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Excess iron promotes emergence of foamy macrophages that overexpress ferritin in the lungs of silicosis patients

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Abstract

Background and objective: Inhalation of high concentrations of respirable crystalline silica (RCS) can lead to silicosis. RCS contains varying levels of iron, which can cause oxidative stress and stimulate ferritin production. This study evaluated iron-related and inflammatory markers in control and silicosis patients.

Methods: A cohort of stone benchtop industry workers ($n = 18$) were radiologically classified by disease severity into simple or complicated silicosis. Peripheral blood and bronchoalveolar lavage (BAL) were collected to measure iron, ferritin, C-reactive protein, serum amyloid A and serum silicon levels. Ferritin subunit expression in BAL and transbronchial biopsies was analysed by reverse transcription quantitative PCR. Lipid accumulation in BAL macrophages was assessed by Oil Red O staining.

Results: Serum iron levels were significantly elevated in patients with silicosis, with a strong positive association with serum ferritin levels. In contrast, markers of systemic inflammation were not increased in silicosis patients. Serum silicon levels were significantly elevated in complicated disease. BAL macrophages from silicosis patients were morphologically consistent with lipid-laden foamy macrophages. Ferritin light chain (FTL) mRNA expression in BAL macrophages was also significantly elevated in simple silicosis patients and correlated with systemic ferritin.

Conclusion: Our findings suggest that elevated iron levels during the early phases of silicosis increase FTL expression in BAL macrophages, which drives elevated BAL and serum ferritin levels. Excess iron and ferritin were also associated with the emergence of a foamy BAL macrophage phenotype. Ferritin may represent an early disease

Christian Anthony Aloe and Tracy Li-Tsein Leong have contributed equally to this study.

marker for silicosis, where increased levels are independent of inflammation and may contribute to fibrotic lung remodelling.

KEYWORDS
ferritin, foam cells, interstitial lung disease, iron, silicosis

INTRODUCTION

Silicosis is an irreversible and potentially fatal interstitial lung disease caused by repeated inhalation of respirable crystalline silica (RCS). Silicosis can be divided into acute, accelerated and chronic phenotypes depending on the intensity and duration of exposure.[1] The demand for domestic kitchen benchtops fabricated from engineered (also known as artificial or reconstituted) stone—typically >90% of silica content—over the past decades has seen a wave of silicosis cases in stone benchtop industry workers. These workers typically develop disease after exposure to large amounts of silica over a short period of time (5–15 years).[2,3] Radiographic changes of silicosis consist of diffuse nodules and thoracic lymphadenopathy. While these nodules are typically small (<1 cm) and distributed throughout both lung fields in early disease, these changes can transition from a 'simple' to a 'complicated' form as nodules coalesce into large fibrotic masses termed progressive massive fibrosis.[4] With no effective treatments, and an alarming spike in cases, a greater understanding of the cellular and molecular drivers of this disease is urgently needed.

Alveolar macrophages are frontline cellular responders to inhaled irritants including RCS through engagement of pattern recognition and scavenger receptors. Phagocytosis of RCS activates the NALP3 inflammasome pathway, which promotes secretion of the pro-inflammatory cytokines IL-1β and IL-18.[1,5] IL-1β and IL-18 stimulate the production of a broad suite of acute-phase proteins. Ferritin is well recognized as an acute-phase reactant that is elevated in a wide range of inflammatory conditions.[6–9] Of significance, high serum ferritin levels are related to worse prognosis in patients with acute exacerbations of idiopathic pulmonary fibrosis, where immunohistochemical staining identified alveolar macrophages as an important cellular source of ferritin.[10] NALP3 inflammasome activation by silica also stimulates the generation of reactive oxygen species (ROS) in a dose-dependent manner.[11] The extent of ROS production by RCS is dependent on iron complexed with silica, as high amounts of iron promote greater ROS production and lipid peroxidation in pre-clinical models of RCS exposure.[12] Iron can also independently stimulate the production of ferritin, which is a multimeric protein composed of 24 heavy (FTH) and light chains (FTL). The FTL subunit is primarily responsible for removal of excess free iron from the body, where iron–ferritin complexes are sequestered by macrophages to prevent iron from catalysing the production of free radicals via Fenton chemistry.[13,14] Elevated serum ferritin levels have been detected in simple and complicated silicosis compared to individuals exposed to silica without disease.[15] However, little is known about the driver of excess ferritin production in silicosis.

SUMMARY AT A GLANCE

Silicosis is an aggressive and incurable lung disease. In this study, serum iron levels were increased in silicosis patients, and these levels were strongly associated with serum ferritin levels. Lipid-laden bronchoalveolar lavage macrophages were identified as a major source of ferritin, whereas markers of inflammation were not increased.

