Role of the endothelium and nitric oxide synthases in modulating superoxide formation induced by endotoxin and cytokines in porcine pulmonary arteries

S Muzaffar, J Y Jeremy, G D Angelini, K Stuart-Smith, N Shukla

Background: The interactive roles of cytokines, endotoxins, superoxide (O$_2^-$) and nitric oxide (NO) in the pathogenesis of adult respiratory distress syndrome (ARDS) have not been fully elucidated. The effects of tumour necrosis factor-α (TNF-α), interleukin 1α (IL-1α), and lipopolysaccharide (LPS) and the role of NO and the endothelium in mediating O$_2^-$ formation were therefore investigated in intact porcine pulmonary arteries in vitro.

Methods: Intrapulmonary artery (PA) segments were obtained from White Landrace pigs (25–35 kg) and incubated with LPS, IL-1α, and TNF-α and O$_2^-$ release was measured by the superoxide dismutase (SOD) inhibitable reduction of ferricytochrome c. The source of O$_2^-$ formation was determined using a number of enzyme inhibitors. The role of NO was explored using NO synthase (NOS) inhibitors and the distribution of NOS isoforms and peroxynitrite (ONOO$^-$) assessed by immunocytochemistry.

Results: LPS, IL-1α, and TNF-α promoted the formation of O$_2^-$ from PA compared with untreated controls in a time and dose dependent manner, an effect markedly enhanced by removal of the endothelium but completely inhibited by the NADPH oxidase inhibitor diphenylene iodonium chloride (DPI). t-NAME and the eNOS inhibitor N'-(1-iminoethyl)-ornithine (iNIO) enhanced O$_2^-$ formation from PA (with endothelium) in response to IL-1α and TNF-α but had no effect on LPS meditated O$_2^-$ formation, whereas L-NAME and the iNOS inhibitor L-Nω-(1-iminoethyl)-lysine-HCl (L-NIL) enhanced O$_2^-$ formation only in response to LPS.

Conclusions: LPS, IL-1α, and TNF-α promote O$_2^-$ formation through an upregulation of NADPH oxidase activity which is augmented by removal of the endothelium, as well as the inhibition of eNOS (in the case of cytokines) and iNOS (in the case of LPS). The concomitant expression of NOS isoforms and NO formation with that of NADPH oxidase may therefore constitute a protective system designed to remove O$_2^-$ through the formation of ONOO$^-$. If this is so, the integrity of the endothelium may be axiomatic in the progression and severity of ARDS.

Oxidative stress plays a central role in the aetiology of adult respiratory distress syndrome (ARDS), a condition characterised by a time dependent worsening of intrapulmonary inflammation and hypertension. Principal among the reactive oxygen species (ROS) generated by oxidative stress is superoxide (O$_2^-$) which promotes vasoconstriction, damage to the endothelium, adhesion molecule expression, and thus perpetuation of inflammation. O$_2^-$ also reacts with nitric oxide (NO) to produce peroxynitrite (ONOO$^-$) which, apart from reducing endogenous NO bioavailability, is inflammatory in its own right. In turn, a reduction of NO availability promotes not only vasoconstriction, but also the adhesion of leucocytes and platelets which release a battery of vasoconstrictors and cytokines. The negation of NO by O$_2^-$ therefore plays a key role in the pathogenesis of ARDS through the exacerbation of ongoing inflammatory cascades and the development of pulmonary hypertension. The importance of NO in ARDS has been confirmed by studies which have shown a beneficial effect of inhaled NO in ameliorating a reduction in leucocyte activity and oxidant stress.

In ARDS there are several potential sources of O$_2^-$. In the initial stages of the syndrome endotoxins activate neutrophils, monocytes and platelets and promote their adhesion to the pulmonary vessel walls. Neutrophils, through the respiratory burst reaction, then release large amounts of O$_2^-$ which reduces NO availability. Adherent neutrophils and monocytes also release tumour necrosis factor-α (TNF-α) and interleukins (ILs), the blood levels of which are markedly raised in patients with ARDS. In turn, lipopolysaccharide (LPS), TNF-α and ILs upregulate enzymes that generate O$_2^-$ in cultured vascular tissues (particularly NADPH oxidase) and there is a causal relationship between increased O$_2^-$ formation and impaired vasodilation. It is therefore reasonable to suggest that endotoxins and cytokines augment O$_2^-$ in the pulmonary vasculature in ARDS.

