

Peer Review File

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Reviewer

Abstract:

1. The conclusions rehash the findings. The conclusions should provide the significance of the findings and its implications, and give a take-home message.

Conclusions: An increased plasma NOX4d7 level was associated with weaning failure and 28-days mortality in patients with mechanical ventilation. Our results suggest that NOX4-directed management may lead to improved outcomes in patients with mechanical ventilation. (line 52-54)

Introduction:

1. Was the work hypothesis-driven? State the hypothesis.

Based on the hypothesis that plasma NOX4 may predict clinical outcomes such as successful weaning from mechanical ventilation and 28-days mortality, we performed serial assessment of plasma NOX4 levels in intubated patients. (line 82- 84)

2. Explain “STROBE”.

We present the following article in accordance with the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) reporting checklist. (line 85- 86)

3. All acronyms should be spelled out at first mention throughout the manuscript.

Extubation failure is associated with high mortality and prolonged intensive care unit (ICU) stay (1, 2). (line 60-61)

Methods:

1. Provide a narrative on the variables and ranges for all scores used in the study.

APACHE score includes 12 physiological variables, age and previous health status and can give a single score with a maximal of 71. The SOFA score uses the severity of six organ system (liver, lung coagulation, cardiovascular, renal and neurologic system) and offers a final score from 6 to 24. (line 109-112)

Each comorbid condition is assigned a weight ranging from 1 to 6 based on the relative mortality risk and charlson comorbidity index was calculated by totaling the assigned weight for each comorbid condition(16). (line 114-117)

Glasgow Coma Scale (GCS) score is a neurologic scale to assess the patient's consciousness

in the range from 3 to 15. (line 117-118)

Rapid shallow breathing index (RSBI) score was obtained by dividing respiratory rate by tidal volume

(line 132-133)

2. Was the scoring conducted in a masked manner and how many scorers were there to eliminate bias?

The scoring was conducted by two clinicians with more than 5 years of experience in ICU and the investigators were blinded to patient identity. (line 134-135)

3. Page 5, line 116: SBT was already explained on page 3, line 65.

We revised the manuscript.

4. Page 6, lines 144-146 states that patients were stratified according to low and high NOX4 concentrations, however, Table 1 shows no stratification based on those criteria. What specifically was stratification done for?

Patients were stratified into groups according to low or high NOX4 concentrations using the median value for each as the cut-off point (Day 1 NOX4 level: 16.8 ng/mL, Day 7 NOX4 level: 18.2 ng/mL).

(line 154-156)

5. If weaning failure was indeed associated with NOX4 levels, then provide a separate table with the stratifications (low and high levels) in the two extubation groups (failure and success).

We revised table 1.

	Failed extubation (n=87)	Successful Extubation (n=97)	P
Age§	76 (66,83)	69 (57,78)	0.003
Male*	66 (75.9)	66 (68)	0.255
Severity			
APACHE score¶	23(20,28)	19 (15,23)	<0.001
SOFA score¶	9 (7,12)	6 (5,9)	<0.001
Comorbidity*			
ESRD	5 (5.7)	2 (2.1)	0.258
Heart failure	16 (18.4)	15(15.5)	0.694
Diabetes mellitus	31 (35.6)	27 (27.8)	0.27
Cancer	13(14.9)	6 (6.2)	0.057
Hypertension	40 (46.0)	47 (48.5)	0.769

