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Altered Nrf2/Keap1-Bach1 equilibrium in pulmonary emphysema

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ABSTRACT

Oxidative stress, resulting from increased oxidative burden and decreased level of anti-oxidant proteins, plays a role in the pathophysiology of smoking-related pulmonary emphysema. Expression of several antioxidant proteins, such as heme oxygenase-1 (HO-1), glutathione peroxidase2 (GPX2) and NAD(P)H:quinone oxidoreductase1 (NQO1), results from an equilibrium created by positive or negative regulation by the transcription factors Nrf2, Keap1 and Bach1 respectively. However, whether the expression of these transcription factors is altered in emphysema and could account for decreased expression of antioxidant proteins is not known.

We aimed to investigate the expression and subcellular localisation of Nrf2, Keap1 and Bach1 as potential regulators of HO-1, GPX2 and NQO1 in alveolar macrophages, a key cell in oxidative stress, in lung surgical specimens of non-smokers without emphysema and smokers with and without emphysema.

Western blot, immunohistochemical and laser scanning confocal analysis revealed Nrf2 protein level significantly decreased in whole lung tissue and alveolar macrophages (cytosol and nucleus) in patients with emphysema as compared with those without emphysema. Conversely, Bach1 and Keap1 were increased in patients with emphysema. These modifications were associated with a parallel decrease in expression of HO-1, GPX2 and NQO1 at the cellular level, which was inversely correlated with airway obstruction and distension indexes, and increased macrophage expression of the lipid peroxidation product 4-hydroxy-2-nonenal. Silencing RNA experiments in vitro in THP-1 cells were done to confirm cause effect relation between the loss of Nrf2 and the decrease of HO-1, NQO1 and GPX2 expression. Nrf2/Keap1-Bach1 equilibrium was altered in alveolar macrophages in pulmonary emphysema, which points to a decreased stress-response phenotype. This finding opens a new view of the pathophysiology of emphysema and could provide the basis for new therapeutic approaches based on preservation and/or restoration of such equilibrium.

INTRODUCTION

Chronic cigarette smoking is the most important risk factor for lung emphysema, a major component of morbidity and mortality in chronic obstructive pulmonary disease (COPD) [1]. Several mechanisms by which chronic exposure to cigarette smoke (CS) leads to emphysema include lung inflammation and imbalance in level of proteases/antiproteases and oxidants/antioxidants (namely oxidative stress) [2]. However, the precise nature of these alterations is still unknown.

Alveolar macrophages are important components of lung inflammation in emphysema. Studies of emphysematous lung tissue from human subjects have shown a direct relation between alveolar macrophage density in the parenchyma and severity of lung destruction [3]. Moreover, in the emphysematous lung, alveolar macrophages can release an array of matrix metalloprotease enzymes and reactive oxygen species involved in protease/antiprotease imbalance and oxidative stress [4].

In oxidative stress, phase-2 enzyme systems such as NAD(P)H:quinone oxidoreductase1 (NQO1), glutathione peroxidase2 (GPX2) and heme oxygenase-1 (HO-1) are induced to provide anti-oxidant and anti-inflammatory effects [5]. This process is mediated by binding of nuclear factor erythroid 2-related factor 2 (Nrf2) to antioxidant response element (ARE) sequence [6]. Under basal conditions, Nrf2 is sequestered in the cytoplasm and binds to the actin cytoskeleton by the Kelch-like ECH-associated protein 1 (Keap1), which facilitates its proteasomal degradation [6]. In the presence of oxidative or xenobiotic stimuli, Nrf2 and Keap1 dissociate, and Nrf2 migrates into the nucleus. Deficiency of Nrf2 was recently shown to enhance susceptibility to neutrophil elastase and CS-induced emphysema in mice [7,8]. However, no data exist on the involvement of Nrf2, Keap1 in human lung emphysema. Furthermore, expression of phase-2 genes like HO-1 is also under the control of the transcription factor Bach1, which represses the transcription of HO-1 [9].

Among oxidative stress-induced phase-2 enzymes, HO-1 is mainly expressed in macrophages within the lung [5] and plays a central role in the defence against lung oxidative and inflammatory insults, including CS exposure [10,11]. In patients with severe COPD, the expression of HO-1 was significantly decreased in alveolar macrophages [12,13] but the molecular mechanisms are unknown. This phenomenon has important implications, since it can potentiate the inflammatory and oxidative capacities of macrophages.

Given the critical role of Nrf2/Keap1 and Bach1 transcription factors in controlling phase-2 enzymes, an altered level of Nrf2/Keap1 and Bach1 in lung macrophages could account for a decreased HO-1 expression in human emphysema. We aimed therefore to investigate the expression and subcellular localisation of Nrf2/Keap1 and Bach1 as potential regulators of HO-1 expression in macrophages in lung surgical specimens of non-smokers without emphysema and smokers with and without emphysema. To further examine the consequences of altered expression of Nrf2/Keap1 and Bach1, we also analysed the expression of NQO1 and GPX2 other antioxidant phase-2 enzymes induced by CS in the lung [14,15]. Immunostaining with 4-hydroxy-2-nonenal (4-HNE), a specific and stable end product of lipid peroxidation and a marker of oxidative stress [16], was used to assess oxidative damages in the lungs of patients [17].

Finally, we performed silencing RNA experiments in vitro in THP-1 macrophages incubated with Nrf2 siRNA to verify that Nrf2 participates in HO-1, NQO1 and GPX2 genes expression.

METHODS (See Supplement online**)**

Patients

This study was approved by the local ethics committee of Saint Germain en Laye and stored biopsies were reported to our institutional board (Délégation à la Recherche Clinique, Assistance Publique - Hôpitaux de Paris).

Patients with emphysema

Ten patients with severe emphysema (SE) requiring surgery for lung transplantation (n=2) or lung volume reduction (n=8) were included (Table I). Pulmonary function tests demonstrated severe airflow obstruction and lung distension (Table II). According to the GOLD classification of severity (www.goldcopd.com), SE patients belonged to stage III (severe) or IV COPD (very severe).

Patients without emphysema

Normal lung tissue was obtained from 20 patients without radiological, spirometric and histological features of emphysema. Nine patients were smokers (NE-S) and 11 were nonsmokers (NE-NS).

Processing of lung samples

Lung tissue fragments were immediately frozen in liquid nitrogen and stored at –80°C. The histopathology of biopsies was evaluated on paraffin-embedded sections to verify features of emphysema or normal lung.

Table I: Clinical characteristics

	Nonsmokers without emphysema (NE-NS)	Smokers without emphysema (NE-S)	Patients with severe emphysema (SE)	Between group differences (p)
n	11	9	10	
Age (yr)	58 [32-72]	58.5 [30-68]	57.5 [49-65]	0.99
Sex ratio (F/M)	2/9	0/9	1/9	0.28
Smoking (median no. of packs smoked [range])	0	37.5 [10-50]‡	52 [20-100] †,‡	10^{-4}
Time since smoking cessation (yr)	NA	0 [0-20]	1 [0-8]	0.88
Current smokers/ex-smokers	NA	7/2	2/8	10^{-4}

†: vs. NE-S, $p=10^{-3}$; ‡: vs. NE-NS, $p=10^{-4}$; as assessed by Mann-Whitney U-test; NA: not applicable; ns: nonsignificant.

Table II: Pulmonary function tests

	Nonsmokers without emphysema (NE-NS)	Smokers without emphysema (NE-S)	Patients with severe emphysema (SE)	Between group differences (p)
n	11	9	10	
FEV1% predicted value (% pred)	79 [68-101]	78 [60-110]	22 [13-47]†,‡	10^{-4}
FEV1 /FVC	90 [71-105]	68 [64-85]‡	43.5 [35-50] †,‡	10^{-4}
RV (% pred)	95 [55-117]	117 [96-132]	241 [207-349]†,‡	10^{-4}
TLC (% pred)	82.5 [76-99]	100 [84-116]‡	130 [110-153]†,‡	10^{-4}
PaO2 (kPa)	11.4[10.1-12.4]	11.3[10.1-13.3]	9[7.3-10.9]†,‡	8.10^{-4}
PaCO2 (kPa)	5.5[5.1-6]	5.3[4.8-6.1]	5.7[4.5-6.7]	0.36
DLCO (% pred)	Not done	Not done	43.5 [15-67]	Non relevant
6 min walk test (meter)	Not done	Not done	350 [70-540]	Non relevant

†: vs. NE-S, $p=10^{-4}$; ‡: vs. NE-NS, $p=10^{-4}$; as assessed by Mann-Whitney U-test. ns: non significant

Pa_{O2}: arterial oxygen pressure, Pa_{CO2}: arterial carbon dioxide pressure, FEV_1: forced expiratory volume in one second, RV: residual volume, FVC: forced vital capacity, TLC: total lung capacity, DLCO: diffusion lung capacity for carbon monoxide.

Quantitative RT-PCR analysis

HO-1, HO-2, GPX2 and NQO1 mRNA expression was quantified by RT-PCR (MX3000P, Stratagene, La Jolla, CA) as described [18] and expressed as a ratio to Ubiquitin-c (Table III in Supplement online).

Preparation of lung homogenates for western blot analysis

Lung biopsy samples were homogenised in 10 mM Tris-HCl, pH 6.8, 1% SDS, 5% glycerol. Aliquots were stored at -80°C with 10% Protease Inhibitor Cocktail (Sigma, France). Western blot analysis was performed as described [19] with anti-Nrf2, Keap1, Bach1 (Santa-Cruz Biotechnology, Santa Cruz, CA), and anti-HO-1, HO-2 antibodies (Stressgen, Le-Perray-en-Yvelines, France). Results are expressed as a ratio to β-actin expression (Sigma, St. Louis, MO).

Immunohistochemical analysis

Immunohistochemistry with the primary antibodies anti-Nrf2, Bach1, Keap1, HO-1, and anti-CD68 [macrophage marker] (Dako, Glostrup, Denmark, Clone PG-M1) was performed as previously described [20]. On adjacent sections, positive cells with characteristic macrophage morphology were evaluated by two independent observers in 10 different high power fields at x200 magnification, and results were expressed as the number of positive cells per mm^2 of lung tissue.

Confocal laser scanning microscopy

Double-immunofluorescence labeling was performed to colocalize CD68, HO-1 and 4-HNE, with Nrf2, Bach1 and Keap1 as previously described [21]. The intensity of nuclear Nrf2, Bach1 and cellular HO-1, Keap1 and 4-HNE immunofluorescence was quantified in macrophages.

siRNA transfection

THP-1 cells were transfected with 300nM Nrf2 siRNA (NM_006164; Dharmacon SMARTpool siRNA reagent) or negative control siRNA (Dharmacon plus non targeting pool) using Transpass R2 transfecting reagent as per the manufacturers' instructions. Cells were lysed, nuclear and cytosolic fractions were prepared as previously described [22].

Statistical analysis

Data were analysed by Statview software (Abacus Concepts, Inc.) and displayed as medians and ranges. Between-group differences were first assessed by nonparametric analysis of variance (Kruskal-Wallis test) and Mann-Whitney U-test. Correlations were assessed by Spearman's rank order test. Categorical data were analysed by Chi-square test. $p < 0.05$ was considered significant.

RESULTS

Nrf2/Keap1 and Bach1 protein expression in the lung

We first analysed the expression of Nrf2, Keap1 and Bach1 at the protein level in whole lung homogenates. Western blot showed decreased Nrf2 protein in SE group as compared with NE groups (Figure 1). By contrast, the level of Keap1 and Bach1 protein was increased in the SE group as compared with NE groups.

Subcellular localisation of Nrf2/Keap1 and Bach1 in alveolar macrophages

We investigated whether our findings of decreased Nrf2 and increased Keap1 and Bach1 protein expression in whole lung homogenates would be reflected at the macrophage level and whether they concerned nuclear localisation of the transcription factors. As macrophages play a critical role in emphysema pathophysiology [3,4] we focused on these cells. We quantified the macrophages in our samples by detecting CD68-positive cells on immunohistochemistry. The number of CD68-positive cells was significantly increased in the SE (per mm^2 166 [range 135-180]) as compared with the NE-NS (81 [80-87]) and NE-S groups (120 [100-121]) (p=0.04 vs. NE-S and p= 0.01 vs. NE-NS; Figure E1 See Supplement online) as was found previously [3]. Alveolar macrophages, alveolar epithelial cells and endothelial cells were positive for Nrf2 among NE-NS and NE-S groups. However, semi-quantitative analysis revealed higher staining of macrophages than other cell types (p=0.04). The number of CD68 positive cells with positive Nrf2 staining was decreased in SE (19.9% [16.3-25.6]) as compared with the NE-NS (98.8% [98.3-100]) and NE-S groups (99% [98.9-99.6]; p=0.029). By contrast, the number of CD68 positive cells with positive Bach1 or Keap1 staining was increased in SE as compared with the NE-NS and NE-S groups (for Bach1: 89.2% [70.5-98.9] vs 15% [8.6-24.1] and 10.8% [9.1-11.1]; p=0.03; and for Keap1: 92.8% [89.2-95.8] vs 12.3% [10-18.4] and 11.7% [10.1-11.7]; p=0.03). Confocal laser microscopy with double-immunofluorescence labelling confirmed the colocalisation of CD68 with Nrf2, Bach1 or Keap1 expression in NE-NS, NE-S and SE alveolar macrophages (Figure 2). Nrf2 expression was profoundly decreased in both the cytosol and nucleus of alveolar macrophages of SE as compared with NE groups (Figure 2A), with no difference between NE-NS and NE-S groups. Bach1 and Keap1 expression was increased in both cytosol and nucleus of alveolar macrophages of SE as compared with NE groups (Figure 2B and 2C), with no difference in expression between NE-NS and NE-S groups. Quantification of nuclear Nrf2, Bach1 and cellular Keap1 immunofluorescence confirmed decreased Nrf2, increased Bach1 and increased Keap1 in the SE as compared with NE groups (Figure 2D).