The effect of LPS, IL-1α, and TNF-α on O$_2^-$ formation in whole porcine pulmonary artery (PA) segments was investigated in vitro, and the interactions between LPS, IL-1α, and TNF-α were examined. The effects were also studied over a 16 hour time course since ARDS is a dynamic time dependent process. The source of O$_2^-$ was determined with enzyme inhibitors of NADPH oxidase, xanthine oxidase, and mitochondrial respiration. Since little is known about the relative contributions of endothelial cells and vascular smooth muscle cells to overall O$_2^-$ formation in pulmonary arteries, O$_2^-$ formation was also measured in PA segments in which the endothelium had been removed. In addition, the role of inducible NO synthase (iNOS) and endothelial NO synthase (eNOS) in mediating overall O$_2^-$ production by PA was studied using specific inhibitors of these enzymes as well as immunocytochemistry.

METHODS

Dissection and incubation of pulmonary arteries

Lungs were obtained from White Landrace male pigs of 20–25 kg body weight. All animal experiments were conducted in accordance with the rules and regulations of Bristol.
University and the Home Office regarding the care and use of experimental animals. Pigs were anaesthetised with an intravenous injection of ketamine hydrochloride (10 mg/kg; Ketaset Injection, Fort Dodge Animal Health, Southampton, UK) and inhaled oxygenated halothane. The internal carotid artery was exposed, a cannula was placed in the carotid artery, and the animals were then exsanguinated. The chest was opened by median sternotomy and the lungs excised from the chest. Pulmonary arteries (PA) of 3–4 mm diameter (1st order) or 200–400 mm (4th order) were dissected from the lungs within 30 minutes and placed in Dulbecco’s Minimum Essential Medium supplemented with Glutamax-1, 100 U/ml penicillin, and 100 μg/ml streptomycin (DMEM; GibcoBRL, Paisley, UK). The arteries were cut into 2–3 mm± segments for experimentation. In some studies the endothelium was removed by gently rubbing the luminal surface with a cotton wool bud.

The segments were then incubated for up to 16 hours in serum-free DMEM containing LPS (E coli O26:B6; Sigma, Poole, Dorset, UK), human recombinant IL-1α (R&D Systems, Abingdon, UK), and human recombinant TNF-α (R&D Systems) alone and in combination with each other. After incubation the segments were washed in Dulbecco’s phosphate buffered saline (PBS, GibcoBRL) and the formation of O2·− was measured.

**Measurement of superoxide (O2·−)**

The measurement of O2·− release by arterial segments was performed by detection of ferricytochrome c reduction. Following incubation, arterial segments were rinsed three times with PBS and equilibrated in DMEM without phenol red for 10 minutes at 37°C in a 95% air/5% CO2 incubator (Heraeus, Hera Cell). The formation of O2·− was determined at 550 nm using a spectrometer (Lab-tech International, Ringmer, East Sussex, UK) and converted to nmol O2·− using ΔEmax = 21.1/mM/cm as the extinction coefficient for (reduced-oxidised)
cytochrome c. The reduction of cytochrome c that was inhibitable with SOD reflected actual \( \text{O}_2^- \) release. Segments were blotted, dried and weighed, and the data were expressed as nmol \( \text{O}_2^- \)/mg tissue/h.

**Effect of enzyme and NOS inhibitors on \( \text{O}_2^- \) release**

To determine the source of the \( \text{O}_2^- \) formed, PA segments were preincubated with 10 \( \mu \)M diphenylene iodonium chloride (DPI, Sigma), an NADPH oxidase inhibitor; 10 \( \mu \)M rotenone (Sigma), an inhibitor of mitochondrial respiration; and 100 \( \mu \)M allopurinol (Sigma), an inhibitor of xanthine oxidase, for 2 hours before measurement of \( \text{O}_2^- \).

To study a possible role for NOS derived NO in modifying \( \text{O}_2^- \) formation (\( \text{O}_2^- \) + NO = ONOO\(^-\)), the effect of NOS inhibitors was studied using (1) the non-specific NOS inhibitor \( \text{N}^\text{-nitroarginine methyl ester} \) (\( \text{N}^\text{-NAME}, 100 \mu\text{M; Sigma}), an inhibitor of mitochondrial respiration; and 100 \( \mu \)M allopurinol (Sigma), an inhibitor of xanthine oxidase, for 2 hours before measurement of \( \text{O}_2^- \).

