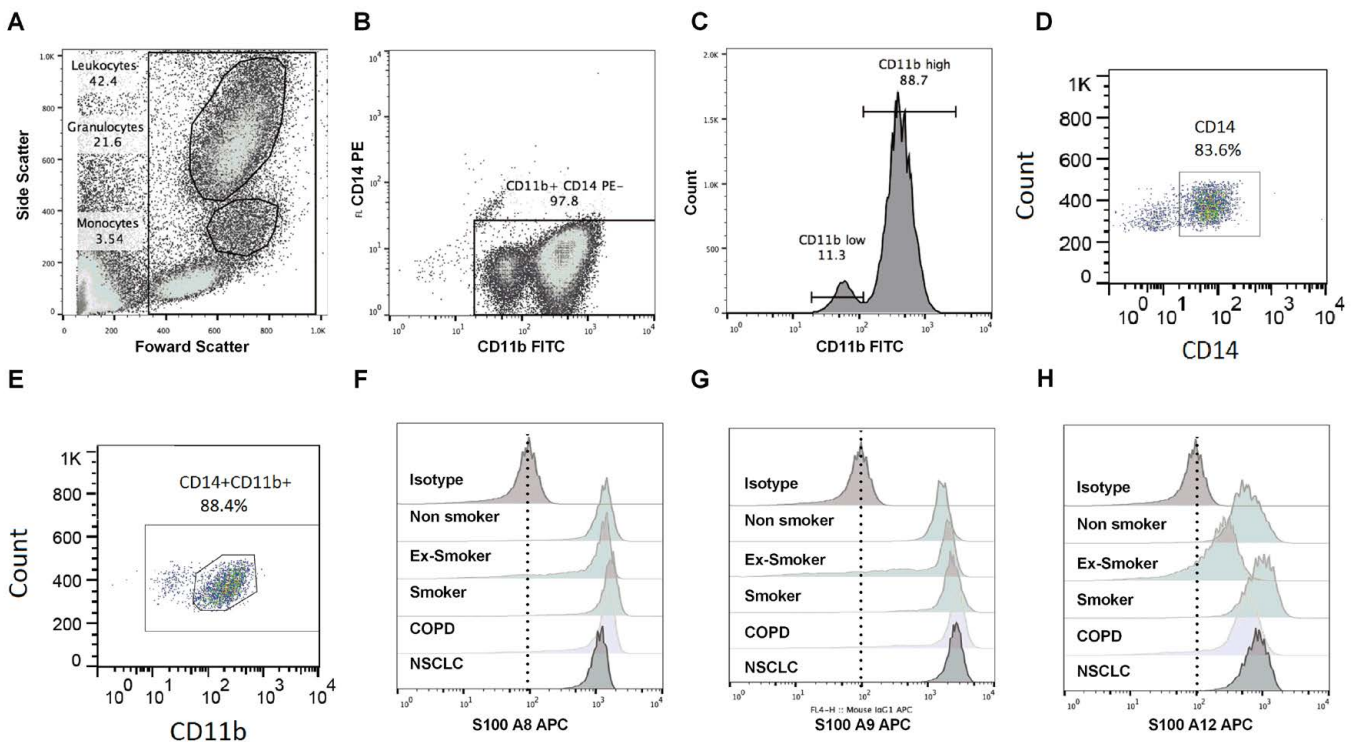
### Flow cytometric analysis

Data were acquired on a FACS Calibur (BD Biosciences, California, USA) and compensations were applied using single stained tubes. Forward and side scatter of lymphocyte, monocyte, and granulocyte populations allowed gating based on size and granularity (Figure 1A). Granulocytes comprised CD11b<sup>+</sup>CD14<sup>+</sup> gated populations (Figure 1B). Among these, subpopulations of granulocytes expressing high (CD11b<sup>hi</sup>) or low (CD11b<sup>lo</sup>) expression of CD11b were identified and

**Table 1.** Subject demographics. Age, smoking pack-years, Mean FEV<sub>1</sub>% predicted and Mean FEV<sub>1</sub>/FVC% are presented as means ± SD.

	Non-smoker	Smoker	COPD	NSCLC	p-values
<b>n</b>	14	5	12	12	NA
<b>Median age (range)</b>	55 (21-85)	43 (36-49)	67 (45-82)	68 (59-80)	0.003
<b>Female/ Male</b>	6/ 8	2/ 3	7/ 5	4/ 8	NA
<b>Median pack years (range)</b>	0	24.00 (6.50-36.25)	30.50 (0-76.00)	54.25 (0-122.50)	<0.0001
<b>Smoking (current/ former/ never)</b>	0/ 0/ 14	5/ 0/ 0	8/ 4/ 0	3/ 7/ 2	NA
<b>Mean FEV<sub>1</sub> % predicted</b>	101.90 ± 14.71	95.14 ± 17.99	59.75 ± 15.30	63.95 ± 9.64	<0.0001
<b>Mean FEV<sub>1</sub>/FVC %</b>	81.63 ± 7.17	74.61 ± 14.44	64.42 ± 9.66	59.68 ± 10.86	0.0002
<b>Inhaled corticosteroid (yes/ no)</b>	0/14	1/4	7/ 5	3/9	NA
<b>Adenocarcinoma</b>				5*	NA
<b>Large cell carcinoma</b>				1*	NA
<b>Squamous cell carcinoma</b>				4*	NA
<b>Stage I/ II/ III/ IV</b>				1/0/3/3*	NA

\*=Some data missing; NA= not applicable.