In this study, we hypothesized that excess iron in silicosis patients is primarily responsible for elevated serum ferritin levels, and that this acute-phase response is stimulated early in the disease process. To address this, we have analysed a panel of serum inflammatory/acute-phase reactants and iron markers in a cohort of silicosis patients stratified by disease severity (simple vs. complicated). In addition, we investigated the cellular source of ferritin whereby matching bronchoalveolar lavage (BAL) macrophages and transbronchial biopsies (TBBx) were analysed for FTH and FTL expression by reverse transcription quantitative PCR (RT-qPCR).

METHODS

Study participants

This study was conducted at the Austin Hospital, Heidelberg, Victoria, Australia, and at RMIT University, Bundoora, Victoria, Australia. Recruitment of cases and controls occurred between January 2020 and October 2021. Stone benchtop industry workers who had worked with engineered stone were eligible for inclusion. The study groups consisted of patients with (1) radiographic changes characteristic of silicosis and (2) no radiological disease. Patients with silicosis were further stratified into 'simple' and 'complicated' study groups based on radiological findings, where all imaging was read by International Labour Office (ILO)-accredited expert chest radiologists using The International Classification of High-resolution Computed Tomography for Occupational and Environmental Respiratory Diseases (ICOERD) grading scale. The scans were then

TABLE 1 Patient characteristics

	No disease ($n = 5$)	Simple silicosis ($n = 8$)	Complicated silicosis ($n = 10$)
Females/males (n)	4/1	0/8	0/10
Age (years)	55 (46–64)	40 (34–52)	44 (27–55)
BMI (kg/m^2)	34.8 (32–39)	26.7 (21–36)	24.3 (21–38)
Smoking status: (never/ex/current)	4/0/0	1/1/6	2/4/4
FEV_1 (% of predicted)	84	85 (69–106)	71 (60–94)
FVC (% of predicted)	87 (80–94)	79 (68–100)	93 (62–99)
DLCO (% of predicted)	80 (77–83)	72 (40–97)	79 (65–103)
Artificial stone exposure (years)	NA	13 (5–17)	13 (4–29)

Note: All numerical data are presented as median (range), if not indicated otherwise.
Abbreviations: DLCO, diffusion capacity of the lungs for carbon monoxide; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

categorized according to the described changes. Patients referred for bronchoscopy to assess chronic cough and who had normal computed tomography (CT) chest and lung function tests were recruited as healthy controls, forming the 'no disease' group. Clinical information, demographics, detailed occupational history, spirometry, diffusion capacity of the lungs for carbon monoxide (DLCO) and HRCT chest imaging were obtained. Peripheral blood sampling, BAL and TBBx (in the case of the silicosis group) were also obtained.

Biomarker analysis

Peripheral blood sampling was performed to obtain white blood cell counts and erythrocyte sedimentation rate (ESR). Serum was collected to measure C-reactive protein (CRP), serum amyloid A (SAA), procalcitonin (PCT), ferritin, iron, total iron-binding capacity, silicon, total bilirubin, alanine aminotransferase (ALT), alkaline phosphatase, gamma-glutamyl transferase, creatinine and urea (as outlined in Appendices S2–S5 in the Supporting Information). BAL (as collected in Appendix S1 in the Supporting Information) was utilized to perform a differential BAL count and evaluate macrophage morphology (as outlined in Appendices S6 and S7 in the Supporting Information). To investigate the cellular sources of ferritin in the lung, FTH and FTL subunit mRNA expression was evaluated by RT-qPCR in BAL cells and TBBx (as outlined in Appendix S8 in the Supporting Information).

Statistical analysis

Data were presented as mean values ± SEM. One-way non-parametric analysis of variance was used for the comparison among three groups and the Kruskal–Wallis H-test was used for post analysis. Following chest HRCT, patients were divided into their study groups according to their disease severity. GraphPad Prism 9.0 software (GraphPad Software, San Diego, USA) was utilized for graph production and data analysis, where a p-value of less than 0.05 was considered as statistical significance.

RESULTS

Table 1 summarizes the clinical characteristics of the study cohort. Twenty-three participants were recruited; 18 (78%) with silica exposure and radiological features of silicosis; and five without radiological features of silicosis that consisted of five (22%) healthy controls. Of the 18 patients with radiological manifestations, eight (44%) presented with early-stage 'simple' disease, with the remaining 10 (56%) exhibiting late-stage 'complicated' disease. The median time of exposure to dust from engineered stone was 13 ± 6 years, which were not significantly different between patients with simple and complicated disease.