Heme oxygenase expression

Having demonstrated decreased cytosolic and nuclear Nrf2 and increased Bach1 and Keap1 immunostaining in lung macrophages of the SE group, we investigated whether these alterations were associated with decreased expression of HO-1. HO-1 mRNA expression in whole lung homogenates was markedly decreased in the SE group as compared with NE groups (Figure 3A). By contrast, the constitutive isoform HO-2 was expressed similarly in all groups (Figure 3B). HO-1 mRNA level was strongly and inversely correlated with airway obstruction and lung distension (Rho for FEV1, +0.54, p=0.003; FEV1/FVC, +0.51, p=0.0046; RV, -0.51, p=0.005; TLC, -0.49, p=0.008). These correlations persisted when non smokers were excluded (Rho for FEV1, +0.592, p=0.012; FEV1/FVC, +0.618, p=0.009; RV, -0.624, p=0.01; TLC, -0.595, p=0.014). Furthermore, western blot analysis confirmed the decreased HO-1 mRNA expression, with decreased protein expression in the SE group as compared with NE groups (Figure 3C). No significant difference was observed for HO-2 protein between groups.

Colocalisation of Nrf2/Keap1 and Bach1 with HO-1 in alveolar macrophages

We initially verified the expression of HO-1 in alveolar macrophages on immunostaining with a CD68 antibody in sequential slides. Most of the HO-1-positive cells in alveolar spaces were CD68 positive (88.5% [75.6-94.2]), as reported previously [23]. Colocalisation experiments on laser confocal miscroscopy revealed that HO-1 expression paralleled that of Nrf2: HO-1 level was decreased concomitantly with decreased level of Nrf2 in alveolar macrophages of the SE group (Figure 4A). Moreover, in alveolar macrophages from SE patients, increased Bach1 and Keap1 expression was associated with decreased HO-1 expression (Figure 4B and 4C). Immunofluorescence quantification showed decreased HO-1 expression associated with decreased level of nuclear Nrf2, increased level of nuclear Bach1 and cellular Keap1 in macrophages of the SE group as compared with NE groups (Figure 4D). HO-1 was expressed in the cytosol and membrane in macrophages of NE groups.

NQO1 and GPX2 mRNA expression

To further examine consequences of altered expression of Nrf2/Keap1 and Bach1, we investigated the mRNA expression of the Nrf2-regulated antioxidant genes NQO1 and GPX2. NQO1 and GPX2 mRNA level was decreased in the SE group as compared with NE groups (Figure 5). Similar to results for HO-1, NQO1 and GPX2 mRNA level was inversely correlated with airway obstruction and lung distension (for NQO1: Rho for FEV1, +0.39, p=0.02; FEV1/FVC, +0.36, p=0.04; RV, -0.44, p=0.015; TLC, -0.40, p=0.03; for GPX2: Rho for FEV1, +0.368, p=0.027; FEV1/FVC, +0.357, p=0.032; RV, -0.544, p=0.001; TLC, -0.502, p=0.003). These correlations persisted when non smokers were excluded for NQO1 (data not shown).

Colocalisation of Nrf2 with 4-HNE in alveolar macrophages

Immunostaining with 4-HNE was used to assess oxidative damages in the lungs. Colocalisation experiments on laser confocal miscroscopy and immunofluorescence quantification revealed that increased 4-HNE expression was associated with decreased level of nuclear Nrf2 in alveolar macrophages of the SE group as compared with the NE groups (Figure 6). 4-HNE staining was positive both in the cytosol and membrane in macrophages of SE group.

Nrf2 silencing in THP-1 cells (FiguresE2-4 in Supplement online)

Western blot analysis confirmed that Nrf2 siRNA transfection induced a time course inhibition of cellular Nrf2 of 55% of control cells at 8h, associated with subsequent HO-1 decrease to 38 and 41% of control cells at 12h and 24h respectively (Figure E2). Nrf2 decrease was confirmed both in cytosolic and nuclear compartments by western blot and confocal analysis (Figure E3). Moreover, confocal double immunostaining allowed to evidence the concomitant decrease of Nrf2 and HO-1 expression. To further examine consequences of Nrf2 silencing, we showed that the mRNA expression of Nrf2-regulated antioxidant genes HO-1, NQO1 and GPX2 was decreased (Figure E4).

DISCUSSION

Here we show the protein expression of Nrf2 decreased and that of Keap1 and Bach1 increased in alveolar macrophages of lung specimens from patients with smoking-related lung emphysema as compared with smoking and nonsmoking patients without emphysema. These abnormalities have important implications in human emphysema, since Nrf2 has been shown to play a protective role against neutrophil elastase and cigarette smoke-induced emphysema in animals [7,8]. Furthermore, these modifications were associated with a parallel decrease in the antioxidant HO-1, NQO1 and GPX2 expressions which were inversely correlated with airway obstruction and lung distension indexes. Decreased Nrf2 expression was associated with an increased macrophage expression of the lipid peroxidation product 4-HNE. Given the vast array of protective genes regulated by Nrf2/Keap1 and Bach1 in addition to HO-1, GPX2 and NQO1, the abnormalities we describe point to a phenotype of decreased stress response which could account for the excessive oxidative stress in alveolar macrophages of patients with smoking-related emphysema. This finding provides a new view in the understanding of lung emphysema in particular and COPD in general.

Nrf2/Keap1 and Bach1 lung expression has never been described for humans. Indeed, only one study reported the protein expression of these transcription factors in human tissues [24]. Although Nrf2 and Bach1 are ubiquitous, we focused on macrophages as key cells of the pathogenesis of lung emphysema [2,4]. Western blot analysis showing significant alteration of Nrf2 and Bach1 proteins in whole lung homogenates from SE patients were confirmed by immunohistochemistry and laser confocal microscopy analysis in macrophages. Some non-macrophage cells showed positive immunostaining for Nrf2, as was found *in vitro* in endothelial and epithelial cells [25,26]. Although we cannot rule out the participation of non-macrophage cells in lung antioxidant responses *via* Nrf2, the intensity of such staining in these cells was significantly lower than that in macrophages. Furthermore, Bach1 was expressed near only in macrophages. Interestingly, Ishii and coworkers [7] found that transplantation of wild-type bone marrow cells expressing Nrf2 into Nrf2-knockout mice instilled with elastase retarded the development of initial lung inflammation and subsequent emphysema. This improvement was associated with the appearance of macrophages expressing Nrf2-regulated antiprotease and antioxidant genes, stressing the importance of Nrf2 pathway in macrophages.

The decreased nuclear localization of Nrf2 protein in macrophages of SE patients was concomitant with decreased cytosolic expression, thus showing a global decrease in Nrf2 protein expression rather a localized defect in nuclear localization. Furthermore, cellular Keap1 protein was increased in macrophages of SE patients. Decreased Nrf2 protein expression could have resulted from increased Keap1 protein expression, which accelerated Nrf2 cytosolic degradation [6], and/or phosphorylation of nuclear Nrf2 with ensuing nuclear extrusion and cytosolic degradation, as demonstrated in cells exposed to oxidative stress-generating high UVB light [27]. Bach1 protein level was increased in the nucleus and cytosol of macrophages of SE patients. As for Nrf2, increased Bach1 protein expression in macrophages of SE patients probably reflects changes in its degradation. Clearly, examination of these mechanisms deserves further study.

Whatever the process leading to the modification in Nrf2/Keap1 and Bach1 protein expression we showed in alveolar macrophages of emphysema patients, these findings were associated with a significant decrease in expression of the downstream cytoprotective phase-2 genes HO-1, GPX2 and NOQ1. We are confident in the strength of this causal relation, because, at least for HO-1, double immunolabelling and laser confocal microscopy analysis verified, the association between expression of Nrf2, Keap1 and Bach1 and that of HO-1.

Silencing RNA experiments in THP-1 macrophages allowed us to confirm a cause effect relation between the loss of Nrf2 and the decrease of HO-1, NQO1 and GPX2 genes expression.

Consistent with the central role of Nrf2 in the protection of the lungs against oxidative stress, we also showed by double immunolabelling that the decreased expression of Nrf2 was associated with an increased macrophage expression of 4-HNE in the lungs of SE patients. Thus a Nrf2-dependent transcriptional regulation of antioxidant enzymes in the lungs may play an important role in counteracting CS-induced lung oxidative stress to protect against emphysema.

The decreased HO-1 mRNA and protein expression demonstrates a transcriptional repression of this enzyme and fits well with the decreased number of HO-1-positive alveolar macrophages that Maestrelli and coworkers and Slebos and associates observed in severe COPD patients [12,13]. Our finding that HO-1 was not increased in smokers without emphysema is unexpected. However, these results agree with the lack of change of Nrf2/Keap1 and Bach1 in these patients and with other results [23,28]. HO-1 protein was expressed in the cytosol and membrane of macrophages of patients without emphysema, a finding never reported before. Although classically expressed in microsomes [29], HO-1 has been found expressed in mitochondria [30], nucleus [31] and caveolae [32] of different cell types. Our findings agree with this last localisation and with data showing that CO, one of the products of heme degradation by HO, inhibits signaling of cell membrane toll-like receptors in macrophages [33]. This and other antioxidant and anti-inflammatory effects of HO-1 and related products, such as decreased production of reactive oxygen species [21] and/or inhibition of metalloprotease-1 expression and activity [34] can explain its protective role against emphysema in smokers tested experimentally [35] and in genotype-phenotype studies [10,11]. The antioxidant deficit elicited by decreased HO-1 expression in SE patients can be potentiated by decreased expression of NQO1, which is induced by cigarette smoke [14], and detoxifies quinones [36], present in this compound. The pathophysiological importance of decreased HO-1 and NQO1-mediated antioxidant and anti-inflammatory effects in our emphysema patients is stressed by the strong correlation between the decreased mRNA expression of these enzymes and airway obstruction and lung distension indexes.

Our study has some methodological limitations. We studied a limited number of patients in each group. The emphysema group was composed of patients with severe emphysema selected on the basis of surgical recruitment and evaluated at only one time point in the course of the disease. Because only one tissue sample from surgically resected material was available for examination, the expression of transcription factors and HO-1 could reflect regional disease activity and may not represent the entire lung. Indeed, emphysema is well known to affect different lung regions to a varying extent. However, in pilot experiments, we verified the reproducibility of our findings in tissue sampled at different sites from 2 lungs.

In addition, our groups without emphysema were composed largely of patients with localised carcinoma and, although tissue was sampled at distance from carcinomatous lesions, cancer itself may have influenced the results. Nrf2 protein expression has been shown to be increased in cancer tissue [24], which may overestimate the differences between patients with and without emphysema. However, in cancer tissue, Keap1 protein expression is increased [24], a phenomenon not observed in our samples.

As well, another potential bias of this study is related to tobacco consumption, since alveolar macrophage dysfunction in COPD may be attributed to active smoking [37]. However, although, most of our patients with emphysema were ex-smokers, we cannot completely rule out an effect of cigarette smoking on our results.

In conclusion, our results showed abnormalities in the expression of Nrf2/Keap1 and Bach1 transcription factors and the ensuing antioxidant phase-2 genes HO-1, GPX2 and NQO1 in alveolar macrophages with smoking-related lung severe emphysema. These phenomena were associated with increased oxidative burden. A better understanding of the altered expression of these transcription factors is needed to establish new therapeutic strategies for emphysema and COPD that could be based on preservation and/or restoration of the equilibrium between Nrf2/Keap1 and Bach1.

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Competing interests
The authors declare that they have no competing interests.

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Ethics committee approval

This study was approved by the local ethics committee of Saint Germain en Laye hospital (20 rue Amargis, 78100 Saint Germain en Laye, France) and stored biopsies were reported to our institutional board (Délégation à la Recherche Clinique, Assistance Publique - Hôpitaux de Paris, Carré Historique de l'Hôpital Saint-Louis, 1 avenue Claude Vellefaux 75010 Paris, France).