A-fib	13 (14.9)	14 (14.4)	0.998
COPD	9 (10.3)	12 (12.4)	0.817
Liver cirrhosis	4 (4.6)	6 (6.2)	0.751
Stroke	16 (18.4)	13 (13.4)	0.42
Intracerebral hemorrhage	5 (5.7)	9 (9.3)	0.416
Charlson comorbidity index ^a	2(1,3)	2(1,3)	0.225
Cause of intubation [†]			0.504
Cardiac arrest	7 (8.0)	7 (7.2)	
Neurosurgery	15 (17.2)	20 (20.6)	
Stroke	0 (0)	2 (2.1)	
Operation	1(1.1)	4 (4.1)	
Respiratory	62 (71.3)	63 (64.9)	
Sepsis	2 (2.3)	1(1.0)	
Pneumonia ^a	72 (82.8)	77 (79.4)	0.579
CURB-65 ^f	3 (2,4)	2 (2,3)	0.001
PaO2/FiO2	196.7 (137.5,313.3)	260 (178.5,375)	0.013
GCS ^a	6 (5,10)	9 (6,11)	0.001
28-day mortality ^a	59 (67.8)	2 (2.1)	<0.001
ICU mortality [†]	72 (82.8)	6 (6.2)	<0.001
MV duration ^a	15(9,26)	8 (7,15)	<0.001
Crp (mg/dL)	116.8 (41.6, 180)	69.6 (11.4,184.9)	0.061
RSBI ^a	73 (60,83)	62 (49,73)	<0.001
Day 1 plasma NOX4 level (ng/mL) ^a	17.5 (13.8, 27.8)	16.1(12.4,23.3)	0.061
Day 1 NOX4 level above median (16.8ng/mL) [†]	48 (55.2)	44 (45.4)	0.237
Day 7 plasma NOX4 level (ng/mL) ^a	24.2(15.3,32.8)	15.2(12.1,21.2)	<0.001
Day 7 NOX4 level above median (18.2ng/mL) [†]	58 (66.7)	35 (36.1)	<0.001

6. Are the higher levels associated with age since older patients were less likely to be successfully extubated? Studies show that levels of NOX4 generally increases with age (Canugovi C, et al. Redox Biol. 2019).

Patients with extubation failure were significantly older (76 vs.69 years, P=0.003). But we found no significant correlation between the plasma NOX4 level and age (Day 1 NOX4: r = -0.015, P = 0.839, Day 7 NOX4: r = 0.069, P = 0.355)

We revised the manuscript.(The discussion)

While Canugivi et al reported that NOX4 expression and ROS levels increase with age (ref), our data showed no significant correlation between the plasma NOX4 level and age (Day 1 NOX4: $r = -0.015$, $P = 0.839$, Day 7 NOX4: $r = 0.069$, $P = 0.355$).

Patients with extubation failure were significantly older than those with extubation success. However, we found that NOX4 levels >18.2 ng/mL on day 7 showed an independent association with extubation failure and 28-days mortality after adjusting for age and other important clinical variables. (line 245-250)

7. Describe the statistical methods used to compare differences between 7- and 28-day NOX4 levels.

The non-parametric Wilcoxon signed-rank test was used for paired comparisons of the longitudinal change between day 1 and day 7 NOX4 levels. (line 164-165)

Results:

1. Results should be presented according to the order of description to avoid unnecessary confusion. For example, Table 1 is described first on page 7. This should be presented after Figure 1.

The order was rearranged and table 1 was presented after Figure 1.

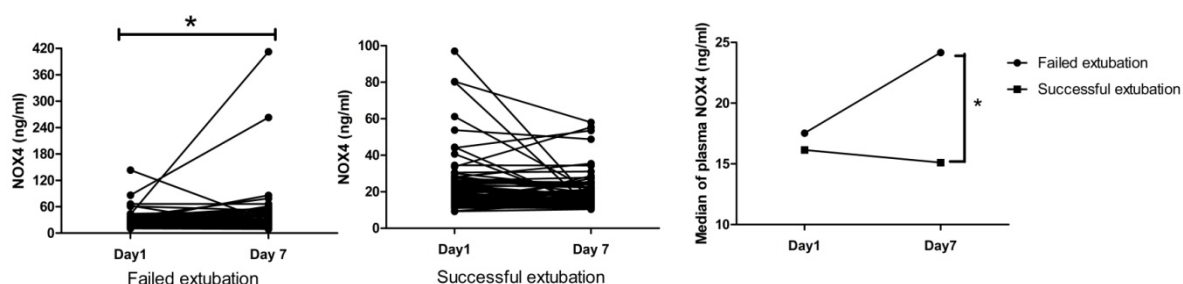
2. Median mortality was 28 days. What was the range?