**Figure 1.** Gating strategy of flow cytometry results. Blood cells from a normal donor were used as an example. Granulocytes and monocytes were gated based on size and granularity (A). Granulocytes constituted 21.6% and monocytes 3.54% of the leukocytes gated (A). Two populations CD11b<sup>+</sup>CD14<sup>+</sup> granulocytes were identified, one with high CD11b, the other with low CD11b expression (B). Among the CD11b<sup>+</sup>CD14<sup>+</sup> granulocytes, the majority expressed high levels of CD11b (88.7%) and 11.3% were CD11b low (C).

gated accordingly (Figure 1C). For monocytes, cells were first gated for CD14 (Figure 1D). Among the CD14<sup>+</sup> monocytes, those that also expressed CD11b were considered positive (Figure 1E); Levels of calgranulin expression were measured as median fluorescence intensity (MFI), and the MFI in calgranulin-positive leukocytes was normalised using the MFI from isotype controls (Figure 1F-H). There was little variability present in the MFI for the isotype controls between groups. A minimum of 10,000 events was recorded and data analysed using FlowJo (Tree Star, Oregon, USA).

Since many studies report results in PBMC or neutrophils separated from whole blood, we compared S100 levels (MFI) in leukocytes separated by conventional density gradient methods with those obtained using whole blood staining, and found similar results in both (data not shown).

**S100A8, S100A9 and S100A12 ELISAs**

To prepare serum, blood collected in Vacutainers (BD Diagnostics) was allowed to clot for 30 minutes in a vertical position at RT then centrifuged at 1000x g for 10 minutes at RT. Serum aliquots (120 µl) separated from the clot by a gel separator were aliquoted into 1.5ml Lobind Eppendorf tubes (Sigma-Aldrich, Sydney, Australia) and stored at -80°C.

Calgranulins in sera (diluted 1:4 v/v in PBS) and EBC were quantified by sandwich ELISAs developed in-house [45]. A 96-well MaxiSorp microplate (Nunc, Denmark) was coated with 50 µl/well coating buffer (0.05M sodium carbonate, pH 9.6) containing 4µg/ml capture antibody (rabbit anti-human S100A8, S100A9 or S100A12 IgG), left at RT overnight, then wells washed three times with 300 µl 0.05% Tween 20 in PBS, blocked with 200 µl 1% skim-milk powder

in PBS at RT for one hour, then wells washed as above. After adding 100  $\mu$ l recombinant S100A8, S100A9 or S100A12 standards (3.9-250 ng/ml PBS) prepared as described, or samples, to duplicate wells, plates were incubated for two hours at RT then washed as above [46]. Detection antibody, 100  $\mu$ l (4  $\mu$ g/ml biotinylated rabbit anti-human S100A8, S100A9 or S100A12 IgG) was added, plates incubated for two hours at RT, washed, then 100  $\mu$ l streptavidin-horseradish peroxidase (HRP) (R&D Systems, USA) added to each well and plates incubated in the dark at RT for 30 minutes. After three washes, 100  $\mu$ l 3,3', 5,5'-tetramethylbenzidine (TMB, Life Technologies, Victoria, Australia) was added, plates incubated for 20 minutes in the dark at RT then the reaction stopped with 50 $\mu$ l sulphuric acid (1M) and  $A_{450nm}$  immediately read on a microplate reader (SpectraMAX, USA).

The limits of detection were 15.6ng/ml, 1.95ng/ml and 1.9ng/ml for S100A8, S100A9 and S100A12 respectively; mean intra-assay coefficients of variation were  $7.4 \pm 7.3\%$ ,  $4.7 \pm 4.7\%$  and  $2.9 \pm 2.7\%$  respectively. Concentrations of calgranulins in samples were estimated by interpolation from the standard curve.

### Exhaled breath condensate (EBC) preparation

After oral lavage with water, subjects breathed tidally for 15-20 minutes through a unidirectional valve into a non-siliconised glass condenser with a saliva trap cooled to 4°C in ice, using a validated method [47]. Aliquots of EBC (120 $\mu$ l) were immediately pipetted into 1.5ml LoBind Eppendorf tubes, de-aerated by flushing with high purity argon (BOC, New South Wales, Australia) at a flow rate of 0.4L/min for 30 seconds then stored at -80°C until analysis.

### Nitrate/nitrite (NOx) measurement

Total NOx concentrations were measured using a modified fluorometric assay based on the Griess method [48]. Standard solutions (0-60 $\mu$ M) were diluted from a stock solution of 1mM sodium nitrate using distilled water. In a 96-well plate, 50 $\mu$ l duplicate standard solutions or subject samples were mixed with nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide and nitrate reductase (Sigma-Aldrich, Australia) to final concentrations of 50 $\mu$ M, 5 $\mu$ M or 50IU/L respectively, then incubated at 37°C for 1 hour; 2, 3-diaminonaphthalene (DAN (0.05mg/ml), 10 $\mu$ l in 0.62M HCl added and plates incubated for 10 minutes in the dark. Reactions were terminated with 10 $\mu$ l 2.8mM NaOH then reactivity read using a Cytofluor 4000 plate reader (Applied Biosystems, Victoria, Australia), excitation 360/40 nm, emission 395/25 nm and gain of 50. The limit of detection was 1.25 $\mu$ M; mean intra-assay coefficient of variation  $6.5 \pm 5.5\%$ ; nitrate concentrations were estimated by interpolation from the standard curve.

### Statistical tests

Statistical analyses were performed using SPSS Statistics version 21 (IBM, New York, USA) and GraphPad Prism version 6 (GraphPad Software, California, USA). The significant probability level was set at  $p < 0.05$  and data presented as mean  $\pm$  SD. The normality of the distributions was tested by the D'Agostino & Pearson omnibus normality test. Differences between groups were tested using Kruskal-Wallis or Mann-Whitney tests followed by Dunn's multiple comparison test.