Author's consent to publication (copyright)

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Footnotes

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Abbreviations

4-HNE: 4-hydroxynonenal
ARE: antioxidant responsive element
Bach1: BTB and CNC homology 1, basic leucine zipper transcription factor 1
COPD: chronic obstructive pulmonary disease
CS: cigarette smoke
FEV1: forced expiratory volume in one second
FVC: forced vital capacity
HO: heme oxygenase
GPX2: glutathione peroxidase 2
Keap1: Kelch-like ECH- associated protein 1
mRNA: messenger ribonucleic acid
NE-NS: Non-emphysema non-smoker
NQO1: NADPH quinone oxidoreductase 1
Nrf2: Nuclear factor erythroid 2-related factor 2
RT-PCR: reverse transcriptase-real-time polymerase chain reaction
RV: residual volume
SE: severe emphysema
TLC: total lung capacity

FIGURE LEGENDS

Figure 1. Expression of Nrf2, Keap1 and Bach1 in lung tissue. Box-and-whiskers plot with median, interquartile range and minimum and maximum values. NE-NS and NE-S patients without emphysema non-smokers and smokers, respectively, SE: severe emphysema patients. Top: representative western blot of Nrf2, Keap1 and Bach1 protein expression of lung homogenates from 3 NE-NS, 3 NE-S and 3 SE patients with the respective βactin controls. Bottom: quantification of Nrf2, Keap1 and Bach1 protein. Results are expressed as a ratio to βactin protein concentration (n=6, Nrf2 and Keap1 *p= 0.004 versus NE groups; Bach1 *p=0.016 vs. NE-S and p=0.049 vs. NE-NS respectively).

Figure 2. Laser confocal microscopy analysis of lung biopsies. Abbreviations are in Figure 1. Immunofluorescent staining was performed with Nrf2, Bach1, Keap1 (in red: left column) and CD68 (in green) and TO-PRO-3 DNA stain (blue). Co-expression is seen by double staining and overlays (Merge column). A: Nrf2; B: Bach1; C: Keap1; D: Quantification of Nrf2 and Bach1 nuclear and cellular Keap1 immunofluorescence in macrophages of NE-NS, NE-S and SE patients (magnification x 1200). Box-and-whiskers plot with median, interquartile range and minimum and maximum values. * $p=10^{-4}$ versus NE groups. Images are representative of all samples.

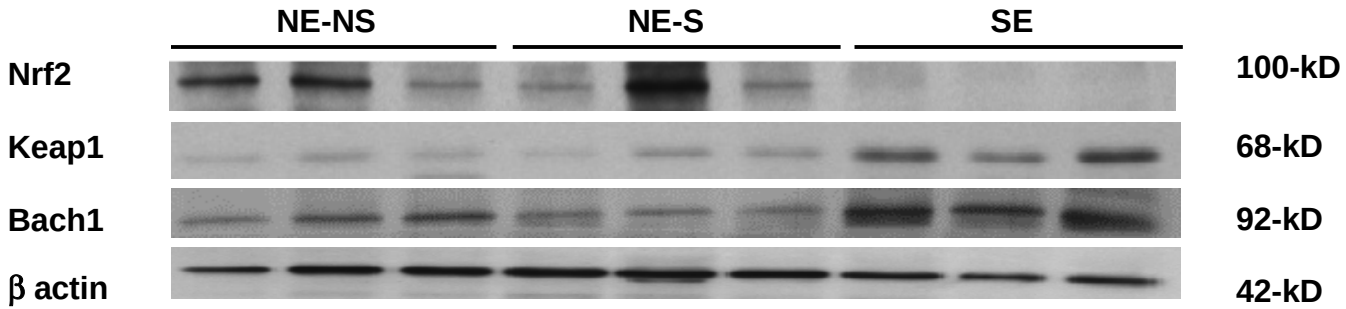
Figure 3. Expression of HO-1 and HO-2 in lung tissue. Abbreviations are in Figure 1. Box-and-whiskers plot with median, interquartile range and minimum and maximum values. Panel A: Expression of HO-1 mRNA. Panel B: Expression of HO-2 mRNA. Results are expressed as a ratio to ubiquitin mRNA levels. *p=0.02 vs. NE-NS and p=0.002 vs. NE-S. Panel C top: representative western blot of HO-1 and HO-2 protein expression of lung homogenates from 3 NE-NS, 3 NE-S and 3 SE patients with the respective βactin controls. Panel C bottom: quantification of HO-1 and HO-2 protein. Results are expressed as a ratio to βactin protein concentration (n=6, * p =0.004 versus NE groups.)

Figure 4. Laser confocal microscopy analysis of lung biopsies. Abbreviations are in Figure 1. Immunofluorescent staining was performed with Nrf2, Bach1, Keap1 (in red, left column) and HO-1 (in green) and TO-PRO-3 DNA stain (blue). Co-expression is seen by double staining and overlays (Merge column). A: Nrf2; B: Bach1; C: Keap1; D: Quantification of nuclear Nrf2, nuclear Bach1, cellular Keap1 and cellular HO-1 immunofluorescence in macrophages (magnification x1200). Box-and-whiskers plot with median, interquartile range and minimum and maximum values. * $p=10^{-4}$ versus NE groups. Images are representative of all samples.

Figure 5. Expression of NQO1 and GPX2 mRNA in lung tissue. Box-and-whiskers plot with median, interquartile range and minimum and maximum values. Panel A: Expression of NQO1 mRNA. Panel B: Expression of GPX2 mRNA. Abbreviations are similar to Figure 1. Results are expressed as a ratio to ubiquitin mRNA levels. * p=0.004 vs NE-NS and 0.0007 vs NE-S; and p=0.018 vs NE-NS and 0.024 vs NE-S for NQO1 and GPX2 respectively.

Figure 6. Laser confocal microscopy analysis of lung biopsies. Abbreviations are in Figure 1. A: Immunofluorescent staining was performed with Nrf2 (in red, left column) and 4-hydroxy-2-nonenal (4-HNE, in green) and TO-PRO-3 DNA stain (blue). Co-expression is seen by double staining and overlays (Merge column). B: Quantification of nuclear Nrf2, and cellular 4-HNE immunofluorescence in macrophages (magnification x1200). Box-and-whiskers plot with median, interquartile range and minimum and maximum values. * $p=10^{-4}$ versus NE groups. Images are representative of all samples.

Figure 1



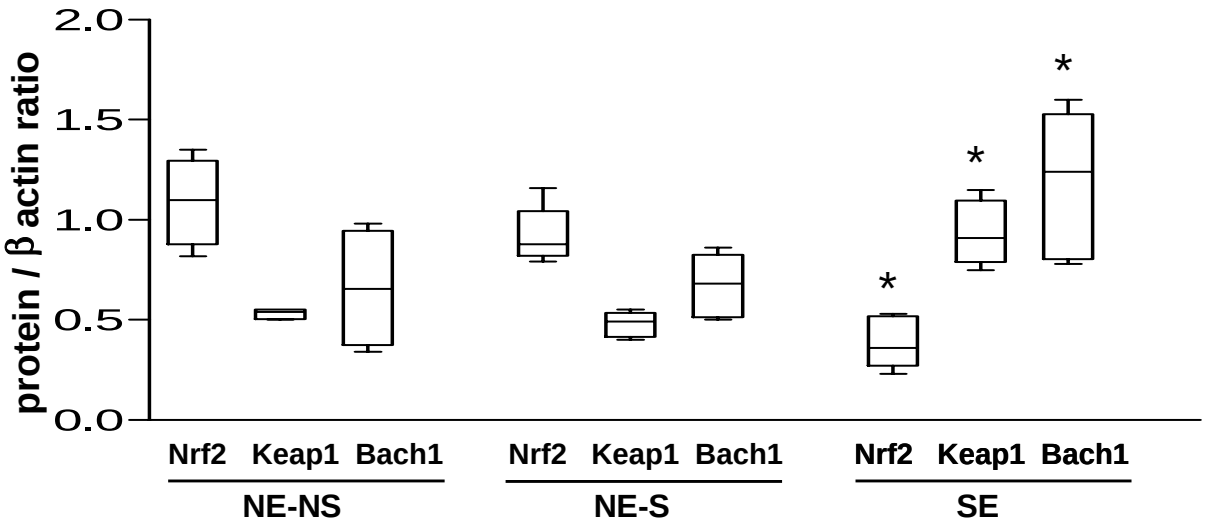
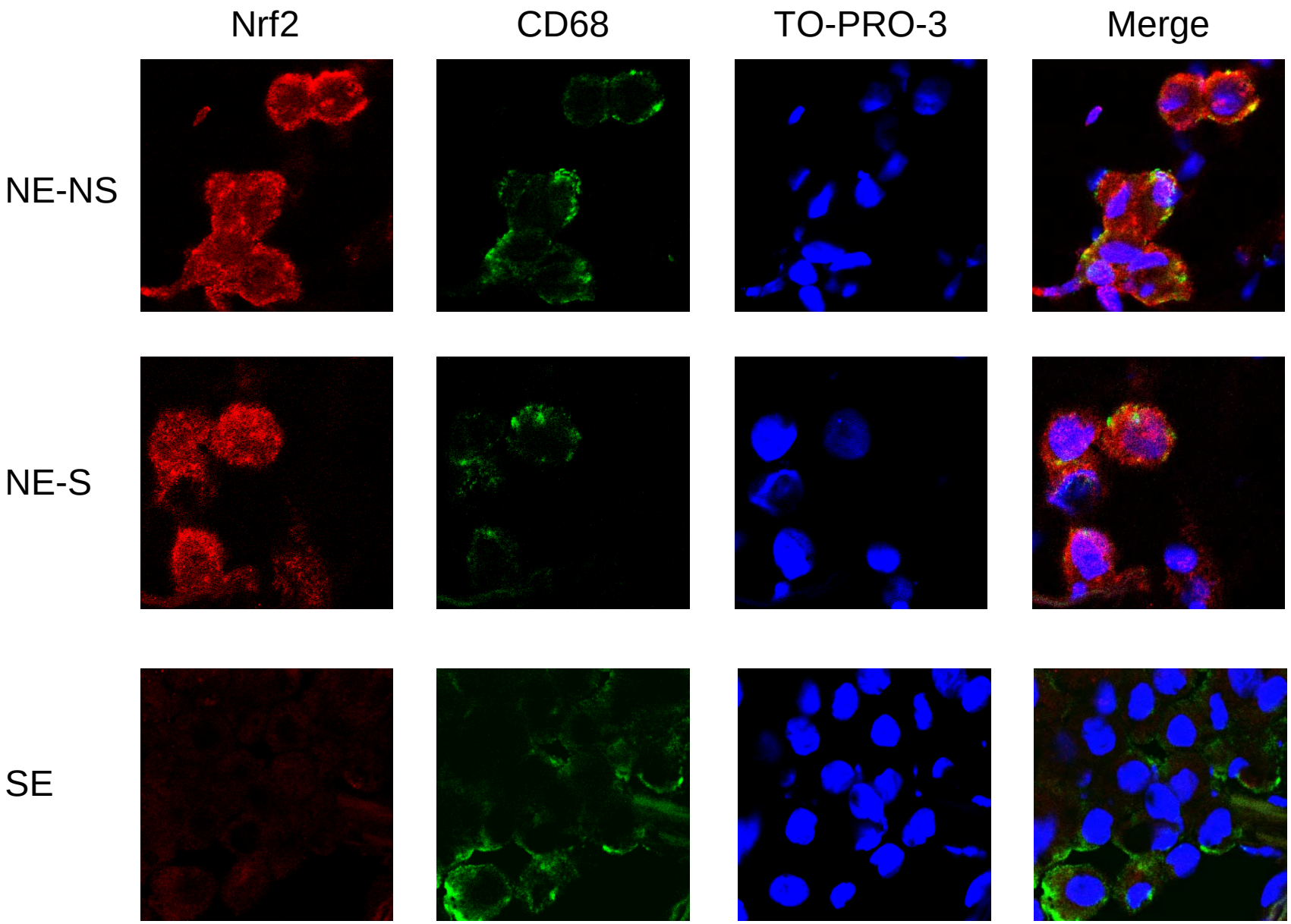