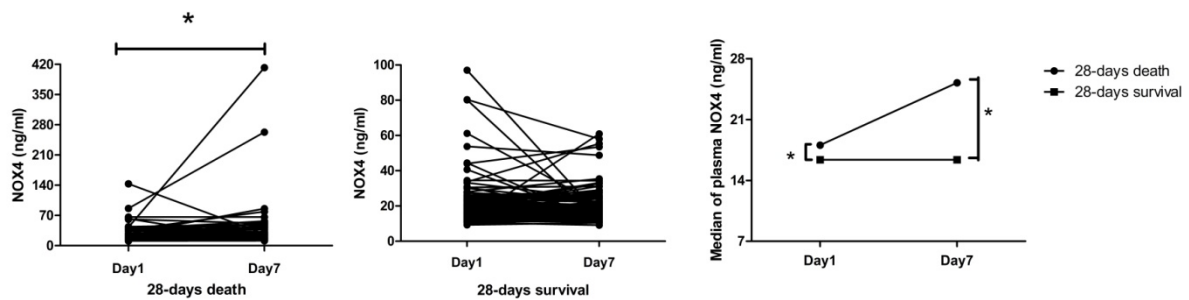
LOS (length of stay) ranges from 7 to 515.

3. Based on the description on page 5, lines 128-129, samples were obtained at 24 hours and at 7 days post mechanical ventilation. Why were samples not collected at later time intervals? Unfortunately, we did not collect samples after 7 days.

4. Figure 4: provide letter to identify the panels.

We provided letter to identify the panels (Lt: day 1, Rt: day7)





5. Figure 4 shows the individual levels of NOX4 for each patient on days 1 and 4. Of the 87 patients who failed extubation, only 2 patients appeared to show increases on day 7, and 1 patient showed a reduction. The remaining 84 patients showed little or no change. Therefore, it is surprising that this is significant. Explain.

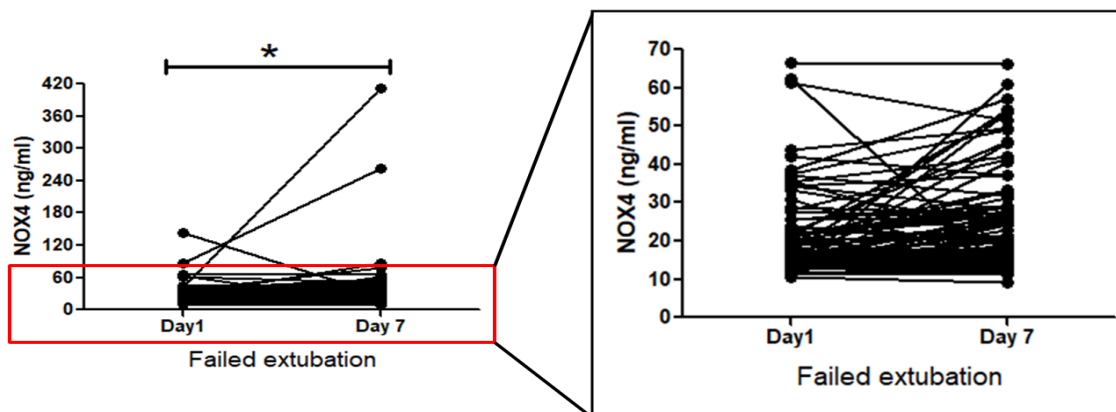
<Figure 4a> Failed extubation

The gap between NOX4 levels of 87 subjects was tight, so the difference seems to be indistinguishable.

If the upper limit of the NOX4 level is lowered, the change trend can be seen in detail.

If we adjust the upper limit of NOX4 level to 70 ng/ml, four are omitted, but the change is clearly visible.