## Results

### Subject demographics

A total of 43 patients were included in this study and the

demographics are summarised in table 1. Smokers were younger than COPD and NSCLC groups as lung disease was an exclusion criterion. As expected, those with COPD and NSCLC had smoked significantly more cigarettes than other groups. Not all subjects were able to provide sufficient EBC or blood for analysis.

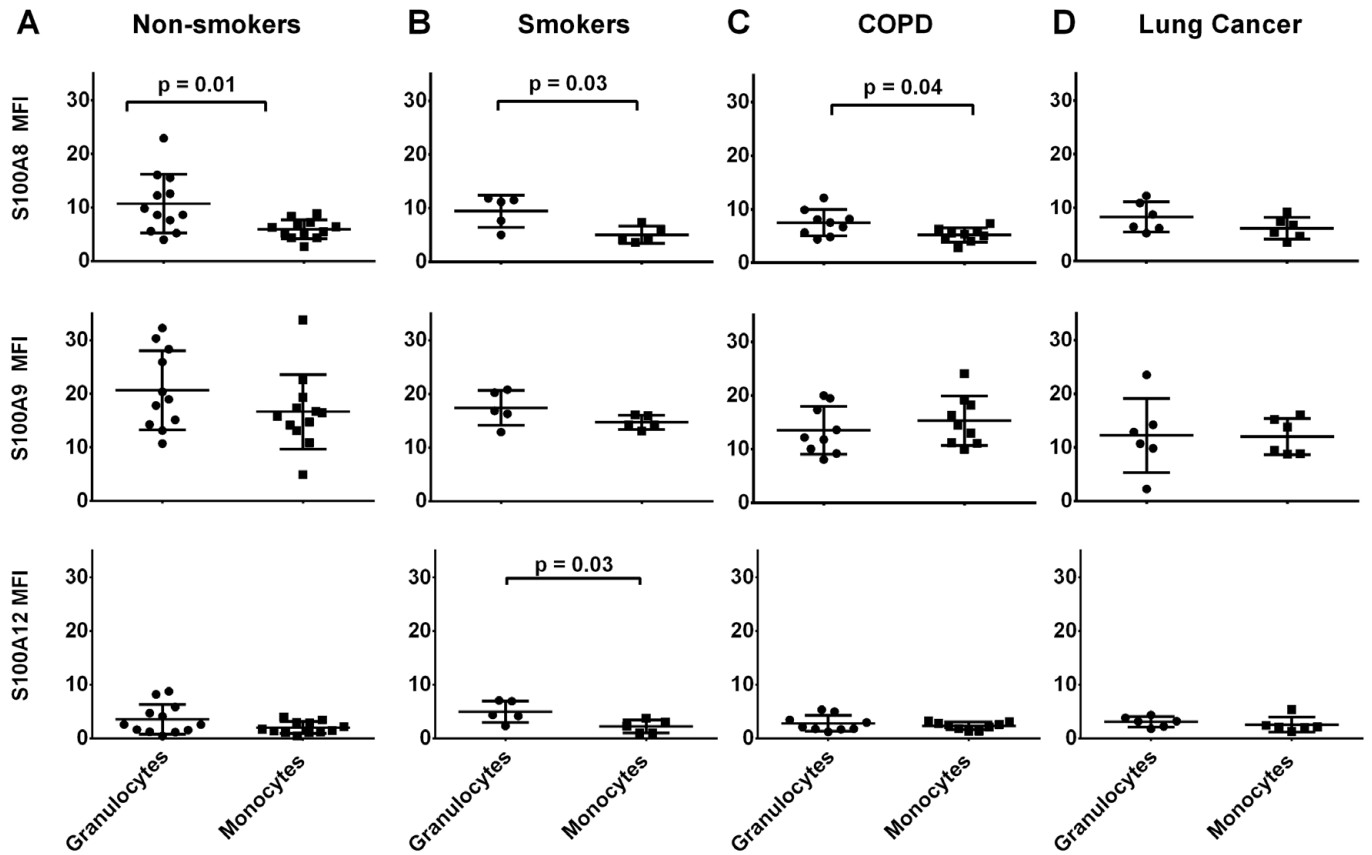
### A8, A9 and A12 expression in whole blood monocytes and granulocytes

Flow cytometry analyses of S100A8, S100A9 or S100A12 expression in monocytes and granulocytes in whole blood samples were performed using samples from 37 individuals (12 non-smokers, 5 smokers, 9 with COPD and 6 with NSCLC) using gating strategies shown in figure 1. Figure 1C shows a typical plot with separation of CD11b<sup>+</sup>CD14<sup>-</sup> granulocytes; two populations with distinct differences in brightness of anti-CD11b reactivity (CD11b<sup>+</sup> high or low) were separated; the CD11b<sup>lo</sup> population comprised 11.3% of the total. S100 expression in these cells was measured as MFI in the various populations.