Figure 2 A



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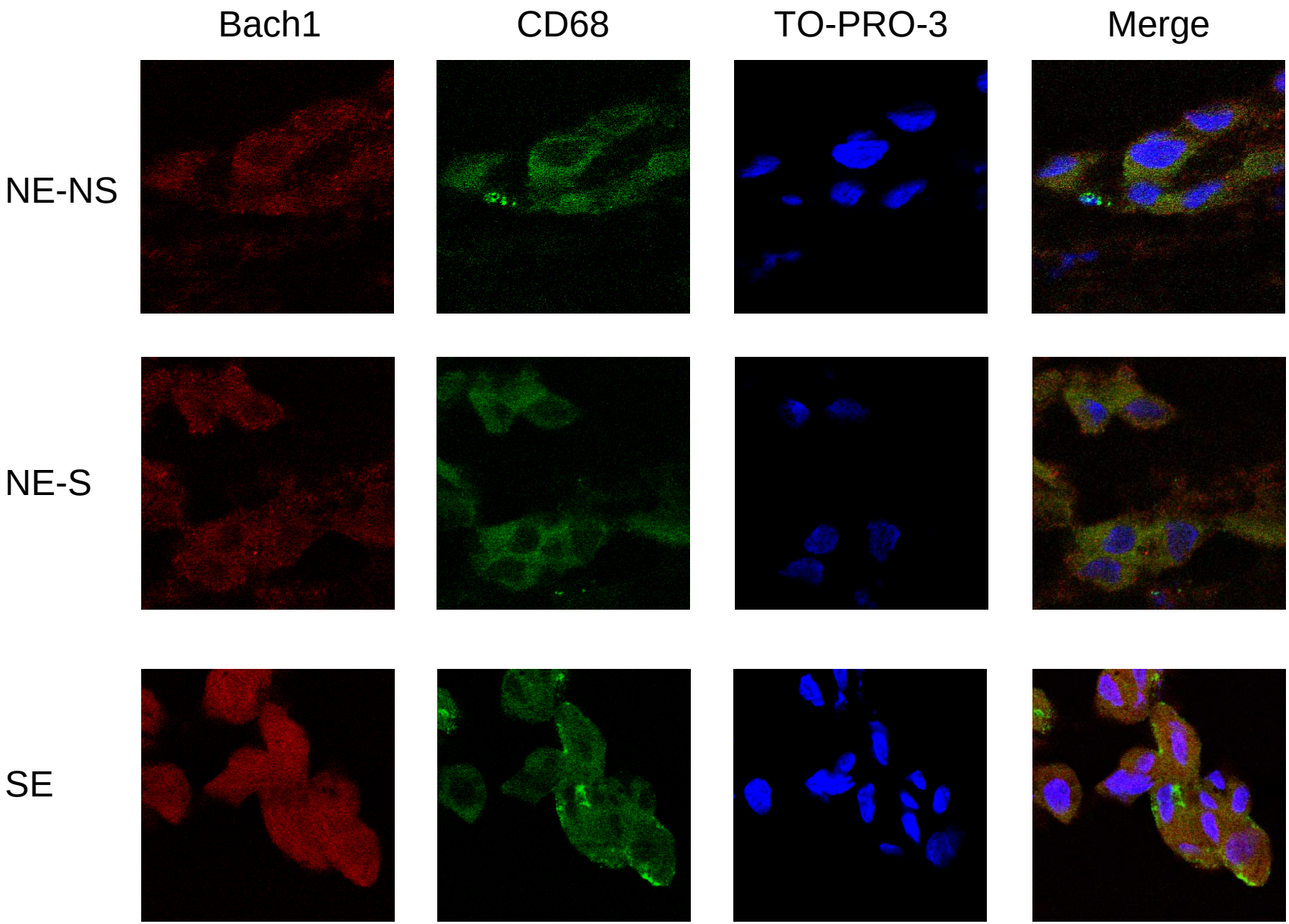

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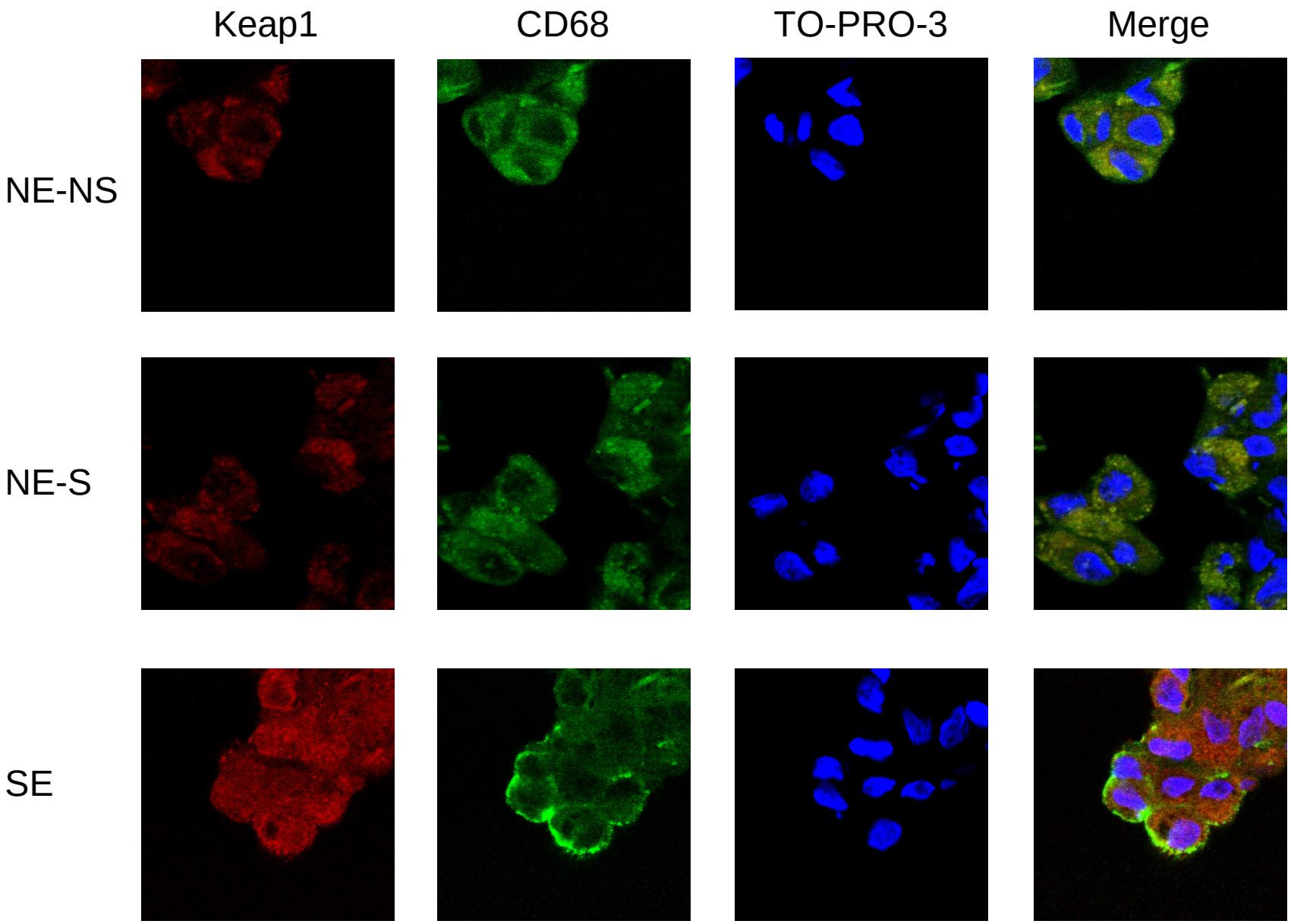

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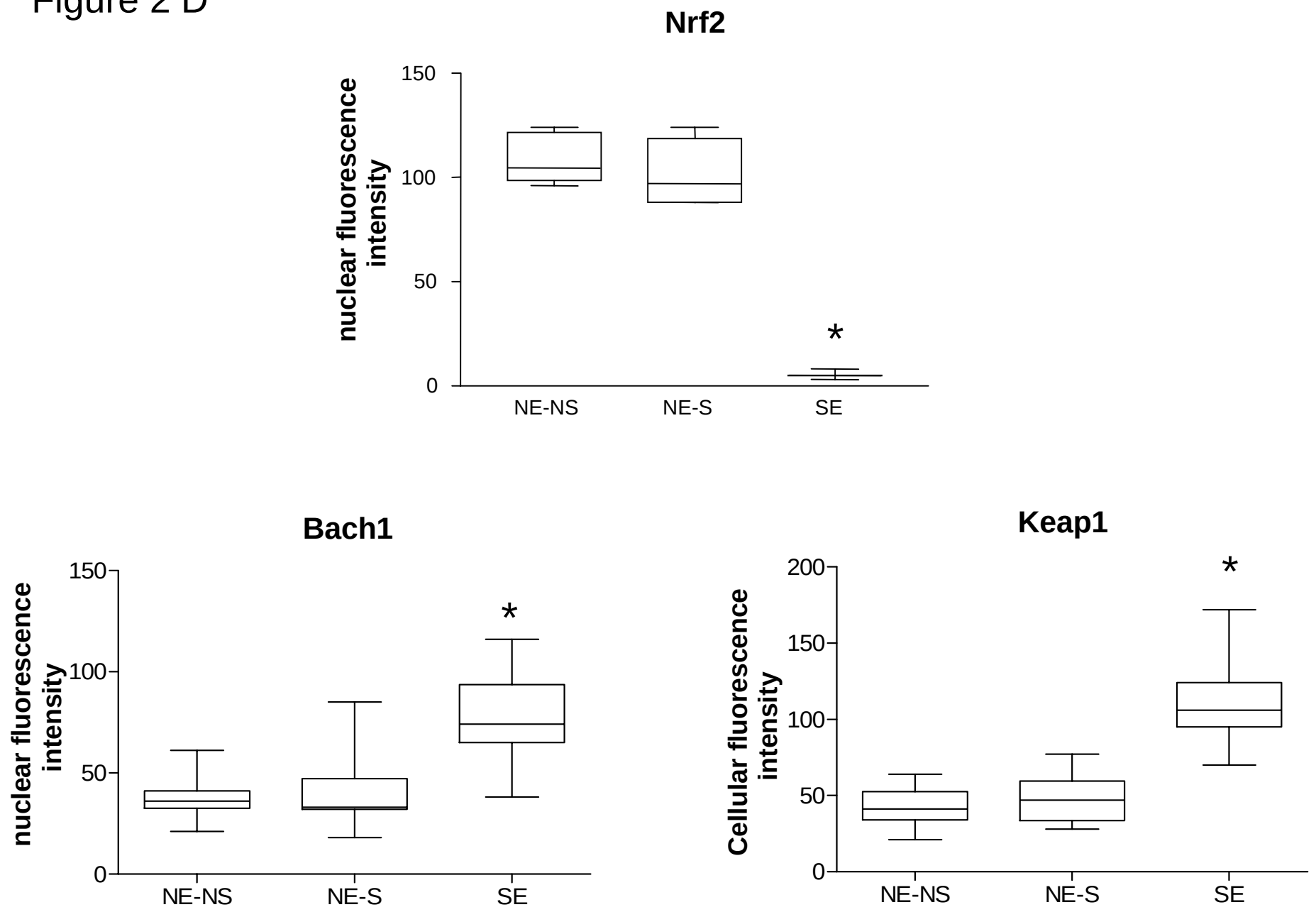