61 is increasing



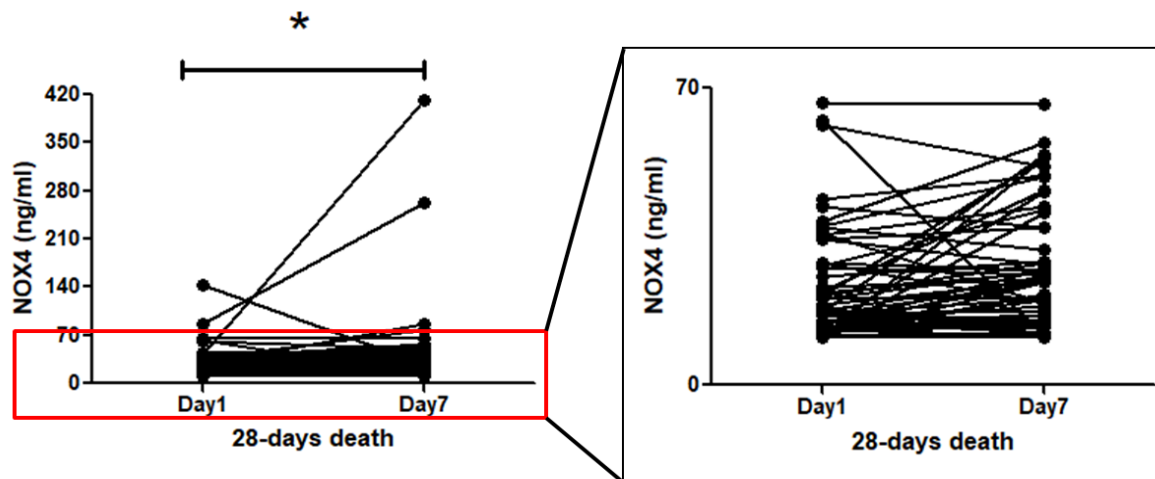
<Figure 4b> 28-days mortality

The gap between NOX4 levels of 61 subjects was tight, so the difference seems to be indistinguishable.

If the upper limit of the NOX4 level is lowered, the change trend can be seen in detail.

If we adjust the upper limit of NOX4 level to 70 ng/ml, four are omitted, but the change is clearly visible.

42 is increasing



6. Same comment for successful extubation.

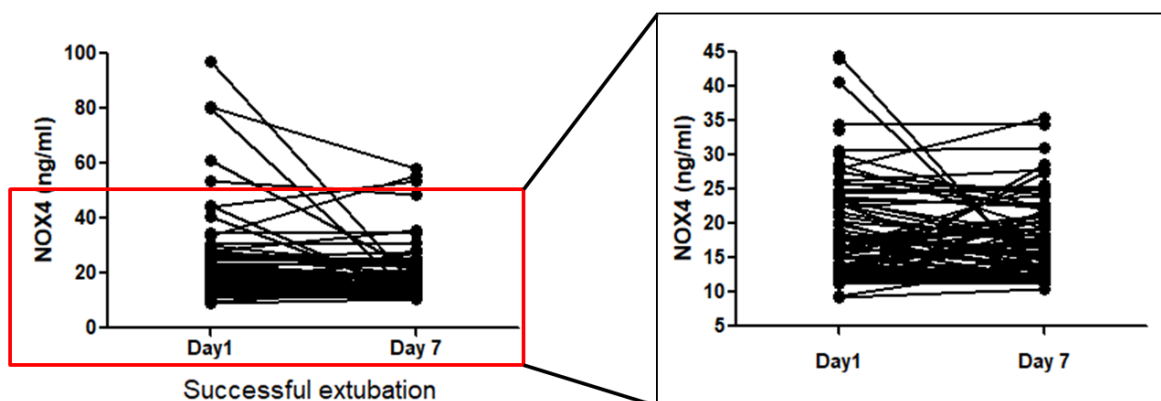
<Figure 4a>successful extubation.

The gap between NOX4 levels of 97 subjects was tight, so the difference seems to be indistinguishable.

If the upper limit of the NOX4 level is lowered, the change trend can be seen in detail.