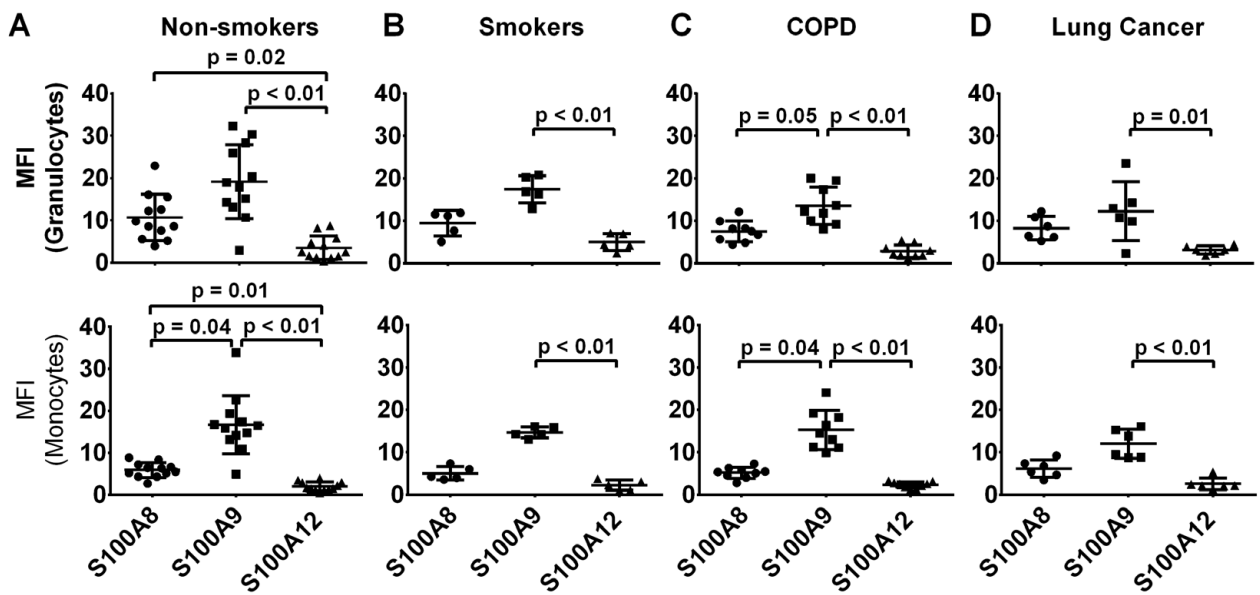
We also tested the differences between age and expression of S100A8, S100A9 and S100A12. Non-smoker subjects were divided into <45 years old and >45 years old and tested for levels of calgranulins. There was no association between age and levels of calgranulins expressed ( $p = 0.57, 0.29, 0.15$  for S100A8, S100A9 and S100A12 in granulocytes;  $p = 0.81, 0.81, 0.11$  for S100A8, S100A9 and S100A12 in monocytes respectively).

Figure 2 shows of reactivity of anti-S100A8, S100A9 and S100A12 antibodies in leukocytes from each clinical group. S100A8 levels in monocytes were significantly lower (approximately half) than in granulocytes from non-smokers ( $p = 0.01$ ). Significant differences were also observed in granulocytes from smokers ( $p = 0.03$ ) and patients with COPD ( $p = 0.04$ ) whereas there was no significant difference in S100A8 levels in granulocytes compared to monocytes from patients with lung cancer (Figure 2B-D). S100A9 levels in granulocytes from all clinical groups were 1.8-fold, and in monocytes 2.7-fold higher than S100A8 (Figure 2). However, S100A9 levels tended to be lower in granulocytes from patients with COPD and NSCLC compared to those from non-smokers (Figure 2A,2C-D). Only monocytes from smokers expressed significantly less S100A12 than granulocytes ( $P = 0.03$ ) (Figure 2B).

The relative abundance of S100A8, S100A9 and S100A12 in granulocytes and monocytes from all clinical groups was compared. Among the granulocytes and monocytes, there were no significant differences in the expression levels of S100A8, S100A9 and S100A12 between various clinical groups ( $p = 0.61$  and  $p = 0.57$  for granulocytes and monocytes respectively). In granulocytes from non-smokers, S100A12 was significantly less abundant than S100A8 ( $p = 0.02$ ) or S100A9 ( $p < 0.01$ ) whereas no significant differences in amounts of S100A8 and S100A9 were found between these cells (Figure 3A). In contrast, S100A9 levels in monocytes from this group were significantly higher than those of S100A8 (2.8-fold;  $p = 0.04$ ) or S100A12 (8.4-fold;  $p < 0.01$ ). Differences in S100A9 and S100A12 expression levels in granulocytes from smokers ( $p < 0.01$ ), or those with COPD ( $p < 0.01$ ) or NSCLC ( $p = 0.01$ ) remained significantly different (Figure 3B-D). The significant differences seen in S100A8 and S100A12 levels in granulocytes could not be replicated in smoking ( $p = 0.41$ ), COPD ( $p = 0.048$ ) and NSCLC ( $p = 0.06$ ) groups, although there was a strong trend. These comparisons were perhaps underpowered to detect a true difference with multiple comparisons, but were significant if unadjusted unpaired t-tests were used (estimated minimum sample size, 1-beta 0.8, alpha 5%,  $n = 16, n = 6$  and  $n = 6$ , respectively). Only monocytes from non-smokers and patients with COPD had significantly more S100A9



**Figure 2.** S100 protein expression in monocytes was lower than in granulocytes. In granulocytes from healthy donors, the MFI of anti-S100A12 reactivity was significantly less than seen for anti-S100A8 or S100A9 ( $p=0.02$  and  $p<0.01$  respectively). No significant differences were seen between S100A8 and S100A12 levels in granulocytes from the clinical groups, although S100A9 levels were significantly higher. Monocytes from these groups reflected similar differences. Levels of S100A9 were significantly higher than S100A8 ( $p=0.01$ ) in monocytes from non-smokers and those with COPD. \*MFI value are normalised to isotype control.