Systemic ferritin is differentially elevated in patients with early-stage silicosis

Serum ferritin levels were significantly elevated in patients with simple silicosis, and there was no significant difference between the simple and complicated silicosis groups (Figure 1A). A similar pattern was observed for serum iron levels and total iron-binding capacity, with levels significantly increased in the simple group and levels remaining elevated in those with advanced disease (Figure 1B,C). There was no evidence for iron overload as transferrin saturation levels fell within normal ranges (Figure 1D). Serum silicon levels were significantly higher in silicosis patients, which increased with disease stage (Figure 1E). Correlation analysis revealed that serum ferritin levels did not associate with serum silicon levels, whereas there was a strong association between serum ferritin and serum iron levels (Figure 1F,G).

Peripheral blood analysis (Figure 2A–D) revealed the absence of a persistent systemic inflammatory response in all groups as total white blood cell, lymphocyte, neutrophil and eosinophil counts were within normal ranges. The classic inflammatory acute-phase reactant, CRP, was not significantly increased in silicosis patients, nor was SAA (Figure 2E,F). Similarly, ESR levels were not significantly altered across the groups and fell within the normal ranges (Figure 2H). Serum PCT levels were also observed to fall

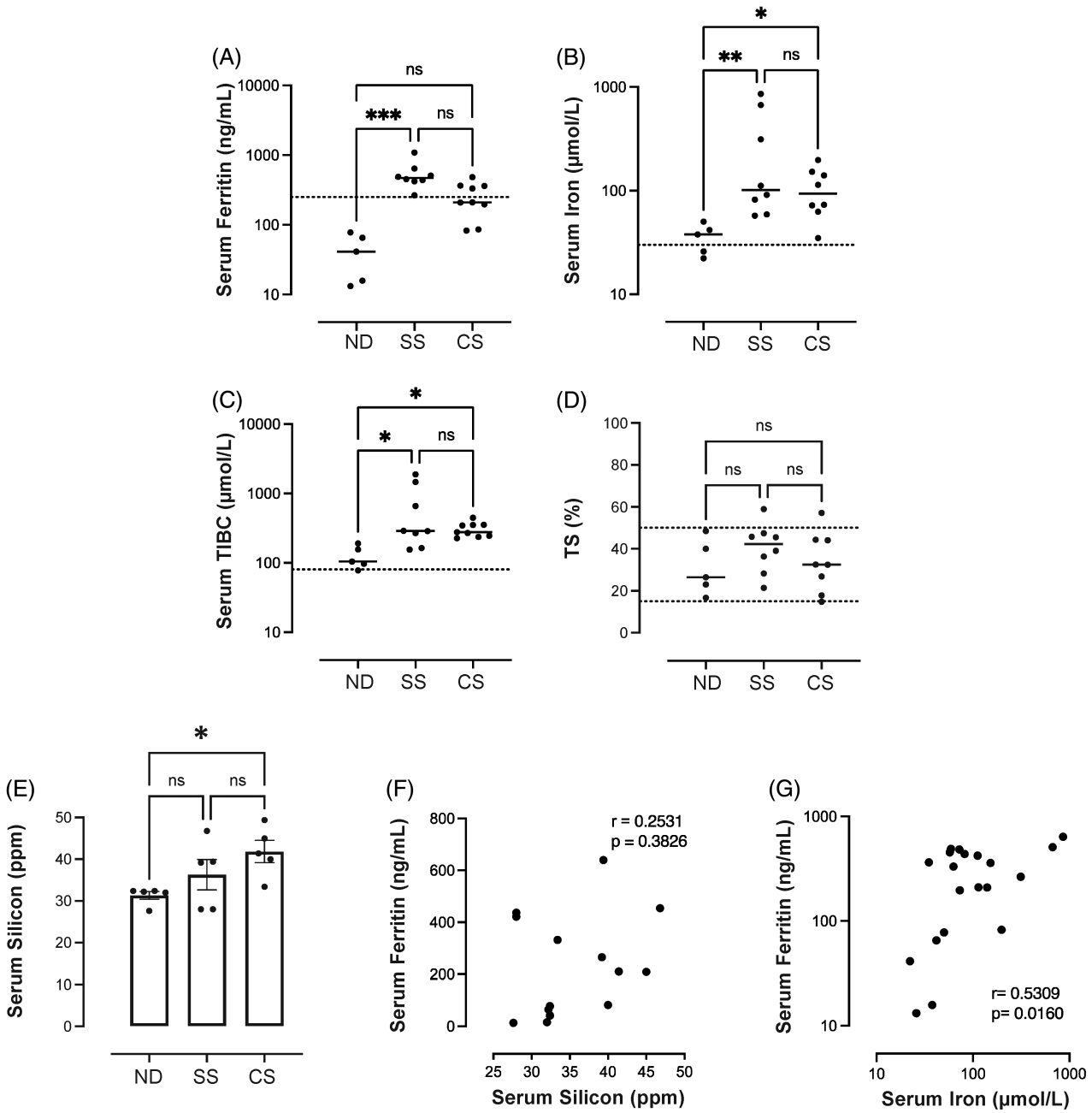

FIGURE 1 Serum ferritin is significantly elevated in patients with simple silicosis (SS) compared to patients with complicated silicosis (CS) and those with no disease (ND) (A). Similarly, serum iron and total iron-binding capacity (TIBC) levels were elevated in patients with SS, with levels remaining elevated in patients with complicated disease (B, C). However, iron overload is not evident as the transferrin saturation % (TS%) levels are within the normal