Immunocytochemical analysis of eNOS, iNOS, and nitrated tyrosine

Immunocytochemical analysis of eNOS, iNOS, and nitrated tyrosine (NT) was carried out in selected samples following incubation for 16 hours with LPS (1 \( \mu \)g/ml), IL-1\( \alpha \), or TNF-\( \alpha \) (both 10 \( \mu \)g/ml) which were then snap frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). Cryostat sections (8 \( \mu \)m) were prepared and fixed in acetone for 10 minutes. The endogenous peroxidase activity was inhibited with 1.2\% \( \text{H}_2\text{O}_2 \) in methanol for 10 minutes. Sections were then treated with horse (for NT staining) or goat serum (for eNOS and iNOS) diluted 1:3 with Tris-buffered saline (TBS; Sigma), pH 7.4, drained and incubated with monoclonal antibodies against eNOS, iNOS (Transduction Laboratories, Oxford, UK) and nitrated tyrosine (Upstate Biotechnology, Buckinghamshire, UK) at a dilution of 1:200 for iNOS and eNOS and 0.3 \( \mu \)g/ml for NT overnight at 4\(^\circ\)C. After washing in TBS the sections were treated with either biotinylated goat anti-rabbit (for NT; 1:200 dilution) or biotinylated goat anti-mouse (for eNOS/iNOS; 1:200 dilution) for 1 hour at room temperature, washed and further treated for an hour with avidin-biotin-peroxidase complex (DAB, Dako) and 0.03\% hydrogen peroxide in PBS, which formed an insoluble brown precipitate (positive staining). The nuclei were counterstained using Mayer's haematoxylin, dehydrated, and mounted. Control slides (an irrelevant isotype matched antibody in place of the primary antibody) were prepared to test the specificity of staining.

**Statistical analysis**

Statistical analysis was carried out using Instat (Graphpad Software Inc, San Diego, USA). Before undertaking the study, power analysis was carried out from which it was determined that an \( n \) of 6 was required for statistical assurance. A Kolmogorov-Smirnov test showed that the data were normally distributed. The data are thus expressed as mean (SE), \( n = 6 \). Analysis was performed using ANOVA and a post hoc unpaired two tailed Student's \( t \) test with Bonferroni's adjustment.

**RESULTS**

LPS, IL-1\( \alpha \), and TNF-\( \alpha \) promoted the formation of SOD inhibitable \( \text{O}_2^- \) from porcine PA segments compared with untreated controls in a time and concentration dependent manner (figs 1 and 2). Since maximal \( \text{O}_2^- \) formation was observed after 16 hours incubation, this time point was used in all subsequent studies. Similarly, the concentrations at which optimal

![Figure 3](http://thorax.bmj.com/content/45/7/600/F1a)

**Figure 3** SOD inhibitable \( \text{O}_2^- \) formation by fourth order (200 \( \mu \)m diameter) porcine pulmonary artery segments with (+E) and without (–E) endothelium in response to lipopolysaccharide (LPS, 1 \( \mu \)g/ml), interleukin 1\( \alpha \) (IL-1\( \alpha \), 10 \( \text{ng/ml} \)), and tumour necrosis factor \( \alpha \) (TNF-\( \alpha \), 100 \( \text{ng/ml} \)) strain segments for 2 hours before measurement of \( \text{O}_2^- \). Data are shown as means with bars representing standard error of the mean. The \( * \) indicates a significant difference between the control and either LPS or LPS + TNF-\( \alpha \). \( p < 0.001 \) compared with LPS or LPS + TNF-\( \alpha \). *p < 0.01 significantly inhibited compared with LPS or cytokine treated segments.