**Figure 3.** Levels of S100A8 were significantly higher in granulocytes than monocytes in all clinical groups except for those with lung cancer group ( $p=0.01$ ,  $p=0.03$  and  $p=0.04$  respectively). In contrast, S100A9 and A12 levels in granulocytes and monocytes were similar in all clinical groups except for smokers where the intensity of anti-S100A12 reactivity in granulocytes was significantly higher than reactivity in monocytes ( $p=0.03$ ). \*MFI value are normalised to isotype control.

than S100A8 ( $p=0.04$  for both), even though a trend was also observed in smokers ( $p=0.19$ ) and NSCLC ( $p=0.19$ ) group, likely underpowered (minimum sample size for unadjusted comparisons both  $n=5$  for both). In contrast, differences in levels of S100A9 and S100A12 remained statistically significant in smokers and those with COPD or NSCLC ( $p<0.01$  for the 3 groups, Figure 3A-D).

We next assessed whether potential differences in S100 expression levels in the CD11b<sup>+</sup> CD14<sup>+</sup> neutrophil subpopulations may identify a particular clinical group. Among the CD11b<sup>hi</sup> and CD11b<sup>lo</sup> subpopulations, there were no significant differences in the expression levels of S100A8, S100A9 and S100A12 when compared between the various clinical groups ( $p=0.84$ ,  $p=0.67$  respectively). Figure 4A shows that among the individual clinical groups, the CD11b<sup>hi</sup> subpopulation contained similar S100 levels to the total granulocyte population (Figure 3A), and significant differences in S100A9 and S100A12 reactivity in this subpopulation were seen in all groups (Figure 4B-D). In contrast, significantly higher amounts of S100A9 compared to S100A12, were only found in the CD11b<sup>lo</sup> subpopulation from non-smokers or patients with COPD (Figure 4A, C,  $p=0.01$ ,  $p<0.01$ ). S100A8 levels were similar in these cells from all clinical groups

CD11b<sup>hi</sup> granulocytes contained significantly more S100A8 (2.6-fold in cells from non-smokers,  $p<0.01$ ; 2.5-fold in cells from patients with NSCLC,  $p=0.02$ ) and S100A9 (3.2-fold in cells from non-smokers,  $p<0.01$ ; 2.6-fold in cells from patients with NSCLC,  $p=0.04$ ) than the CD11b<sup>lo</sup> subpopulation from all subjects (Figure 5). In contrast, amounts of S100A12 were similar in both subpopulations of granulocytes from non-smokers and those with COPD, but lower in CD11b<sup>lo</sup> granulocytes from smokers or those with lung cancer.

**S100A8, S100A9 and S100A12 in serum**

A8, A9 and A12 were detected in serum from the 38 subjects (12 non-smokers, 5 ex-smokers, 5 smokers, 9 with COPD and 7 with NSCLC) but no statistical differences in levels were found between the

groups (overall mean serum levels of S100A8, S100A9 and S100A12 were 78.2, 55.6 and 73.0 ng/ml respectively,  $p=0.48$ , 0.53, and 0.42 for A8, A9 and A12, Kruskal-Wallis, Figure 6A-C).

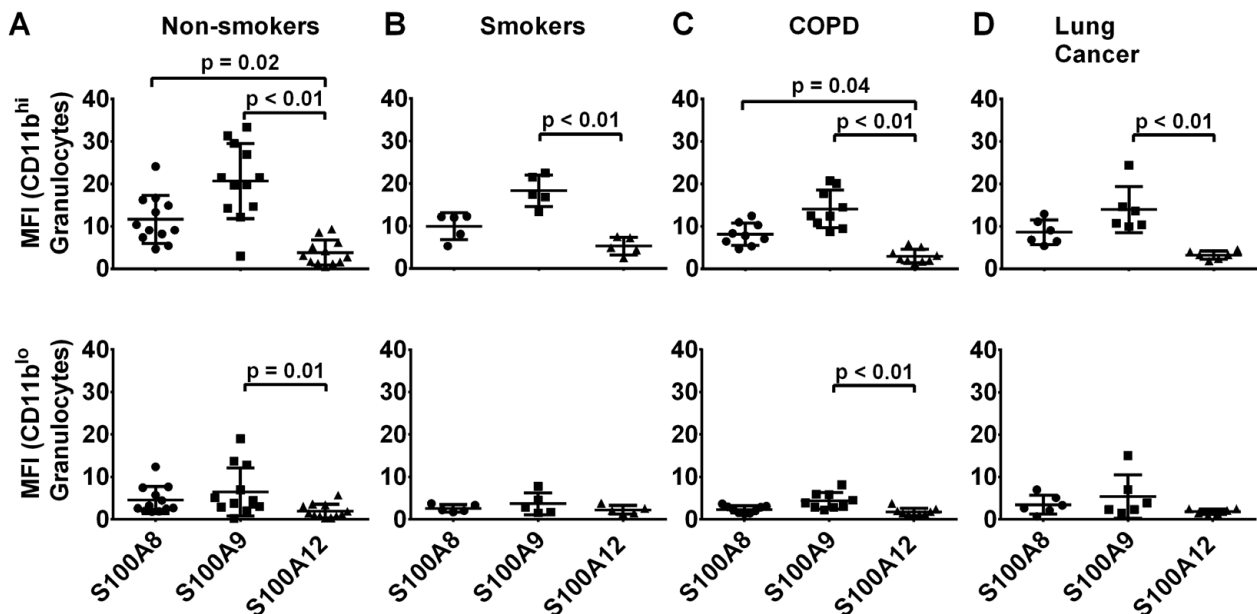
**NOx in Serum and EBC**

NOx was measured in 27 serum samples (8 non-smokers, 5 smokers, 7 with COPD and 7 with NSCLC). Serum from subjects with NSCLC contained significantly more NOx ( $9.8 \pm 1.5 \mu\text{M}$ ) than serum from non-smokers ( $6.9 \pm 0.8 \mu\text{M}$ ,  $p=0.0003$ , Mann Whitney test,) but not more than in samples from smokers or subjects with COPD (Figure 6D).