range of 15%–50% (D). Quantification of silicon levels in serum shows significantly higher silicon levels in patients with CS (E). Correlation analysis reveals that ferritin does not associate with serum silicon levels (F), whereas there was a strong association between serum ferritin and serum iron levels (G). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way non-parametric analysis of variance (Kruskal–Wallis test), data represent mean ± SEM. Horizontal dashed lines represent the limit/s of the normal range

within the normal range in control and silicosis patients (Figure 2G). Ferritin levels can also be altered because of liver or kidney dysfunction; however, assessment of liver and renal functions fell within the normal range in both patients with simple and complicated silicosis (Figure 3), with the exception of serum ALT that was increased in complicated silicosis patients.

Foamy macrophages produce FTL, which increases circulating ferritin levels

BAL cell differential analysis revealed similar profiles in patients with no disease versus patients with simple and complicated silicosis (Figure 4A–D), where BAL

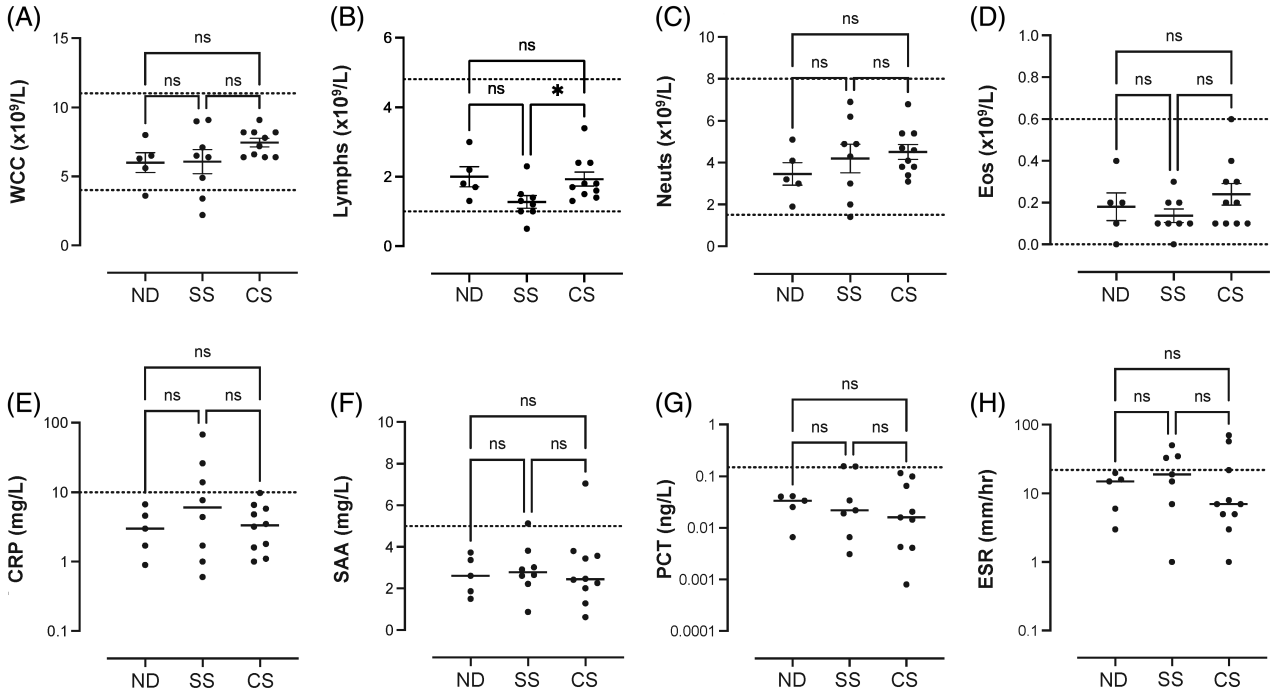

FIGURE 2 Haematological analysis of white blood cell counts and an acute-phase reactant panel demonstrated the absence of a persistent systemic inflammatory response in non-diseased (ND), and in simple (SS) and complicated silicosis (CS) patients. This is evident as the total WCC, blood lymphocyte (Lymphs), neutrophil (Neuts), and eosinophil (Eos) numbers were within normal ranges (A–D). Markers of systemic inflammation, C-reactive protein (CRP), serum-amyloid A (SAA), procalcitonin (PCT) and erythrocyte sedimentation rate (ESR) are not significantly altered across the groups (E–H). *$p < 0.05$, one-way non-parametric analysis of variance (Kruskal–Wallis test), data represent mean ± SEM. Horizontal dashed lines represent the limit/s of the normal range