If we adjust the upper limit of NOX4 level to 45 ng/ml, four are omitted, but the change is clearly visible.

55 is decreasing.

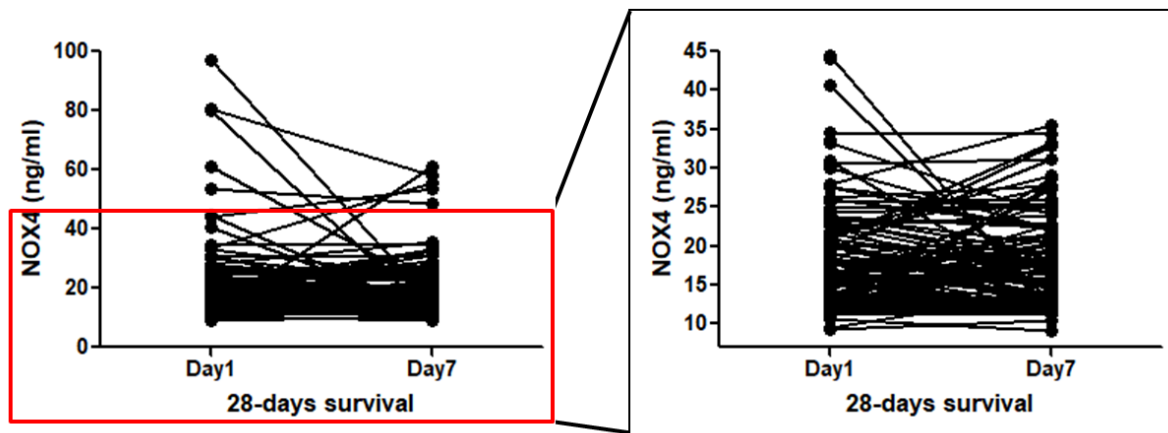


<Figure 4b> 28-days survival

The gap between NOX4 levels of 123 subjects was tight, so the difference seems to be indistinguishable.

If the upper limit of the NOX4 level is lowered, the change trend can be seen in detail.

If we adjust the upper limit of NOX4 level to 45 ng/ml, the change is clearly visible. 63 is decreasing.



Discussion:

1. The authors neglected to discuss that increasing age is associated with NOX4 levels. The findings of elevated NOX4 and reduced weaning success may therefore be a consequence of age.

While Canugivi et al reported that NOX4 expression and ROS levels increase with age (ref), our data showed no significant correlation between the plasma NOX4 level and age (Day 1 NOX4: $r = -0.015$, $P = 0.839$, Day 7 NOX4: $r = 0.069$, $P = 0.355$).

Patients with extubation failure were significantly older than those with extubation success. However, we found that NOX4 levels >18.2 ng/mL on day 7 showed an independent association with extubation failure and 28-days mortality after adjusting for age and other important clinical variables. (line 245-250)

2. The discussion could be greatly improved with more detailed analysis of the figures and results, and less rehashing of the literature.

We added the discussion about the role of NOX4 and shortened the literature review.

Biologically, NOX4 is believed to play a role in endothelial signal transduction, cytoskeletal reorganization, and apoptosis of endothelial cells (13, 28). NOX4 is upregulated in several pulmonary diseases including tuberculous fibrosis, idiopathic pulmonary fibrosis, and lung cancer (29, 30). The previous studies demonstrated that NOX4 was shown to play a distinct role in ventilator-induced lung injury and *Pseudomonas aeruginosa*-induced lung inflammation (11, 31).

While Canugivi et al reported that NOX4 expression and ROS levels increase with age (32), our data showed no significant correlation between the plasma NOX4 level and age (Day 1

NOX4: $r = -0.015$, $P = 0.839$, Day 7 NOX4: $r = 0.069$, $P = 0.355$).

Patients with extubation failure were significantly older than those with extubation success. However, we found that NOX4 levels >18.2 ng/mL on day 7 showed an independent association with extubation failure and 28-days mortality after adjusting for age and other important clinical variables. (line 239-250)