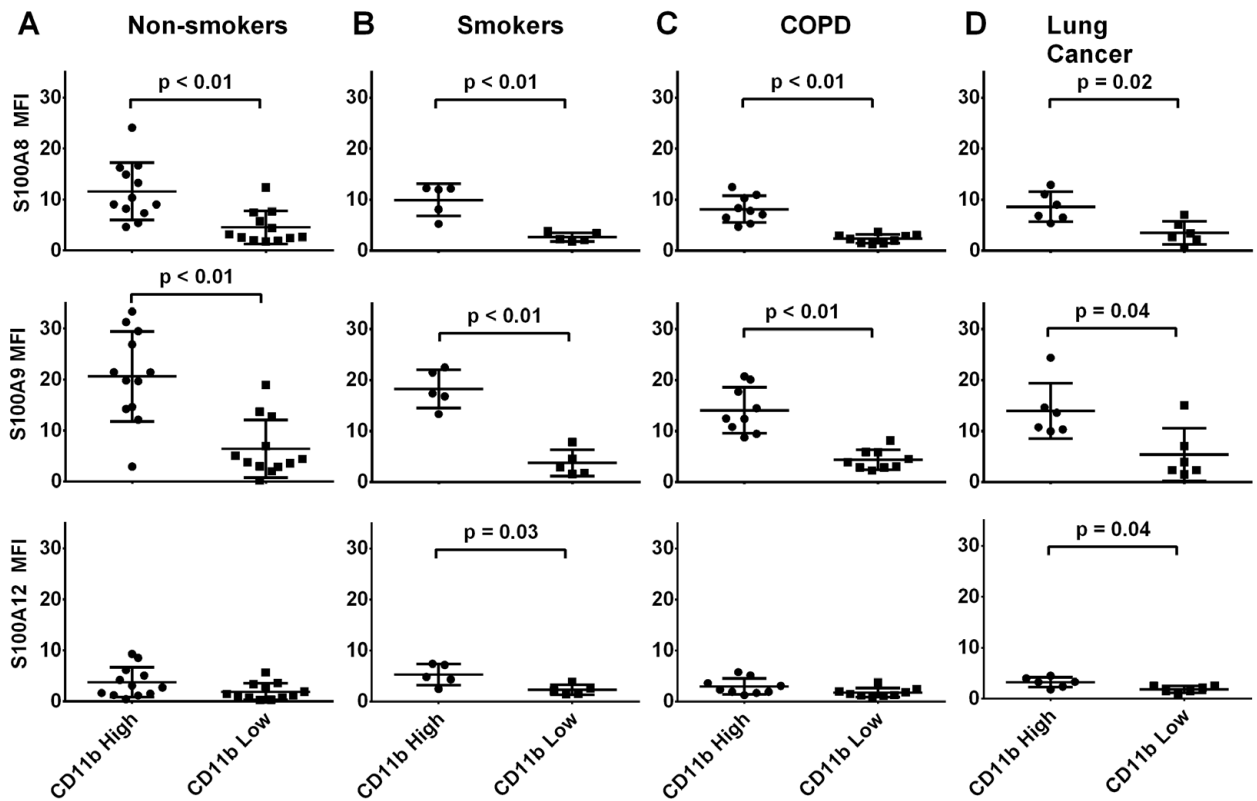
NOx was detected in the 32 EBC samples tested (11 non-smokers, 4 smokers, 10 with COPD and 7 subjects with NSCLC). No significant differences were found between the groups ( $p=0.91$ , one-way ANOVA, Figure 6E).

**Discussion**

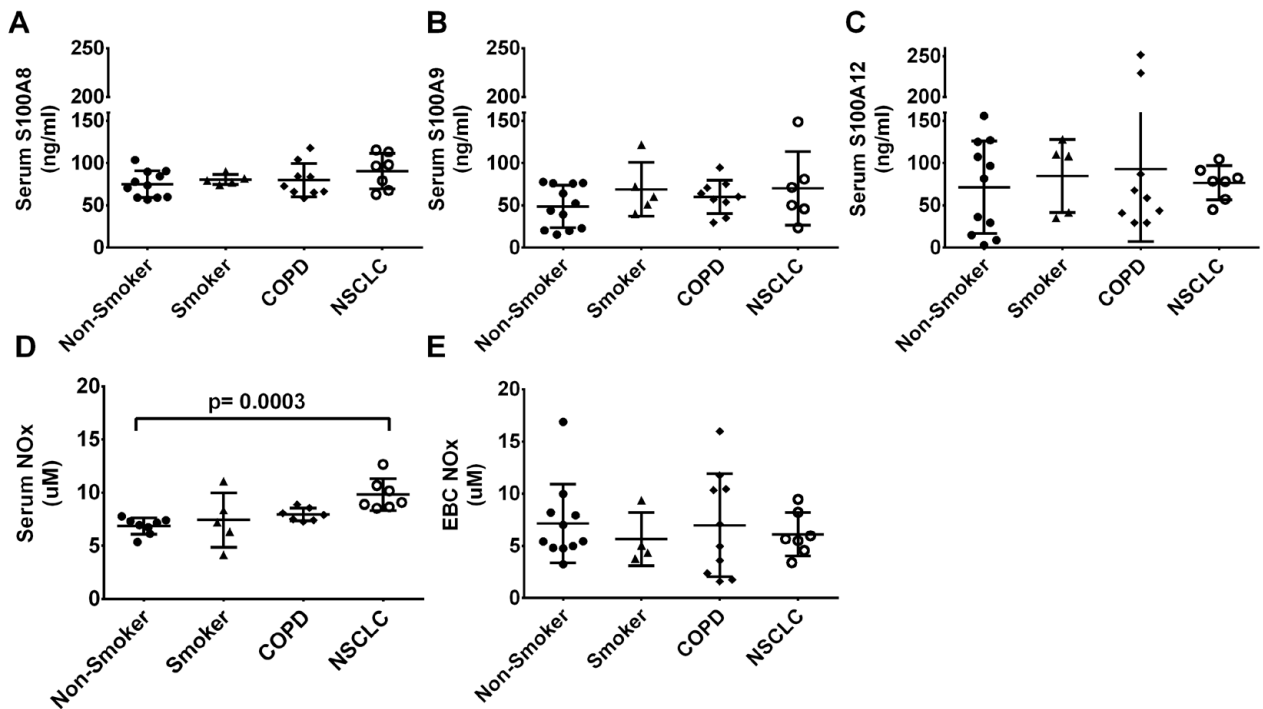
S100A8 and S100A9 are abundantly expressed in blood neutrophils and monocytes; in neutrophils, the S100A8/A9 complex is estimated to comprise ~45% of total cytosolic proteins, and in monocytes, around 1% [49]. S100A12 expression is less, and mainly in granulocytes, with low levels in monocytes, that may represent a later stage of maturation [50]. Our earlier studies found some 4-fold more constitutive S100A9 mRNA than S100A8 mRNA in human neutrophils; expression of both genes in monocytes was approximately 20-fold less. Both cell types contained substantially less S100A12 mRNA [51]. The calgranulins are not expressed in lymphocytes [52-54]. These proteins are also expressed in monocytic and granulocytic MDSC, although characterisation of the three calgranulins in these subpopulations has been scant. One study using flow cytometry identified the proteins in CD14<sup>+</sup>HLA-DR<sup>low</sup> cells and the authors suggested that CD14<sup>+</sup>S100A9<sup>+</sup> MDSC from peripheral blood differentiated between healthy donors and those with colon cancer, on the basis of concentrations of S100A9 [21]. Based on this, we compared calgranulin expression in CD11b<sup>+</sup> CD14<sup>+</sup> and CD11b<sup>lo</sup>