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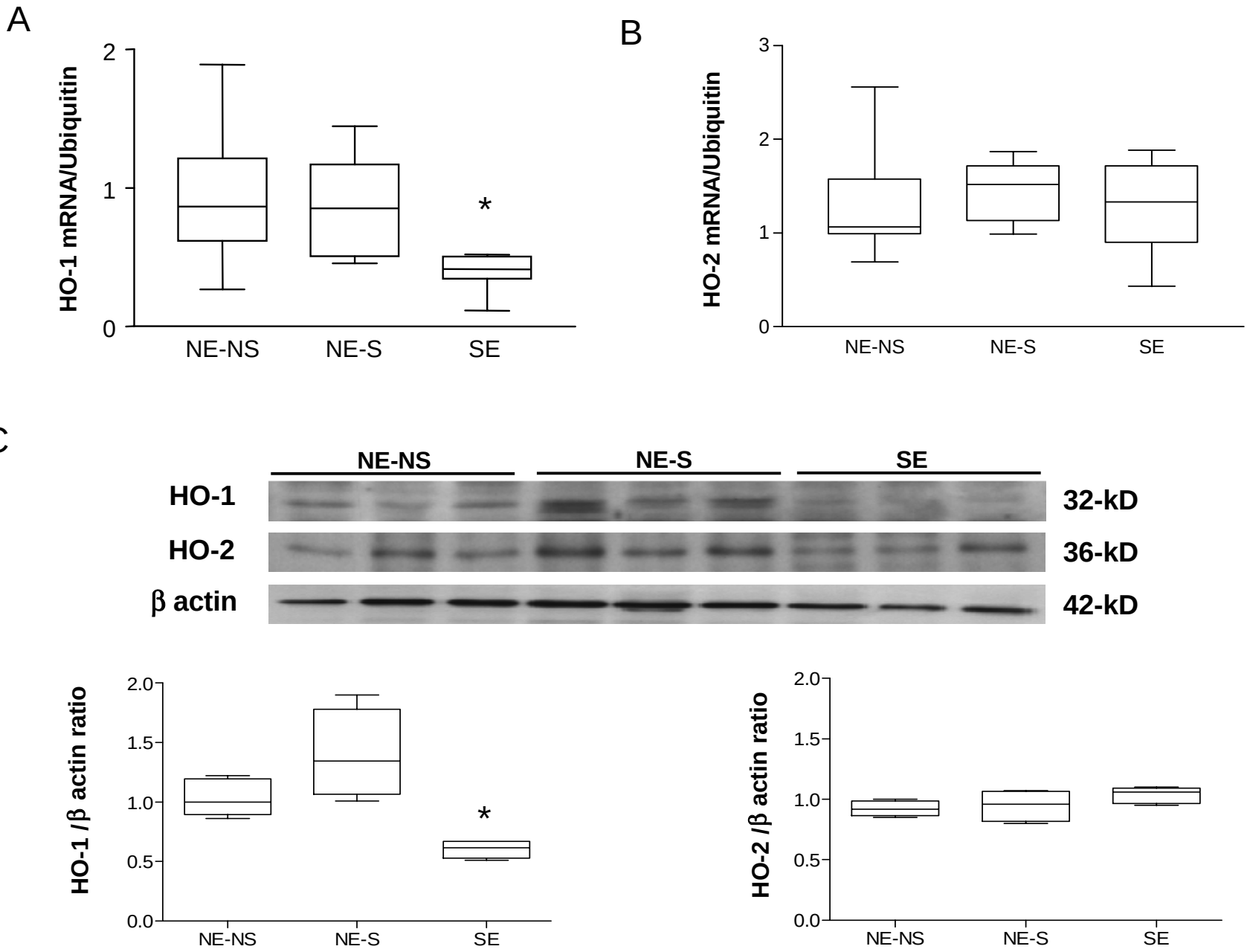
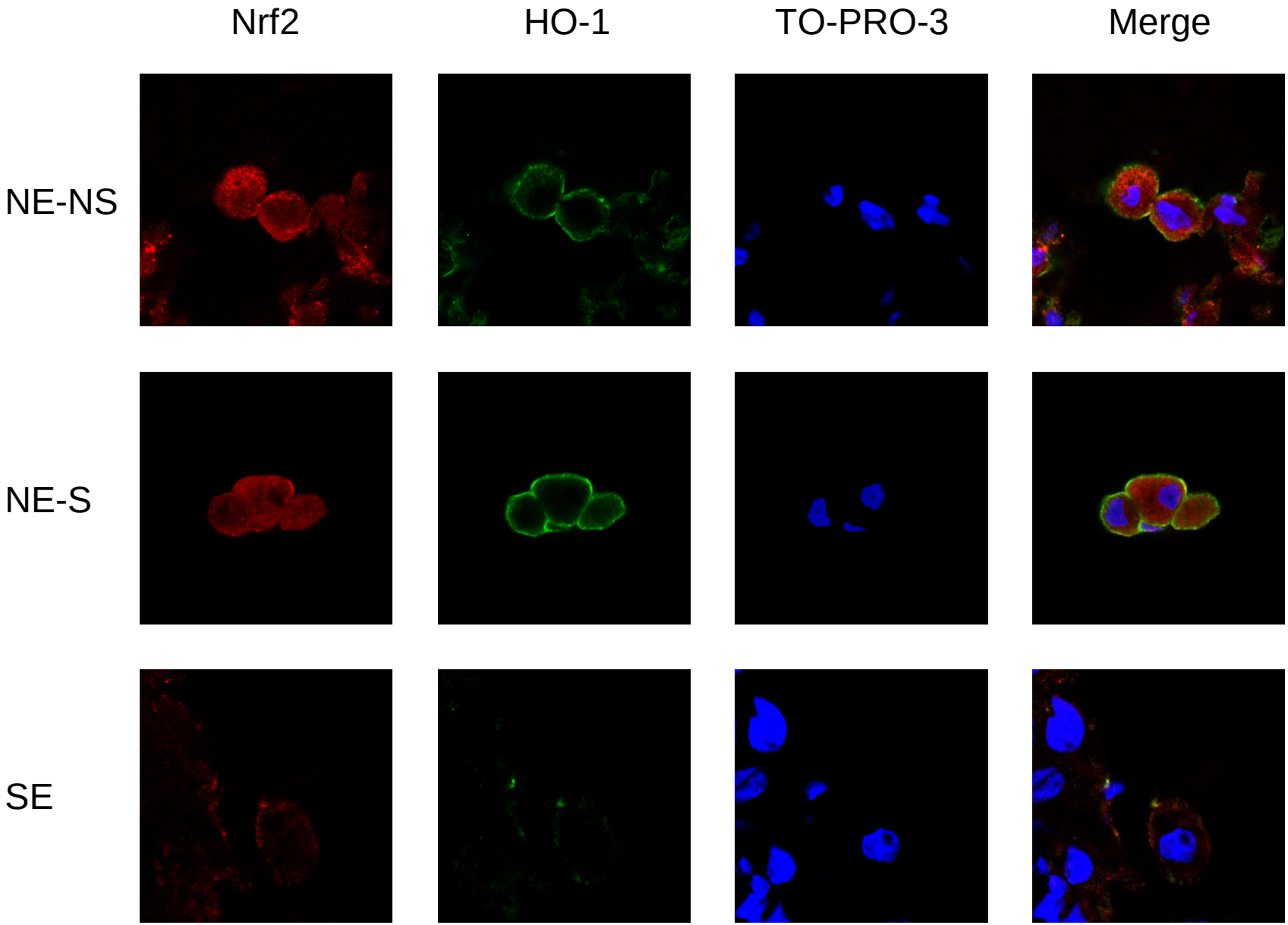


Figure 4 A



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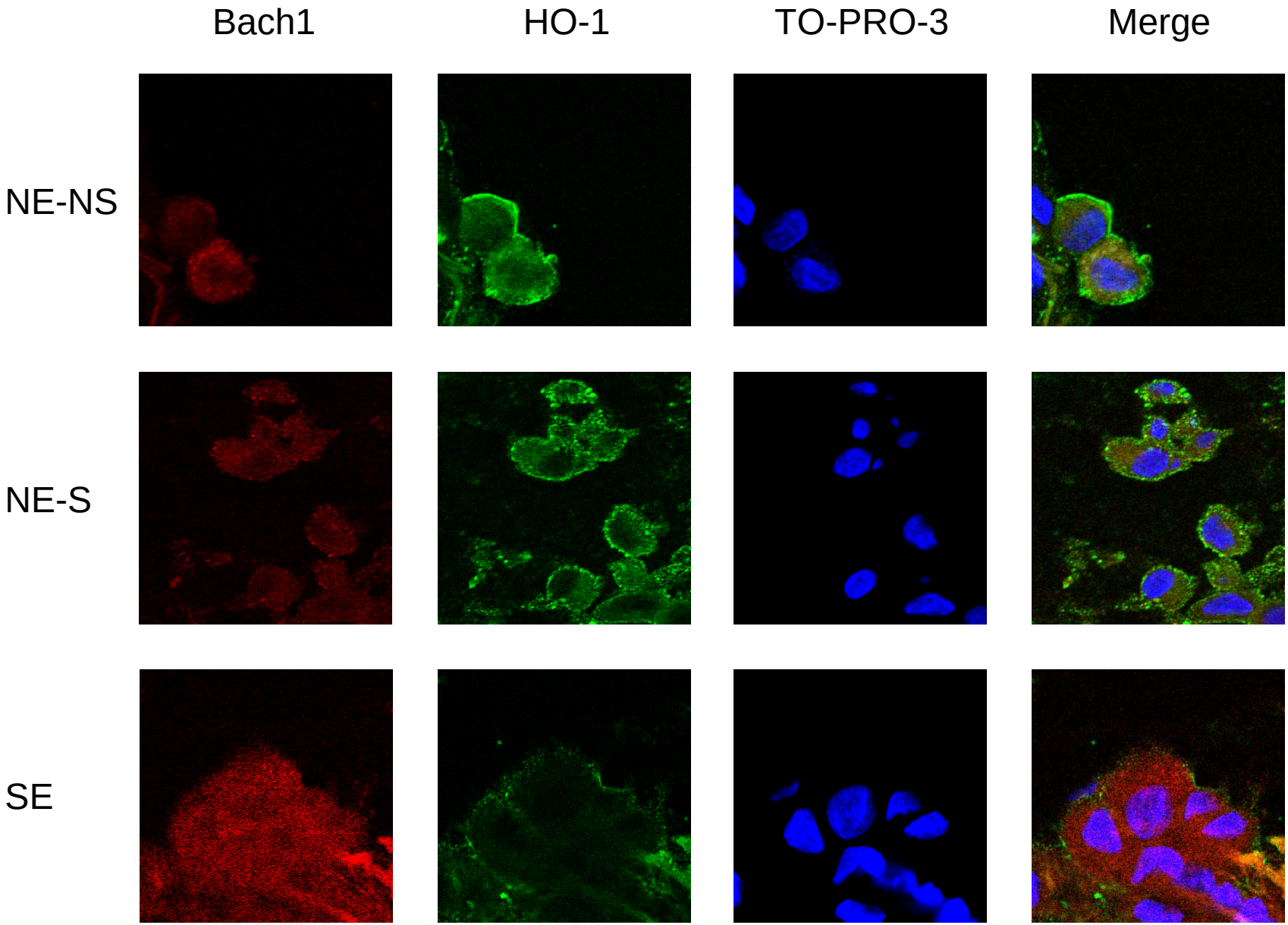

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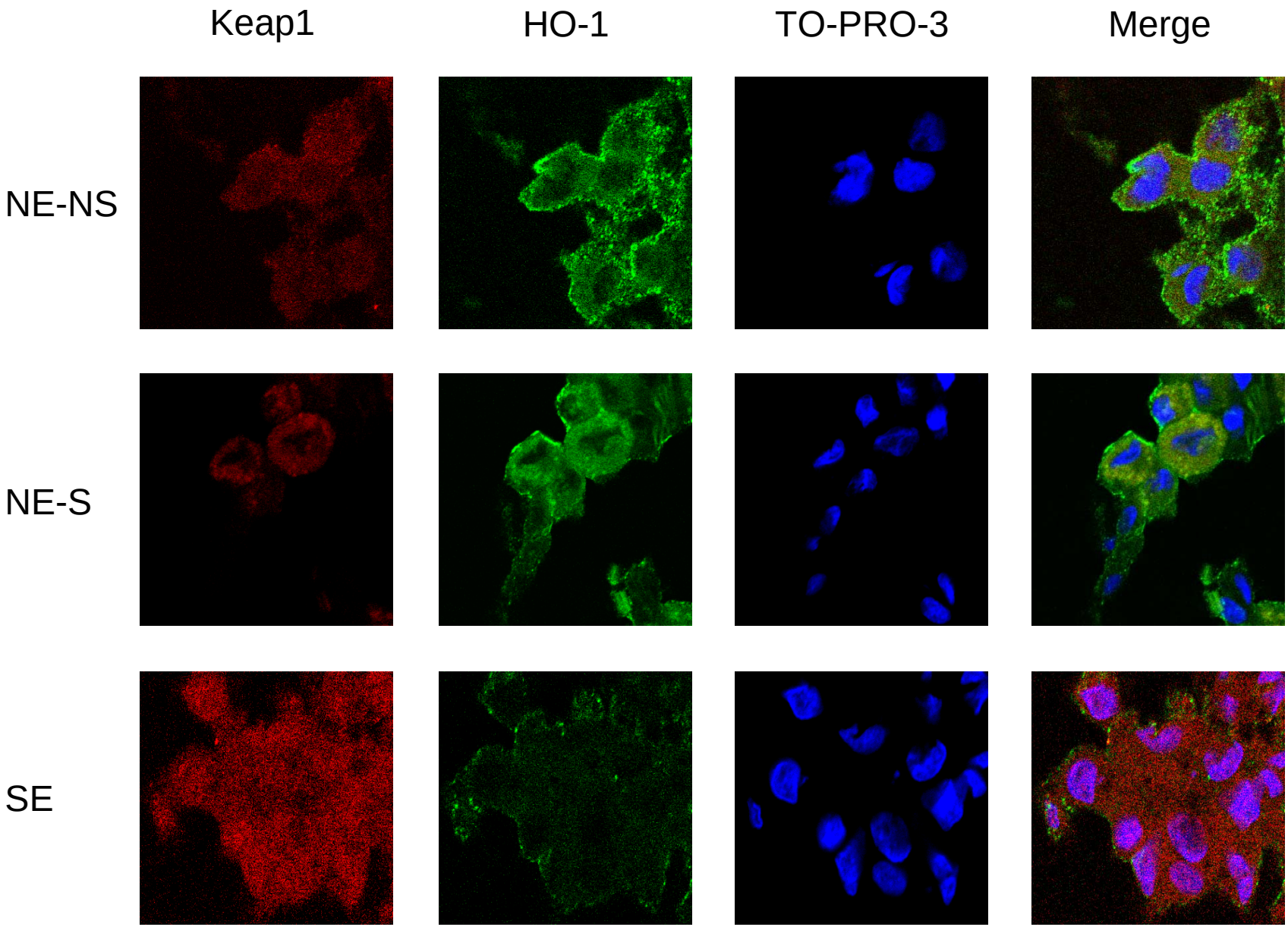