![Figure 4](http://thorax.bmj.com/content/45/7/600/F1b)

**Figure 4** Effect of diphenylene iodonium chloride (DPI; 10 \( \mu \)M), allopurinol (100 \( \mu \)M), and rotenone (10 \( \mu \)M) on SOD inhibitable \( \text{O}_2^- \) formation by porcine pulmonary artery segments with (+E) and without (–E) endothelium in response to (A) LPS (1 \( \mu \)g/ml), (B) IL-1\( \alpha \) (10 \( \text{ng/ml} \)), and (C) TNF-\( \alpha \) (10 \( \text{ng/ml} \)) following 16 hours incubation. Values are mean (SE); \( n = 6 \). *p < 0.001 significantly inhibited compared with LPS or cytokine treated segments.
responses were obtained were used in subsequent experiments (10 ng/ml for both IL-1α and TNF-α, and 1 µg/ml for LPS). Removal of the endothelium enhanced the formation of O$_2^•$ from porcine PA segments compared with untreated controls in response to LPS, IL-1α, and TNF-α following incubation for 16 hours (fig 2). When comparing first order arterial segments (2–4 mm diameter) with fourth order arterial segments (200–300 µm diameter), with and without endothelium, O$_2^•$ formation and release in response to LPS, IL-1α and TNF-α was similar between the two different sized vessels, although the absolute release of O$_2^•$ was greater in the smaller vessels (fig 3).

DPI, an inhibitor of NADPH oxidase (but not rotenone) inhibited the formation of O$_2^•$ formation and release from porcine PA segments (with and without endothelium) in response to LPS, IL-1α, and TNF-α (fig 4). Allopurinol had a slight but still significant effect on O$_2^•$ formation induced with LPS and TNF-α but had no effect on IL-1α induced effects (fig 4).

The non-specific NOS inhibitor L-NAME enhanced O$_2^•$ formation and release from porcine PA segments (with and without endothelium) in response to LPS, IL-1α, and TNF-α but only when the endothelium was present in response to LPS (fig 5). The eNOS inhibitor l-NIO enhanced O$_2^•$ formation from porcine PA segments with endothelium only in response to IL-1α and TNF-α and had a lesser effect on LPS mediated O$_2^•$ formation (fig 5). The iNOS inhibitor, on the other hand, enhanced O$_2^•$ formation from porcine PA segments with endothelium only in response to LPS, but had no effect on IL-1α and TNF-α mediated O$_2^•$ formation (fig 5).

Following incubation for 16 hours the immunoreactive distribution of eNOS, iNOS, and nitrated tyrosine (index of ONOO$^•$ formation) in response LPS, IL-1α, and TNF-α was assessed. Nitrated tyrosine was located principally in the endothelium (fig 6A–E), indicating that both NO and O$_2^•$ are present at high concentrations in this region. Similarly, eNOS was located in the endothelium and was markedly upregulated by IL-1α and TNF-α and, to a lesser extent, by LPS (fig 6F–J), confirming the effects of the NOS inhibitors. In contrast, iNOS was upregulated only in response to LPS and was located ubiquitously through the arterial segment, and not to IL-1α or TNF-α (fig 6K–O), again confirming the observations with the NOS inhibitors.

**DISCUSSION**

This study shows that TNF-α, IL-1α, and LPS promote the formation of O$_2^•$ in PA segments (with and without intact endothelium) in a time dependent manner and at concentrations that have been reported to appear in the blood of patients with ARDS.$^{18}$ These responses were not confined to large pulmonary arteries but were seen also in arterial microvessels, indicating that these effects occur ubiquitously throughout the pulmonary vasculature. Furthermore, DPI completely inhibited the generation of O$_2^•$ in response to TNF-α, IL-1α and LPS, indicating that an increase in NADPH oxidase activity mediates these effects. The slight reduction in O$_2^•$ formation by allopurinol suggests that xanthine oxidase may also be upregulated.

These data are in agreement with previous observations. IL-1β has been shown to induce O$_2^•$ production from cultured rat pulmonary microvascular smooth muscle cells, human internal mammary artery smooth muscle cells, and human cord vein endothelial cells.$^{15}$–$^{18}$ TNF-α activated O$_2^•$ producing NADH oxidase in cultured rat aortic smooth muscle cells in a time and dose dependent manner.$^{19}$ Increased O$_2^•$ has also been reported in whole lungs from guinea pigs following the injection of TNF-α.$^{20}$ LPS challenge also increases lung O$_2^•$ generation in anaesthetised rats, which was found to coincide with the alterations in cardiovascular function measured in these animals.$^{21}$ Clinically, it has long been recognised that intrapulmonary oxidative stress is present in patients with ARDS.$^{22}$–$^{23}$