**Figure 4.** Comparisons of S100A8, S100A9 and S100A12 levels in CD11b<sup>hi</sup>CD14<sup>+</sup> and CD11b<sup>lo</sup>CD14<sup>+</sup> granulocytes from all clinical groups. CD11b<sup>hi</sup>CD14<sup>+</sup> granulocytes expressed substantially more S100A8 and S100A9 than the CD11b<sup>lo</sup>CD14<sup>+</sup> subpopulation. S100A12 levels in granulocytes were substantially less than levels of S100A8 or S100A9. \*MFI value are normalised to isotype control.



**Figure 5.** Among the granulocyte subpopulations, MFI of S100A8 was significantly higher in CD11b<sup>hi</sup>CD14<sup>+</sup> compared to CD11b<sup>lo</sup>CD14<sup>+</sup> granulocytes from all clinical groups ( $p < 0.01$ ,  $p < 0.01$ ,  $p = 0.03$  and  $p = 0.02$  respectively). Differences in S100A9 expression were similar ( $p < 0.01$  in all groups except for lung cancer where  $p = 0.04$ ). However no significant differences in intensities of anti-S100A12 reactivity were observed in CD11b<sup>hi</sup>CD14<sup>+</sup> granulocytes from non-smokers or patients with COPD compared to levels in CD11b<sup>lo</sup>CD14<sup>+</sup> granulocytes ( $p = 0.03$  and  $p = 0.04$  respectively). \*MFI value are normalised to isotype control.



**Figure 6.** Serum levels of S100A8 (A), S100A9 (B) and S100A12 (C) did not differ significantly between the six subject groups. Serum nitrite/nitrate (NOx) (D) was significantly higher in the lung cancer group compared to the never-smoker group ( $p = 0.0019$ , Kruskal-Wallis test) but the same trend was not observed in the EBC NOx (E).

CD14<sup>+</sup> monocyte and granulocyte populations in healthy non-smokers, smokers and patients with COPD or NSCLC.

We used whole blood flow cytometry to confirm the high expression of S100A8 and S100A9 in granulocytes from non-smokers, and lower expression in monocytes (Figure 2A). Monocytes from smokers and patients with COPD contained significantly less S100A8 than granulocytes. In marked contrast to the significant differences seen in these groups, granulocytes and monocytes from patients with NSCLC contained similar amounts of S100A8 (Figure 2D). Unexpectedly, and in contrast to earlier reports we found no significant differences in S100A9 levels in granulocytes and monocytes between any clinical group and NSCLC [50]. S100A9 expression had a tendency to be lower in granulocytes from those with respiratory diseases, compared to controls. As expected, S100A12 expression was low in both cell types and similar amounts were found in each, although the S100A12-expressing monocytes from smokers had a lower MFI than S100A12<sup>+</sup> granulocytes. Emerging evidence indicates protective rather than pro-inflammatory roles for S100A8 in the lung. S100A8 and S100A9 scavenge hypohalous acid oxidants in human asthma and intranasal treatment with S100A8 suppresses asthma and acute lung injury in murine models [15,17,18]. S100A8 also reduces mast cell activation and signalling that is redox-dependent [17]. Asthmatic inflammation is also reduced in transgenic mice expressing human S100A12, indicating immunosuppressive properties [55].