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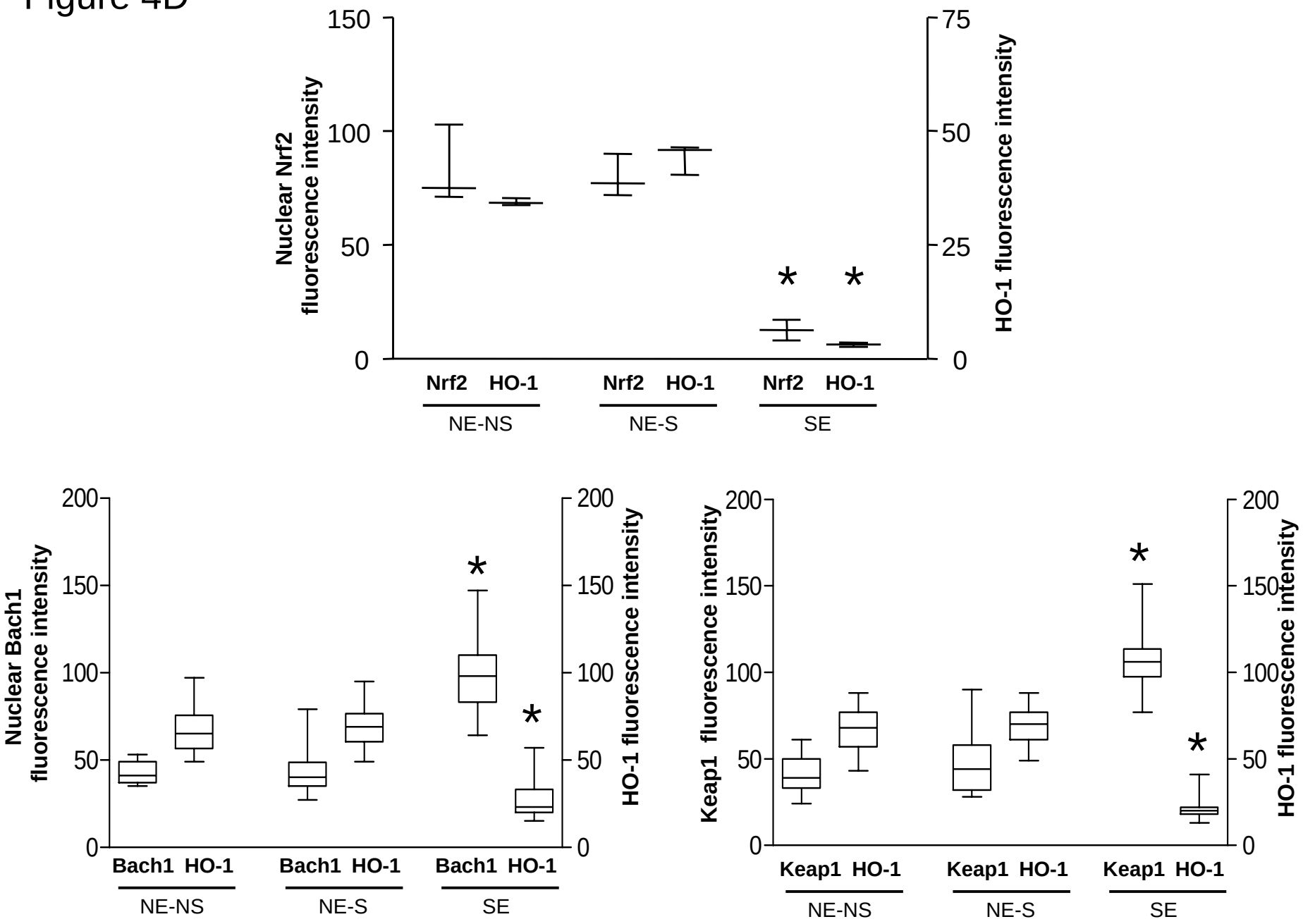


Figure 5

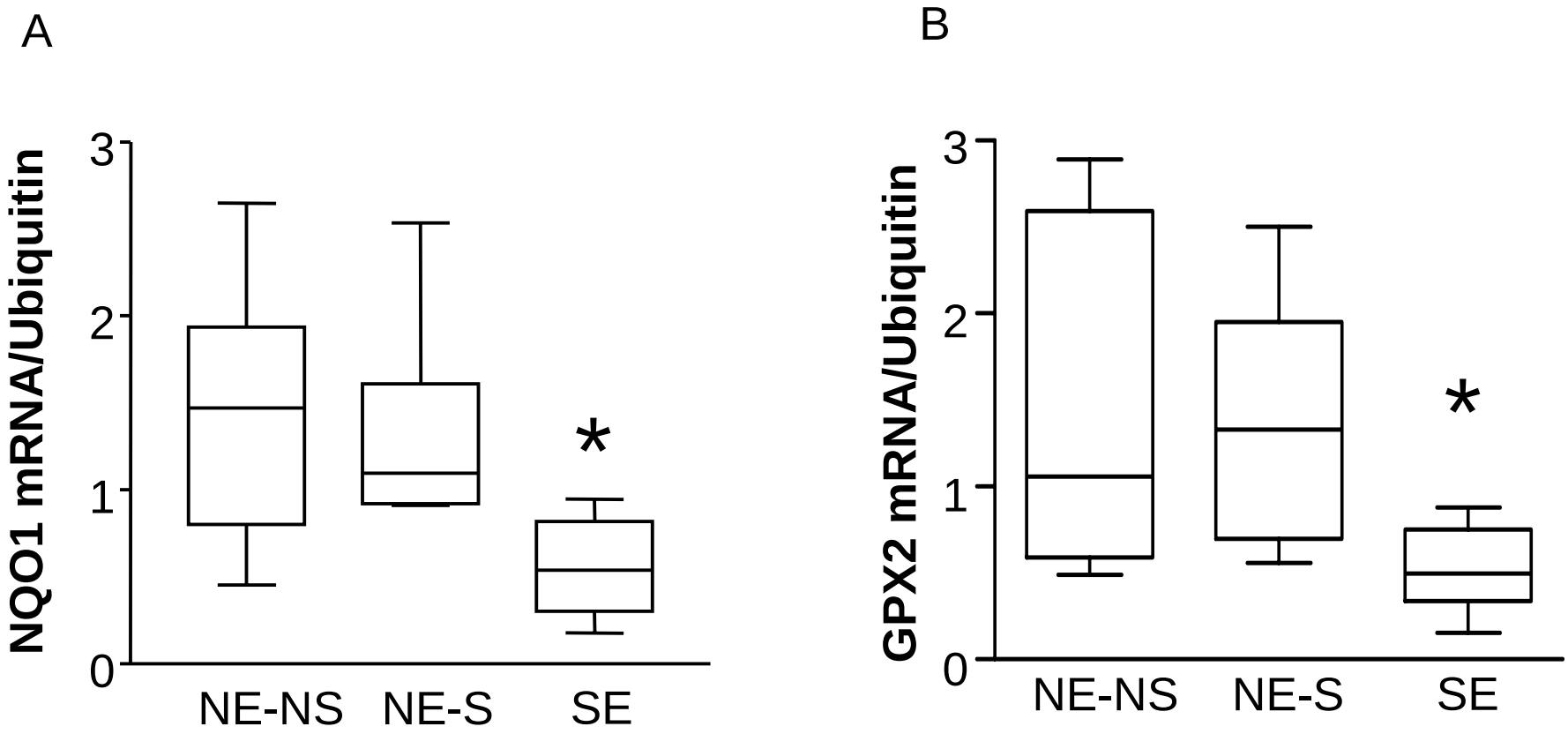


Figure 6 A

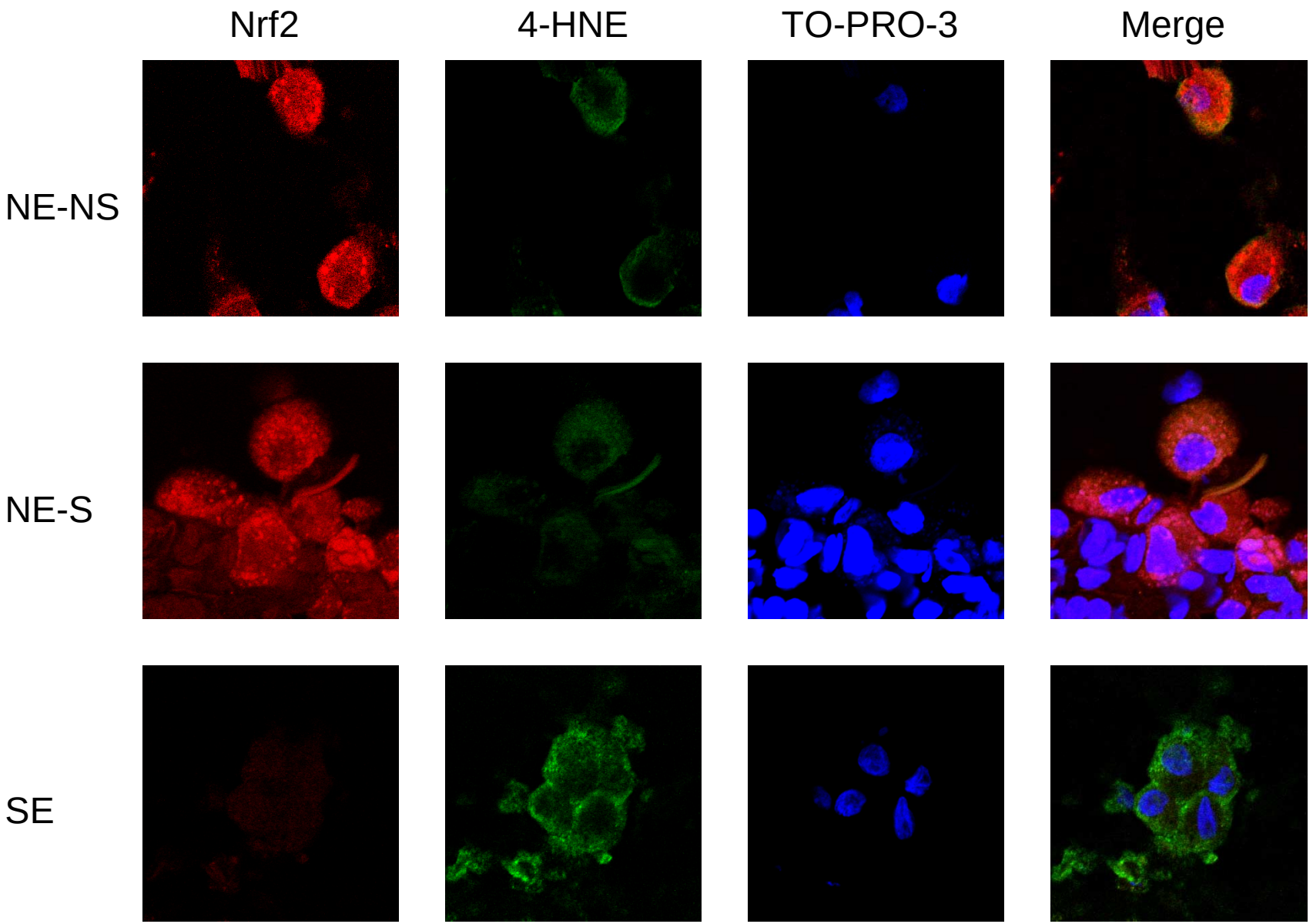
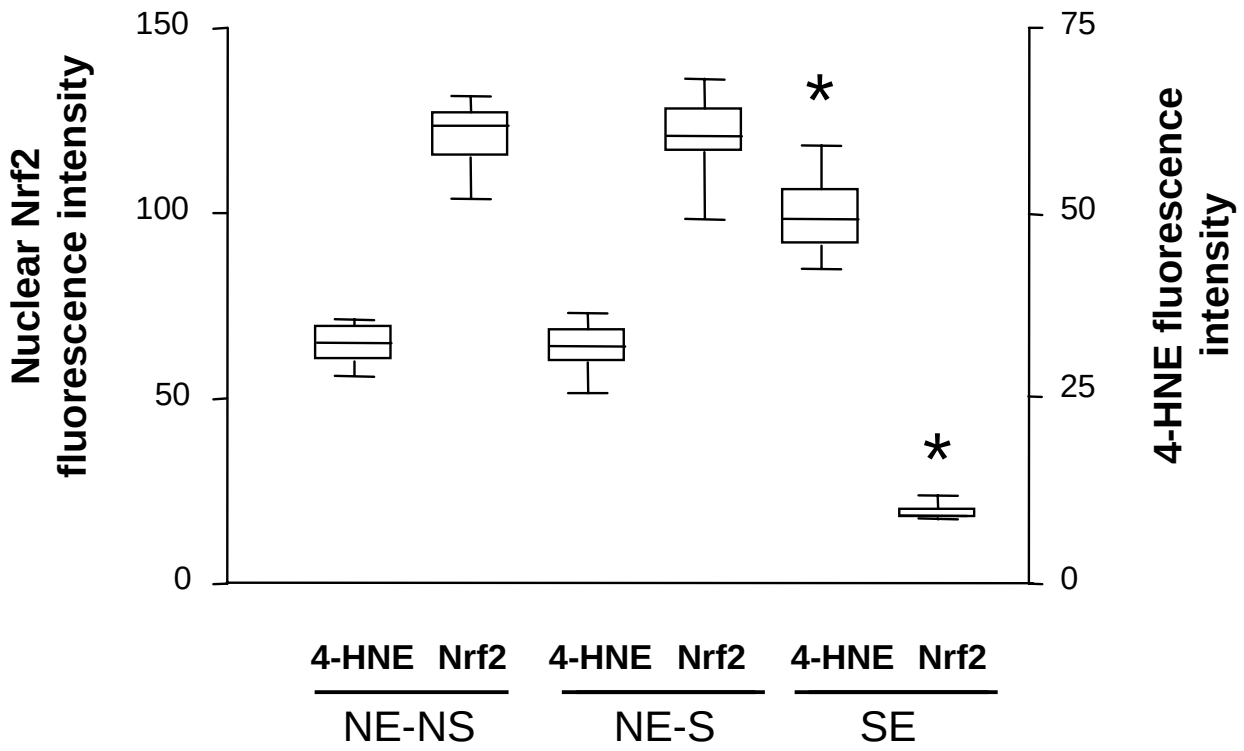


Figure 6 B



ONLY DATA SUPPLEMENT

Altered Nrf2/Keap1-Bach1 equilibrium in pulmonary emphysema

Delphine Goven and Anne Boutten, Véronique Leçon-Malas, Joelle Marchal-Sommé, Nadia Amara, Bruno Crestani, Michel Fournier, Guy Lesèche, Paul Soler,
Jorge Boczkowski and Marcel Bonay

METHODS

Patients

This study was approved by the local ethics committee of Saint Germain en Laye and stored biopsies were reported to our institutional board (Délégation à la Recherche Clinique, Assistance Publique - Hôpitaux de Paris).