macrophages were the dominant immune cell. However, BAL macrophages were morphologically different across the study groups (Figure 4E–G). Macrophages in the silicotic lung appeared both enlarged and vacuolated, where a foam cell-like appearance was attributed to lipid loading, confirmed by Oil Red O Staining (Figure 4H–J). Quantitative assessment demonstrated that there was a 32 ± 7% and 26 ± 9% increase in lipid-loaded macrophages in patients with simple and complicated silicosis, respectively, relative to those with no disease (Figure 4K).

Local lung ferritin levels in the BAL fluid were significantly increased in both simple and complicated groups, and positively correlated with circulating serum ferritin levels (Figure 5A,B). FTH mRNA expression in BAL cells from patients with simple silicosis was not increased (Figure 5C), whereas FTL subunit expression was significantly increased (Figure 5D). Median FTL levels were lower in the complicated silicosis group; however, they were not significantly different to the simple silicosis group (Figure 5D). FTH expression in BAL cells did not correlate with serum ferritin levels, whereas there was a strong positive association between FTL mRNA expression in BAL cells and serum ferritin levels (Figure 5E,F). FTH and FTL expression in TBBx were not significantly different in simple versus complicated silicosis (Figure 5G). Paired analysis of FTH and FTL mRNA expression in matching BAL and TBBx revealed that BAL cells were the main ferritin source in the lung (Figure 5H,I).

DISCUSSION

Our study demonstrated that serum ferritin levels were increased in patients with silicosis, with the highest levels observed in simple silicosis, suggesting that levels rise early during the disease process. Although seven (78%) of nine patients with complicated silicosis displayed serum ferritin levels above those in the no disease control group, a larger sample size may be required to attain significance. In contrast, markers of inflammation and hepatic/renal dysfunction were not increased in patients with simple silicosis, which can also drive increased ferritin production. Our findings are in contrast with a recent study by Blanco-Pérez, where increased serum ferritin levels were detected in silicosis patients in conjunction with elevated levels of inflammatory markers including CRP.[15] However, this study was characterized by high incidence of active tuberculosis infection, which is an independent cause of systemic inflammation and confound findings of elevated ferritin.[16] In our study, there were no cases of active tuberculosis and a single case of latent tuberculosis as evidenced by a positive QuantiFERON-TB Gold assay.