![Figure 5](http://thoraxjnl.com) Effect of the NOS inhibitors L-NAME (L-NAME, 100 µM), N£{prime}-(1-iminoethyl)-L-ornithine (L-NIO, 10 µM), and L-N£{prime}+[1-iminoethyl]-lysine-HCl (L-NIL, 10 µM) on SOD inhibitable O$_2^•$ from porcine pulmonary artery segments in response to (A) lipopolysaccharide (LPS, 1 µg/ml), (B) interleukin 1α (IL-1α, 10 ng/ml), and (C) tumour necrosis factor α (TNF-α, 10 ng/ml) following 16 hours incubation in intact arteries (left) and without endothelium (right). Values are mean (SE); n=6. *p<0.001 v LPS or cytokine treated only.
Figure 6  Distribution of immunoreactive nitrated tyrosine and expression of eNOS and iNOS in pig pulmonary arteries. Freshly prepared pulmonary arterial segments were treated with vehicle (A, F, K), 1 µg/ml lipopolysaccharide (B, G, L), 10 ng/ml IL-1α (C, H, M), 10 ng/ml TNF-α (D, I, N) or with control IgGs (E, J, O). Frozen sections of these segments were then immunostained for either nitrated tyrosine (A–E), eNOS (F–J) or iNOS (K–O). Dark brown staining represents positive staining. The endothelium is indicated by arrowheads.
Removal of the endothelium resulted in a marked increase in $O_2^-$ release from PA segments both before and after incubation with LPS, TNF-$\alpha$, and IL-1$\alpha$, indicating that the endothelium of PAs possesses a system for removing $O_2^-$ radicals. Although there are reports that the endothelium produces $O_2^-$, our results show that the endothelium quenches the output of $O_2^-$ by the arterial segment as a whole. Since the endothelium is a principal source of NO, the reaction of $O_2^-$ with NO may account for the removal of $O_2^-$ by the endothelium and, as such, was investigated using NO synthase (NOS) inhibitors. The non-specific NOS inhibitor l-NAME mimicked the effect of endothelium removal—that is, $O_2^-$ release was increased by l-NAMe in segments with intact endothelium—indicating that NO removes $O_2^-$ from the system through the reaction: NO + $O_2^-$ → ONOO-, thereby reducing that which reacts with ferricytochrome c. Indeed, immunocytochemical analysis clearly showed a marked increase in NT (an index of ONOO- formation) in the endothelial region of PA segments after incubation with cytokines and LPS, supporting this proposal.

To differentiate between the roles of eNOS and iNOS in reducing $O_2^-$ formation in intact PAs, the effect of two inhibitors of these isoforms were investigated. The eNOS inhibitor l-NIO, but not the iNOS inhibitor t-NIL, enhanced $O_2^-$ formation in response to IL-1$\alpha$ and TNF-$\alpha$ and, to a lesser extent, to LPS. Immunocytochemical analysis confirmed that eNOS (but not iNOS) is upregulated in the endothelium of the arterial segments in response to these cytokines and that LPS had no effect. It therefore appears that cytokines (principally) elicit a simultaneous upregulation of both smooth muscle NADPH oxidase and endothelial eNOS which negates the increased formation of $O_2^-$, thereby protecting the vessel against oxidative damage. In turn, loss of the endothelium would render the PA susceptible to the pathogenic impact of cytokine induced $O_2^-$ formation. These data are also in agreement with those of Bhagat et al who concluded that IL-1$\beta$ upregulates eNOS but not iNOS in venous tissue of human subjects in vivo.