Human MDSC are a heterogeneous group of cells with variable phenotypes and immunosuppressive features [56,57]. Earlier studies reported increased numbers of CD11b<sup>+</sup>CD14<sup>+</sup>S100A9<sup>+</sup> and CD11b<sup>+</sup>CD14<sup>+</sup>S100A8<sup>+</sup> monocytes in blood from patients with NSCLC, which was not confirmed [31]. Our observations are supported by a recent report showing that the percentage of S100A9<sup>+</sup> CD14<sup>+</sup> cells, and MFI of S100A9 reactivity in whole blood leukocytes was almost identical between NSCLC and healthy controls [58]. In that study, more than 80% of CD14<sup>+</sup> cells expressed S100A9 in both groups. In addition, no differences in expression of CD14<sup>+</sup>HLA-DR<sup>+</sup>CD33<sup>+</sup>CD11b<sup>+</sup> MDSC from patients with COPD compared to smokers or non-smokers were reported [59]. Until now, S100A12 expression levels have not been compared in these cell populations from patients with lung disorders but together, our results indicate that S100 expression levels did not define particular monocyte or granulocyte subpopulations from any cohort tested. Thus, their potential as markers in NSCLC and inflammatory respiratory diseases appears limited. However, comparisons of S100A8<sup>+</sup>CD11b<sup>+</sup>CD14<sup>+</sup> and S100A8<sup>+</sup>CD11b<sup>+</sup>CD14<sup>+</sup> expressing cells may be useful, as S100A8-expressing granulocytes were statistically higher than monocytes from all groups, except for those from patients with NSCLC. It is also feasible that if additional surface markers (for example, CD33, HLA-DR, CD15, IL-4R) had been included, we may have detected additional differences between the groups. In this study, we analysed monocytes expression of the S100 protein levels and additional markers would better reflect the identification of MDSC.

Interestingly, we identified two subpopulations of granulocytes, based on expression of the surface integrin CD11b. Leukocyte expression of this protein increases upon activation, and higher levels on cells from patients with COPD are reported [60]. The CD11b<sup>lo</sup> population have only occasionally been mentioned previously [61]. Percentages of cells expressing S100A8 and S100A9 were much higher than for S100A12 in the CD14<sup>+</sup>CD11b<sup>hi</sup> population. Overall, CD14<sup>+</sup>CD11b<sup>lo</sup> granulocytes contained markedly less S100A8 and S100A9 whereas numbers of S100A12-expressing cells from non-smokers

or patients with COPD were similar in CD14<sup>+</sup>CD11b<sup>hi</sup> cells. Recent evidence points to neutrophil subsets with diverse roles that regulate inflammation, some of which impact on neutrophil transmigration [62]. CD11b<sup>lo</sup> neutrophils are less adhesive to endothelial cells than CD11b<sup>hi</sup> cells and populations with these phenotypes can vary depending on treatment with glucocorticoid therapy [61]. Despite this, we found that expression on granulocytes of each subset was similar in all groups tested even though some smokers, patients with COPD or NSCLC, were on inhaled corticosteroids (Table 1). Interestingly, S100A9-deficient neutrophils have reduced responsiveness to chemoattractants and the significantly lower levels seen in CD11b<sup>lo</sup> neutrophils may indicate a subpopulation with functionally distinct migratory properties (Figure 5) [63]. It would be interesting to perform detailed analysis of CD14<sup>+</sup>CD11b<sup>lo</sup>S100A8<sup>lo</sup>S100A9<sup>lo</sup> granulocyte subpopulations from whole blood preparations that had undergone minimal activation.

Leukocytes secrete S100A8, S100A9 and S100A12 and changes in serum concentrations are considered markers of inflammation in a number of clinical conditions. In this study, no differences in serum levels of these proteins was found between the groups, even though S100 levels in granulocytes tended to be lower in patients with COPD and NSCLC. Furthermore, some patients in our study were using inhaled corticosteroids and this drug enhances calgranulin expression in human monocytes *in vitro* but no evidence of such an effect was seen on circulating levels of the S100s, either in leukocytes or in serum [64]. Sampling from the site of disease, as reported for patients with asthma and COPD may more accurately reflect changes [7,65]. In addition, samples were taken from patients with stable disease, not during exacerbations.

Serum from patients with NSCLC had higher significantly NOx levels than non-smokers, confirming reports of elevated serum nitrated proteins in subjects with lung cancer, and suggesting a role for NO-mediated stress in this disease [66]. In contrast, total NOx levels in EBC did not differ between groups. Elevated nitrite, but decreased nitrate in EBC of patients with lung cancer has been described but here we compared total nitrite concentrations that could mask changes in nitrate to nitrite ratios [66]. Although the source of NO in patients with NSCLC is unclear, and could include several cell types within the lung, circulating MDSC from patients with NSCLC also have increased NO production [66-69]. The role of NOx in MDSC warrants further investigation.

The limitations are the relatively small subject numbers in each group, and the high inter-individual variability of S100 protein expression, possibly accounted for by factors that may affect S100 protein expression not yet identified, e.g. gender, age, co-morbid medical conditions and medications. Another limitation is that eosinophils which are not separated during the gating process are likely to be included in the granulocyte fraction and their effect on the data is unclear.

## Conclusions

We did not confirm studies suggesting that the S100s in monocytes may represent predictive factors in NSCLC. Nonetheless these quantitative comparisons of these individual proteins in CD14<sup>+</sup>CD11b<sup>hi</sup> granulocytes and define a novel CD14<sup>+</sup>CD11b<sup>lo</sup>S100A8<sup>lo</sup>S100A9<sup>lo</sup> subpopulation. Comparison of S100A8 expression in granulocytes and monocytes from patients with NSCLC indicated similar levels in each, where as in the non-NSCLC groups, granulocyte levels were significantly greater. Coupled with the significant increases in NOx concentrations found in serum from these patients, we suggest that



further studies with larger patient cohorts is warranted to determine links between these MDSCs, the S100 proteins, NO pathways and T-cell activation.

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