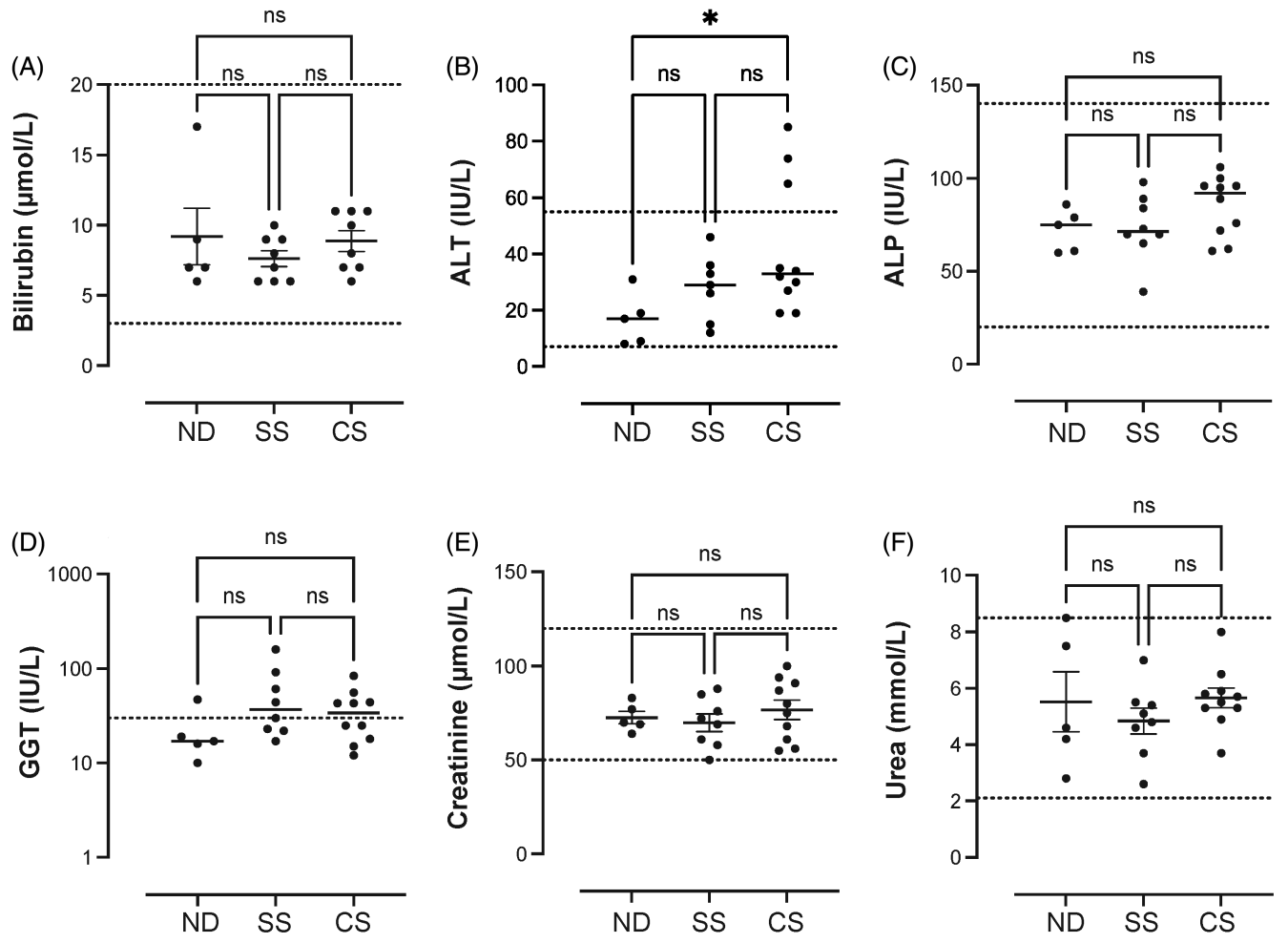

FIGURE 3 Markers of liver and renal dysfunction are not altered in the serum of simple (SS) and complicated silicosis (CS) patients. With the exception of alanine aminotransferase (ALT) in CS, total bilirubin, ALT, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), creatinine and urea levels fell within the normal range in patients with no disease (ND), SS and CS (A–F). *$p < 0.05$, one-way non-parametric analysis of variance (Kruskal–Wallis test), data represent mean ± SEM. Horizontal dashed lines represent the limit/s of the normal range

We also found that serum ferritin levels strongly associated with serum iron levels. Whilst iron is vital in a wide range of metabolic processes, including oxygen transport and DNA synthesis, iron dysregulation can produce free radicals causing oxidative damage in a broad spectrum of diseases.[13,17] Silicates complex iron onto their surfaces, including inorganic iron and iron derived from biological systems.[18,19] Ghio et al. demonstrated that the ability of silicates to catalyse oxidant generation in vitro is proportional to the amount of ferric ion complexed onto their surfaces. As such, surface complexation of iron onto silicates may be an important determinant of their ability to cause silicosis.[12,18] In contrast to iron, serum ferritin levels did not correlate with serum silicon levels. Intriguingly, serum silicon levels were increased in complicated silicosis patients and there was a subset of patients with high serum silicon levels in simple disease. It would therefore be important to establish whether simple silicosis patients with high serum silicon levels progress more rapidly in future studies.

A secondary consequence of excess iron is the generation of oxidative stress including the accumulation of oxidized phospholipids. Intriguingly, we observed a change in macrophage morphology where alveolar macrophages were enlarged and vacuolated in patients with silicosis. This morphological change was attributed to lipid loading, confirmed by Oil Red O staining. Mechanisms mediating foam cell formation in silicosis are not well defined. Hou et al. reported that foam cells were associated with a dramatic increase in levels of oxidized low-density lipoprotein (ox-LDL).[20] Interestingly, both iron and ferritin have been implicated in promoting apoptosis of foam cells in human atheroma,[21] where iron-laden macrophages associate with LDL oxidation, angiogenesis and nitric oxide production.[22] Yuan et al. also demonstrated that iron-laden macrophages exocytosed both iron and ferritin into culture medium, where exposure to ox-LDL, but not high-density lipoprotein, resulted in apoptosis of iron-laden human macrophages.[22] It is plausible that iron loading may be driving lipid accumulation and apoptosis in lipid-laden BAL macrophages in a progressive manner, although further work is needed to confirm this.