Patients with emphysema

Ten patients with severe emphysema (SE) requiring surgery for lung transplantation (n=2) or lung volume reduction (n=8) with emphysema defined radiographically were included (Table I). Ex-smokers were defined as patients who stopped smoking at least 1 year before surgery. Patients with α1-antitrypsin deficiency were excluded. Pulmonary function tests demonstrated severe airflow obstruction and lung distension (Table II). According to the GOLD classification of severity (www.goldcopd.com), SE patients belonged to stage III (severe) or IV COPD (very severe). Seven patients with emphysema were receiving corticosteroids, either oral (n=1) or inhaled (n=7)**.** Tissue samples were taken from the resected parenchyma in a macroscopically emphysematous region.

Patients without emphysema

Normal lung tissue was obtained from 20 patients without radiological, spirometric and histological features of emphysema. Nine patients were smokers (NE-S) and 11 were nonsmokers (NE-NS). NE-S patients were undergoing surgery for resection of a localised primary lung carcinoma (n=8) or a benign lesion (n=1). NE-NS patients were undergoing surgery for resection of a localised primary lung carcinoma (n=6), lung metastases (n=2) or a benign lesion (n=3). Tissue samples were taken at a site distant from the pathological process without macroscopical and microscopical evidence of emphysema. NE-S patients had mild alteration of pulmonary function (Table II). NE-S patients showed lower cumulative exposure to tobacco than SE patients (Table I). No difference was observed between groups for age and sex ratio (Table I).

Processing of lung samples

Lung tissue fragments were immediately frozen in liquid nitrogen and stored at –80°C. The histopathology of biopsies was evaluated on paraffin-embedded sections to verify features of emphysema or normal lung.

Table I: Clinical characteristics

	Nonsmokers without emphysema (NE-NS)	Smokers without emphysema (NE-S)	Patients with severe emphysema (SE)	Between group differences (p)
n	11	9	10	
Age (yr)	58 [32-72]	58.5 [30-68]	57.5 [49-65]	0.99
Sex ratio (F/M)	2/9	0/9	1/9	0.28
Smoking (median no. of packs smoked [range])	0	37.5 [10-50]‡	52 [20-100] †,‡	10^{-4}
Time since smoking cessation (yr)	NA	0 [0-20]	1 [0-8]	0.88
Current smokers/ex-smokers	NA	7/2	2/8	10^{-4}

†: vs. NE-S, p =10^{-3}; ‡: vs. NE-NS, p=10^{-4}; as assessed by Mann-Whitney U-test; NA: not applicable; ns: nonsignificant.

Table II: Pulmonary function tests

	Nonsmokers without emphysema (NE-NS)	Smokers without emphysema (NE-S)	Patients with severe emphysema (SE)	Between group differences (p)
n	11	9	10	
FEV1% predicted value (% pred)	79 [68-101]	78 [60-110]	22 [13-47]†,‡	10^{-4}
FEV1 /FVC	90 [71-105]	68 [64-85]‡	43.5 [35-50] †,‡	10^{-4}
RV (% pred)	95 [55-117]	117 [96-132]	241 [207-349]†,‡	10^{-4}
TLC (% pred)	82.5 [76-99]	100 [84-116]‡	130 [110-153]†,‡	10^{-4}
PaO2 (kPa)	11.4[10.1-12.4]	11.3[10.1-13.3]	9[7.3-10.9]†,‡	8.10^{-4}
PaCO2 (kPa)	5.5[5.1-6]	5.3[4.8-6.1]	5.7[4.5-6.7]	0.36
DLCO (% pred)	Not done	Not done	43.5 [15-67]	Non relevant
6 min walk test (meter)	Not done	Not done	350 [70-540]	Non relevant

†: vs. NE-S, $p=10^{-4}$; ‡: vs. NE-NS, $p=10^{-4}$; as assessed by Mann-Whitney U-test. ns: non significant

Pa_{O2}: arterial oxygen pressure, Pa_{CO2}: arterial carbon dioxide pressure, FEV_1: forced expiratory volume in one second, RV: residual volume, FVC: forced vital capacity, TLC: total lung capacity, DLCO: diffusion lung capacity for carbon monoxide.

Quantitative RT-PCR analysis

Total RNA was extracted from frozen lung tissue and reverse transcribed (Nucleospin RNAII, Macherey Nagel, Düren, Germany). RT-PCR with specific primers (Table III) to quantify the mRNA expression of HO-1, HO-2, GPX2 and NQO1 was performed as described [1]. Ubiquitin-c (UBQ) mRNA served as an endogenous RNA control, with mRNA results expressed as a ratio to UBQ mRNA. Reactions involved use of an MX3000P (Stratagene, La Jolla, CA). All samples underwent all steps in the RT-PCR procedure in the same run for all the samples with blinding to diagnosis.

Table III: Sequence of primer pairs used for amplification of cDNAs.

HO-1	TTCTTCACCTTCCCCAACATTG	CAGCTCCTGCAACTCCTCAAA
HO-2	GGAAACCTCAGAGGGGGTAG	GTGGCCAGCTTAAACAGCTC
NQO1	CAGACGCCCGAATTCAAATC	AGGCTGCTTGGAGCAAAATACA
GPX2	GAACGAGCATCCTGTCTTcG	CCAAATGATGAGCTTGGGATCG
Ubiquitin-c	CACTTGGTCCTGCGCTTGA	TTTTTTGGGAATGCAACAACTTT

Preparation of lung homogenates for western blot analysis

Lung biopsy samples were homogenised in lysis buffer (10 mM Tris-HCl, pH 6.8, SDS 1%, glycerol 5%). The homogenates were centrifuged at 10.000 g for 15 min at 4°C to remove tissue fragments, and supernatants were collected. Aliquots of lung homogenates were stored at -80 °C with 10% Protease Inhibitor Cocktail (Sigma, France). Western blot analysis was performed as described [2]. Primary antibodies were rabbit polyclonal anti-human Nrf2 (1:500 dilution; C-20; Santa-Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-human Keap1 (1:250 dilution; E-20; Santa-Cruz Biotechnology), goat polyclonal anti-human Bach1 (1:250 dilution; C-20; Santa-Cruz Biotechnology), rabbit polyclonal anti-human HO-1 (1:3000 dilution; Stressgen, Tebu, Le-Perray-en-Yvelines, France) and rabbit polyclonal anti-human HO-2 (1:500 dilution; Stressgen, Tebu, Le-Perray-en-Yvelines, France) . Expression of the housekeeping protein β-actin was evaluated with use of a mouse monoclonal anti-β-actin antibody (1:3000 dilution; Sigma, St. Louis, MO). Protein expression was quantified by densitometric analysis under light with use of a charge-coupled device camera and an image analyzer (VisioCapt-Bio1D, Fisher Bioblock Scientific, Illrisch, France). Results are expressed as ratio to β-actin expression.

Immunohistochemical analysis

Immunohistochemistry was performed as previously described [3]. Cryostat sections 4- to 6-µm thick were fixed in acetone and underwent reaction with the primary antibodies rabbit polyclonal anti-human Nrf2 (1:500 dilution), goat polyclonal anti-human Bach1 (1:250 dilution), goat polyclonal anti-human Keap1 (1:50 dilution), rabbit polyclonal antibody anti-human HO-1 (1:1000 dilution), and mouse monoclonal IgG3 anti-human CD68 antibody [macrophage marker] (1:50 dilution; Dako, Glostrup, Denamark, Clone PG-M1). Positive cells were revealed with use of the Vectastain ABC-alkaline phosphatase kit system (Vector Laboratories, Paris, France) and fast red substrate (Dako AAPAAP kit system). To test the specificity of the immunostaining, primary antibodies were replaced by an isotype-matched control antibody and normal serum, with no positive cells identified (data not shown). On adjacent sections, positive cells with characteristic macrophage morphology were evaluated by two independent observers in 10 different high power fields at x200 magnification, and results were expressed as the number of positive cells per mm^2 of lung tissue. Complete agreement in scoring was obtained between two independent observers.

Confocal laser scanning microscopy

Cryostat sections 10-µm thick and THP-1 cells in chamber slide were fixed in 4% paraformaldehyde, saturated with 50 mM NH_4Cl and permeabilised with 0.1% Triton X100. After saturation with normal serum from the species used to produce the secondary antibodies, slides were incubated with appropriate dilution of primary antibodies as used for immunohistochemical staining (1:25 Nrf2; 1:10 Bach1, Keap1 and CD68; 1:100 HO-1; 1:5000 rabbit polyclonal anti-4-HNE antibody [4] (Calbiochem, San Diego, CA)) or normal species-matched serum (control) prior to the addition of secondary fluorescent-labelled antibodies [for Nrf2, Alexa Fluor 546-conjugated antirabbit (1:750 dilution); for Bach1, Keap1 biotinilated antigoat (1:300) + Alexa Fluor 568-conjugated streptavidin (1:400); for CD68 Alexa Fluor 488-conjugated antimouse (1:750) and for 4-HNE and HO-1, Alexa Fluor 488-conjugated antirabbit (1:750)] and treated with RNAse A (1 mg/ml). Cell nuclei were localized by DNA staining with TO-PRO-3 (0.4 µg/ml) (Molecular Probes, Oregon, USA). Slides were mounted with Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA) and examined by confocal laser scanning microscopy (LSM-510-META microscope; Zeiss, Oberkochen, Germany). In parallel, double-immunofluorescence labeling was performed to colocalize CD68, 4-HNE and HO-1 with Nrf2, Bach1 and Keap1. The intensity of nuclear Nrf2, nuclear Bach1 and cellular HO-1, Keap1 and 4-HNE immunofluorescence was quantified in macrophages. No labelling was observed when primary antibodies were replaced by appropriate isotype-matched control antibodies or normal serum.

siRNA transfection

The THP-1 cell line was purchased from the American Type Culture Collection (TIB202, ATCC, Rockville, MD). Cells were grown in RPMI 1640 in the presence of 10% fetal bovine serum and differentiated in Phorbol myristate acetate (PMA) 30 ng/ml for 24h. Cells were transfected with 300nM SMARTpool Nrf2 siRNA (NM_006164; Dharmacon SMARTpool siRNA reagent) or negative control siRNA (Dharmacon plus non targeting pool) using Transpass R2 transfecting reagent as per the manufacturers' instructions. Briefly, Nrf2 or negative control siRNA were added to the transfecting reagent diluted in serum free medium and incubated for 20 min to allow the formation of the transfection complex. The siRNA transfection complexes were added at a final concentration of 300nM to 2 x 10^6 cells/well in T25 flask (for western blot analysis) or 10^5 cells/well in chamber slides (for confocal analysis)

and incubated for 4h following which fresh complete medium was added. Target protein knockdown was assessed between 4 to 24h posttransfection by probing extracts of transfected cells on western blot with anti-Nrf2 antibody. Under these conditions, the transfected cells looked morphologically normal and viability did not differ from untransfected cells (Cytotoxicity detection kit LDH, Roche, Meylan, France).

Preparation of THP-1 protein extracts for western blotting

The whole cell extracts were obtained by lysing THP-1 cells in lysis buffer (150mM NaCl, 10mM HEPES pH 8, 500mM Saccharose, 1mM Na_2EDTA, 1% Nonidet P40). Nuclear and cytosolic fractions were prepared as previously described [5]. Briefly, adherent cells were washed twice and scrapped with phosphate-buffered saline and centrifuged at 7,500 g for 10 min at 4°C. Pellets were suspended in 100 µl MSHE (0.22 M mannitol, 70 mM saccharose, 10 mM Tris-HCl, 0.5mM EGTA, 2mM KHEPES, 0.1% BSA, pH=7.4) and forced 60-fold through 26 Gauge diameter needle and centrifuged 5,000 g 10 min 4°C. 1-Cell pellet containing nuclear proteins was suspended in 100µl wash buffer (10mM Tris pH7.4, 1.5mM EDTA, 0.01% NP-40, 10% Glycerol), centrifuged 5,000 g, 10 min, 4°C. Pellet was resuspended in 30µl nuclear buffer (10mM Tris pH7.4, 1.5mM EDTA, 10% Glycerol, 0.4M KCl), forced 15-fold through 26 Gauge diameter needle and centrifuged at 21,000 g, 30 min, 4°C. Nuclear proteins were contained in the supernatant. 2-Cell supernatant containing cytosolic and mitochondrial proteins was centrifuged 20 min at 15,000g, 4°C. Cytosolic proteins were contained in the supernatant (centrifugation 30 min, 21,000 g, 4°C, the supernatant contains cytosolic proteins) and mitochondrial proteins were in the pellet resuspended in 30µl MSHE. Aliquots were stored at -80 °C with 10% Protease Inhibitor Cocktail (Sigma, France) until western blot analysis. Western blot analysis was performed as described [2]. Expression of the housekeeping proteins β-actin for whole cells and cytosol and lamin B1 (mouse monoclonal anti-lamin B1 antibody (1:1000 dilution; ZL-5; Santa-Cruz Biotechnology, Santa Cruz, CA) for nuclei was evaluated. Results are expressed as ratio to β-actin for whole cells and cytosol or lamin B1 for nuclei.