In contrast, the iNOS inhibitor t-NIL enhanced $O_2^-$ formation in response to LPS but had no effect on IL-1$\alpha$ or TNF-$\alpha$ mediated $O_2^-$ formation. Immunocytochemical analysis again confirmed an upregulation of iNOS in response to LPS but no effect of cytokines on this isoform. It therefore appears that LPS has little effect on the upregulation of eNOS but influences the expression of iNOS at the level of the vascular smooth muscle. Nevertheless, NO produced by iNOS appears to have a protective effect against LPS induced $O_2^-$ formation. Other studies have shown that LPS promotes the upregulation of iNOS while suppressing the expression of eNOS. Additionally, as these data indicate, the excess formation of ONOO- may contribute to the pathophysiology of ARDS directly since ONOO- has been shown to exert pathological effects including endothelial cell apoptosis and the expression of adhesion molecules. Clinically, raised levels of NO metabolites are present in high levels in bronchoalveolar lavage (BAL) fluid from patients with ARDS, which is consistent with an upregulation of eNOS and iNOS in intrapulmonary tissues in this condition. More crucially, increased levels of nitrotyrosine residues (index of ONOO-) have been reported in BAL fluid from patients with acute lung injury. Consolidating the hypothesis that upregulation of both NO and $O_2^-$ generating enzymes occurs in ARDS by the mechanisms proposed in this study.

In conclusion, the present study shows that LPS and two major cytokines additively promote the formation of $O_2^-$ by pig pulmonary arteries in vitro, principally through an upregulation of NADPH oxidase. In turn, this $O_2^-$ would perpetuate and augment inflammation through the induction of adhesion molecules, vascular constriction, and the negation of NO bioavailability. In this situation the endothelium reduces LPS and cytokine induced $O_2^-$ generation, a mechanism that appears to be mediated through NO derived from eNOS (in the case of cytokines) and iNOS (in the case of LPS). The concomitant expression of these NO isoforms with that of NADPH oxidase may therefore constitute an important protection system against oxidative stress. However, at some point in the sequelae of ARDS this defense system may be overwhelmed, rendering the vasculature susceptible to oxidative stress. The integrity of the endothelium may thus be axiomatic in the progression and severity of ARDS. A reduction in oxidative stress coupled with the delivery of NO would therefore seem to be a potentially effective strategy for treating ARDS.

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**References**


LUNG ALERT

The SARS epidemic: emergence of a new respiratory virus?

This clinical study of 138 patients from a Hong Kong hospital suggests that the severity of the severe acute respiratory syndrome (SARS) (intensive care and/or death) was associated with advanced age, high peak lactate dehydrogenase level (LDH), and an absolute neutrophil count that exceeded the normal range at presentation. 78% of patients had consolidation on the chest radiograph at initial consultation.

Nineteen inpatients (6 in Hong Kong, 1 in Canada, 10 in Vietnam, 1 in Taiwan, 1 not stated) with SARS were identified as being infected with the SARS coronavirus determined by virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR), or serology. The virus sequence was obtained either from clinical specimens or virus isolates from 12 of these patients and amplified by RT-PCR for viral identification. None of the patients infected with the SARS coronavirus was infected with human metapneumovirus. Co-infection with another virus (rhinovirus) was found in one patient. Further genetic analysis showed the SARS coronavirus to be a new virus. Initial testing suggests that this virus may never before have infected the US population. A variety of respiratory pathogens were also identified by RT-PCR in patients whose samples were submitted for SARS testing, including 5 with human metapneumovirus and 13 with rhinovirus. None of the patients who were positive for human metapneumovirus had pneumonia.

This was a study of 50 cases of clinically diagnosed SARS from three hospitals in Hong Kong and 280 controls. Evidence of the SARS coronavirus (serology and/or viral RNA from nasopharyngeal aspirate or stool) was found in 45 patients but not in controls. Despite fever, 49 (98%) patients had no evidence of leucocytosis. Older age, severe lymphopenia, and impaired ALT were associated with increased severity of infection requiring intensive care and ventilatory support. Peak LDH was not measured. All patients had radiological evidence of consolidation on the chest radiograph.

This outbreak was a point source outbreak starting with a visitor from southern China on 21 February 2003. The disease spread rapidly from hospital health care workers to the community. Cumulative total cases during March 2003 were as follows: 14th (n = 3), 21st (n = 203), 26th (n = 319), 31st (n = 610). The authors suggest that the incubation period is 2–11 days; lymphopenia and liver dysfunction were found commonly in hospital admitted patients.

Comment

A new respiratory virus, the SARS coronavirus, appears to have been identified and we await further information on its source. The markers of severity of SARS are still unclear, but older age and impaired liver function appear to be consistent between the above studies. However, the clinical spectrum of this disease in the community (those patients not admitted to hospital) is still to be defined. An assessment of the severity of the syndrome in the presence or absence of detection of the SARS coronavirus may be of interest.

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