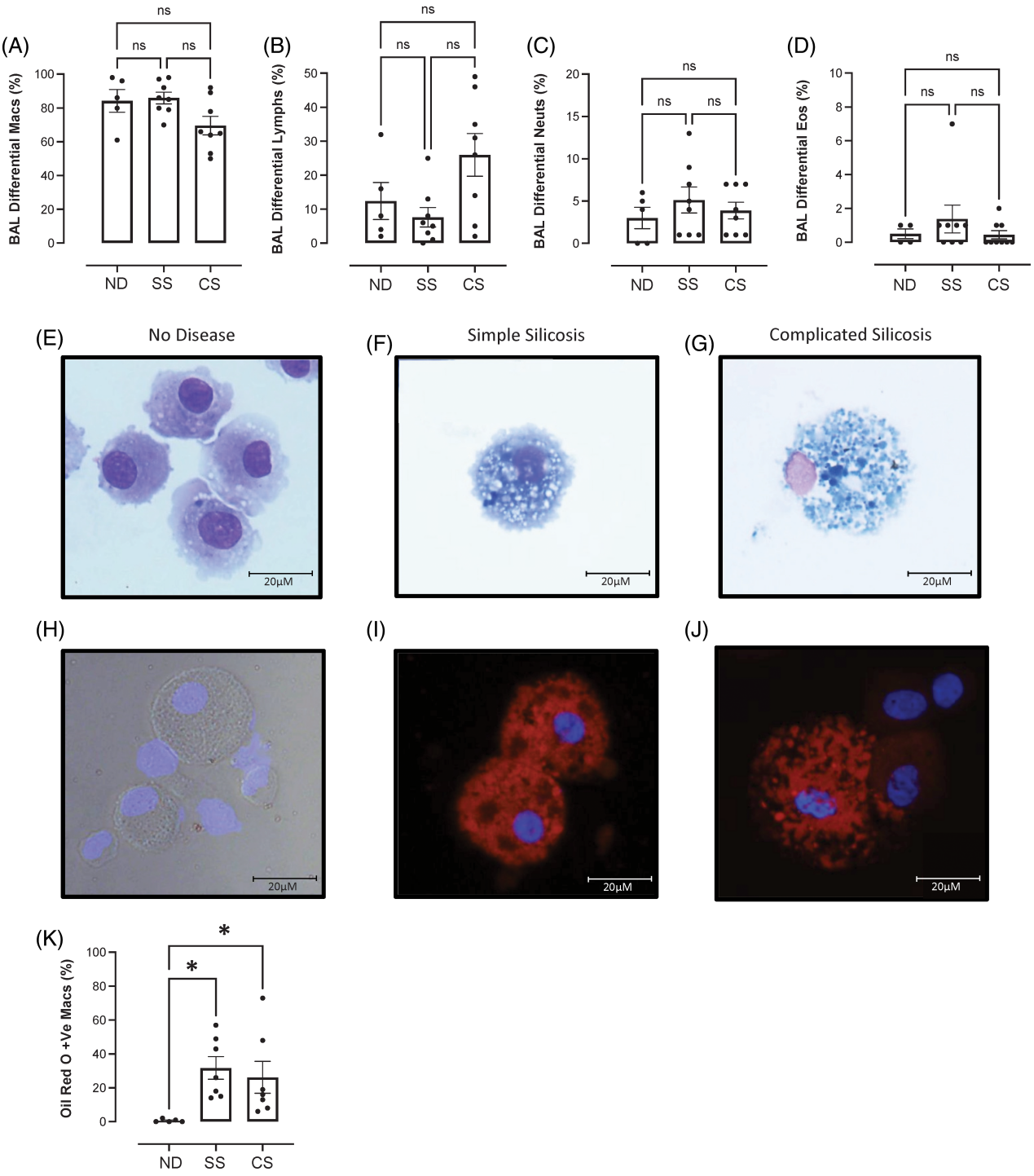

FIGURE 4 Bronchoalveolar lavage (BAL) cell profiles are not altered in patients with simple (SS) and complicated silicosis (CS) compared to patients with no disease (ND). This is evident as no statistical differences in BAL macrophages (Macs), lymphocytes (Lymphs), neutrophils (Neuts) and eosinophils (Eos) are observed across the study groups when BAL cytospots are stained with Kwik-Diff™ (ThermoFisher Scientific) and quantified (A–D). A change in macrophage morphology is observed across the study groups (E–G), where alveolar macrophages in silicosis-diseased patients are found to be lipid-laden when stained with Oil Red O (H–K). *$p < 0.05$, one-way non-parametric analysis of variance (Kruskal–Wallis test), data represent mean ± SEM

Our study is the first to show that BAL macrophages are an important source of ferritin in the silicotic lung where FTL, but not FTH, expression correlates with BAL and serum ferritin levels. Iron is oxidized to the ferric state by FTH, while FTL stabilizes the complex, favouring mineralization during iron storage in macrophages. Hence, ferritin is post-transcriptionally increased during exposure to excess iron through an iron response element (IRE) contained within an untranslated region of the ferritin gene. IRE is regulated by binding of a cytoplasmic protein, iron