Statistical analysis

Data were analysed by Statview software (Abacus Concepts, Inc.) and displayed as medians and ranges. Between-group differences were first assessed by nonparametric analysis of variance (Kruskal-Wallis test). In the case of global significant difference, between-group comparisons were assessed by nonparametric Mann-Whitney U-test. Correlations were assessed Spearman's rank order test. Categorical data were analysed by Chi-square test. $p < 0.05$ was considered significant.

Figure E1. Specific immunohistochemical staining of CD68 positive cells in lung biopsies from NE-NS, NE-S and SE patients. Abbreviations are in Figure 1 (magnification x200 and x1000).

Figure E2. Effect of Nrf2 siRNA transfection on Nrf2 and HO-1 protein expression in THP-1 cells. Panel A: Representative western blot of time course Nrf2 and HO-1 protein expression in THP-1 cells transfected with 300nM siRNA for Nrf2 or nonsilencing siRNA (control) with the respective β-actin controls. Panel B: Quantification of Nrf2, and HO-1 proteins. Results are expressed as a ratio to β-actin protein concentration (% vs. time matched control) (n=4,

Nrf2 and HO-1 *p= 0.03 versus control).

Figure E3. Effect of Nrf2 siRNA transfection on Nrf2 in nuclear and cytosolic fraction of THP-1 cells. Panel A: Representative western blot of Nrf2 protein 8h after transfection with 300nM siRNA for Nrf2 or nonsilencing siRNA (control) with the respective lamin B1 and β-actin controls. Panel B: quantification of Nrf2 protein. Results are expressed as a ratio to lamin B1 and β-actin protein concentrations (% vs. control) (n=4, Nrf2 in nuclear and cytosolic fraction *p= 0.03 versus control). Panel C: Representative laser confocal microscopy analysis of THP-1 cells. Immunofluorescent staining was performed with Nrf2 (in red, left column) and HO-1 (in green) and TO-PRO-3 DNA stain (blue). Co-expression is seen by double staining and overlays (Merge column).Upper panel: Control cells were transfected with nonsilencing siRNA. Lower panel: Cells transfected with siRNA for Nrf2.

Figure E4. Expression of HO-1, NQO1, and GPX2 mRNA in THP-1 cells. Box-and-whiskers plot with median, interquartile range and minimum and maximum values. Results are expressed as a ratio to ubiquitin mRNA levels (% vs. time matched control) (n=4, * p =0.03 vs control).

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Figure E1

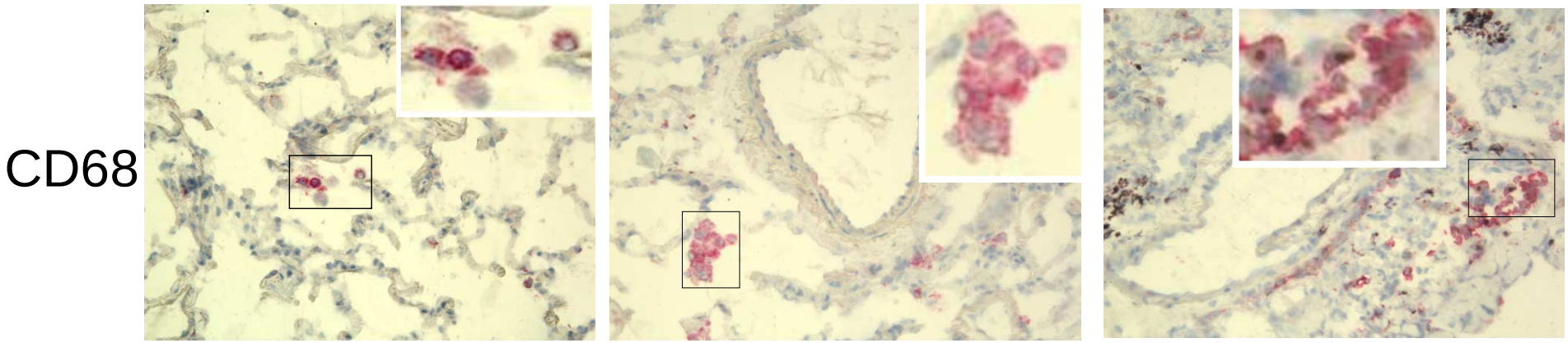
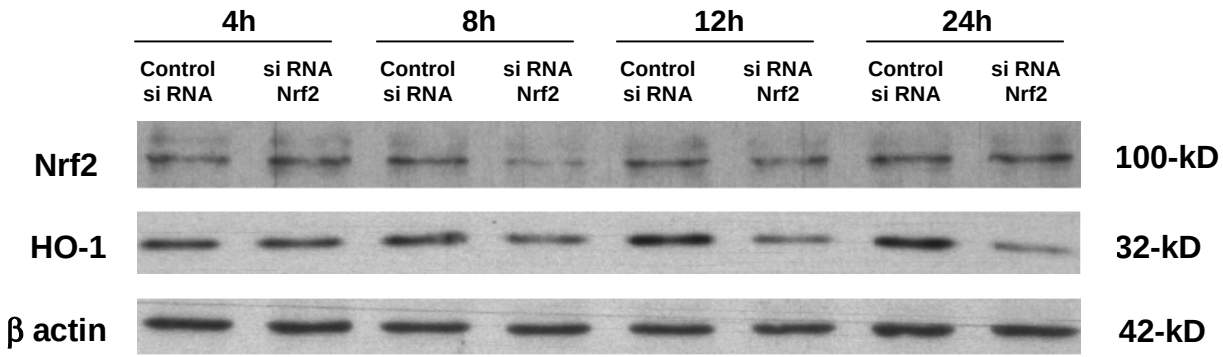


Figure E2

A



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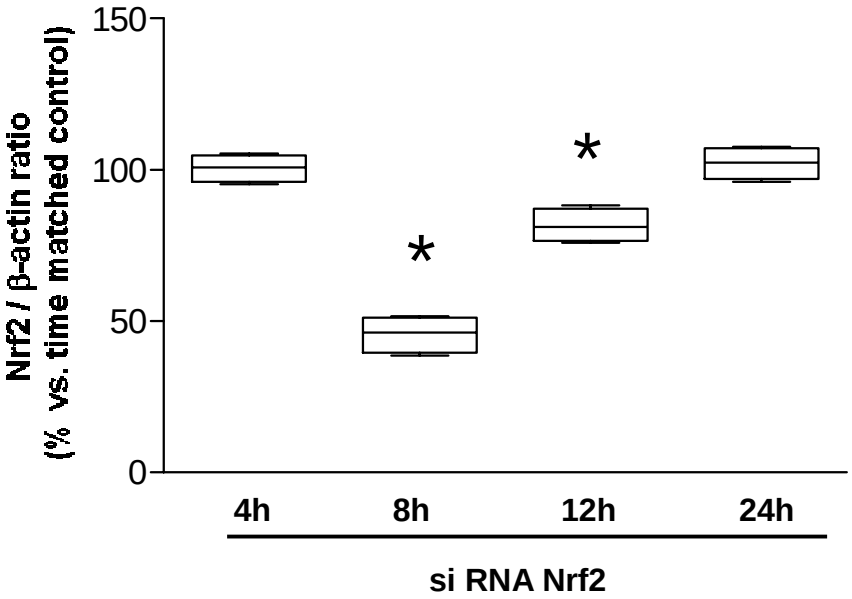

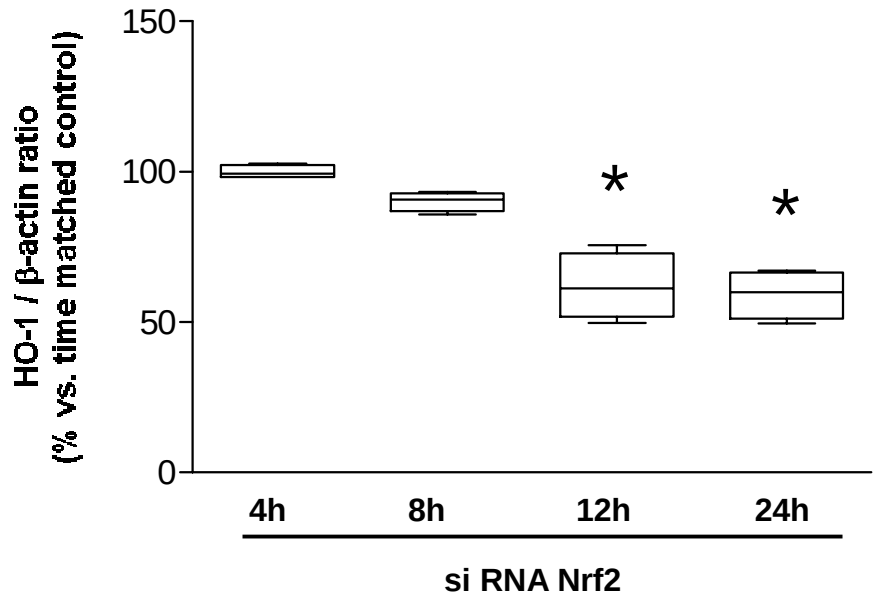

Figure E3

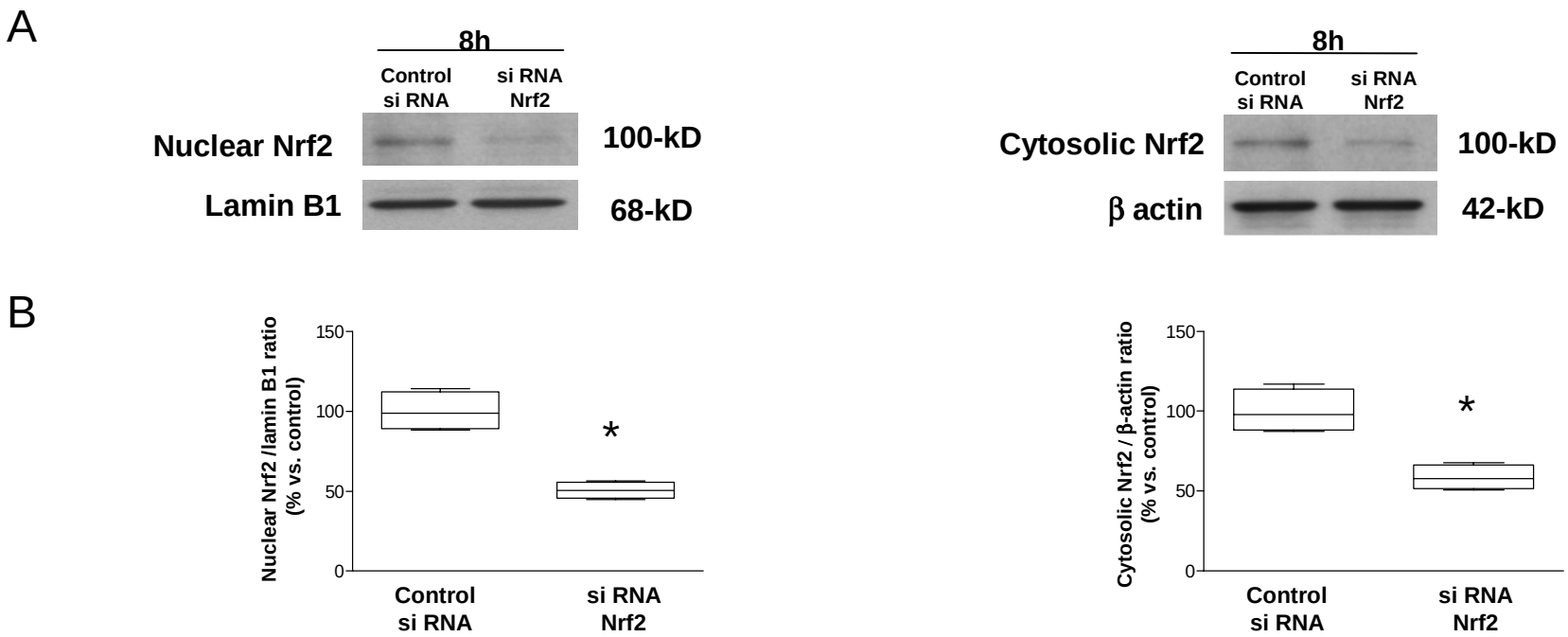

Figure E4