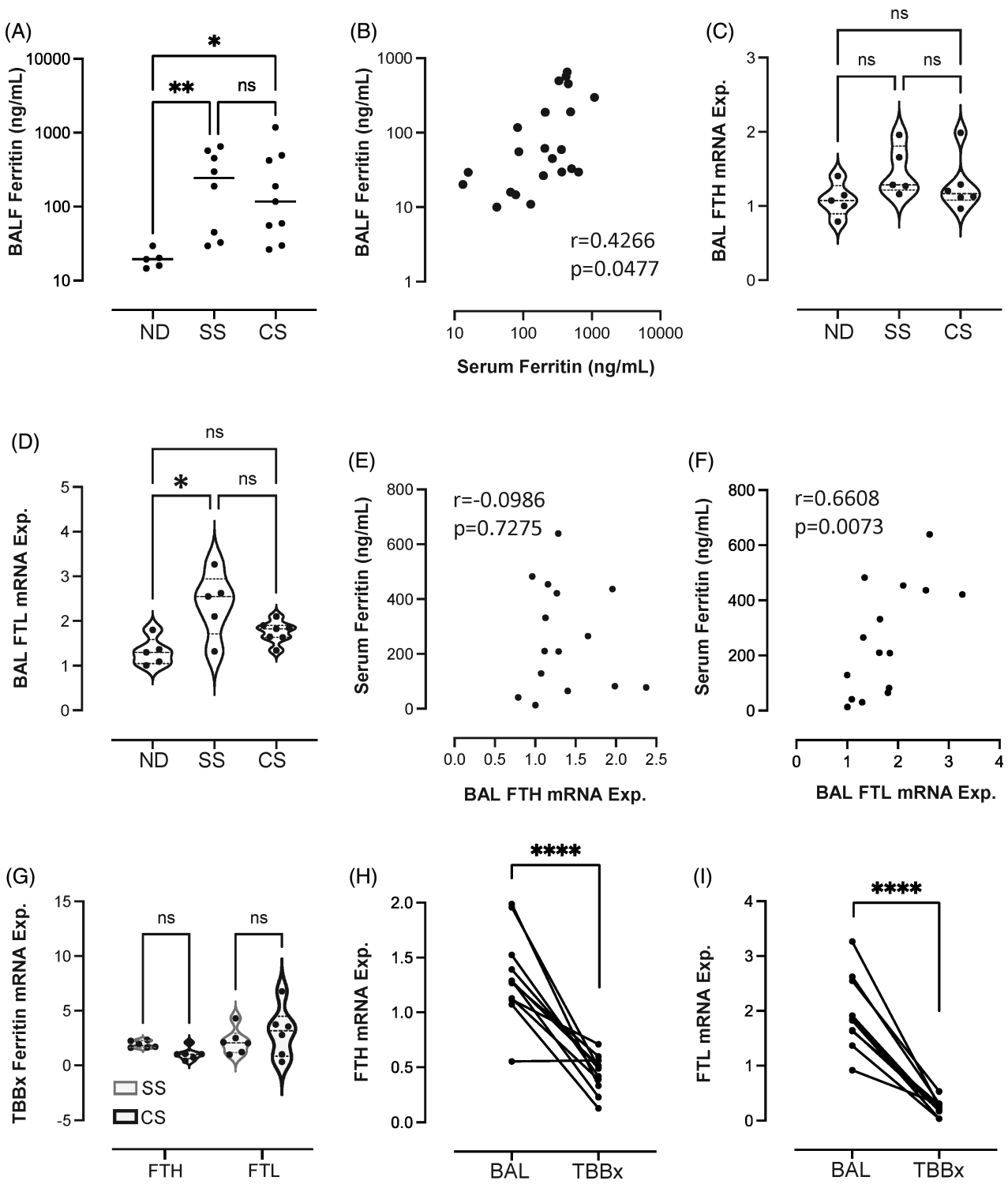

FIGURE 5 Ferritin light chain (FTL) is overexpressed in bronchoalveolar lavage (BAL) macrophages and positively associated with circulated ferritin levels. Ferritin in the BAL fluid (BALF) is elevated in patients with both simple (SS) and complicated silicosis (CS) compared to patients with no disease (ND) (A), where local ferritin levels positively correlated with circulating serum ferritin (B). Evaluation of ferritin heavy chain (FTH) and FTL subunit mRNA expression by RT-qPCR revealed the cellular source of ferritin to be BAL macrophages, where FTL is significantly elevated in patients with SS (C, D). FTH expression in BAL cells did not correlate with serum ferritin levels, whereas FTL mRNA expression in BAL cells revealed a strong positive association with serum ferritin levels (E, F). Ferritin subunit expression was also evaluated in transbronchial biopsies (TBBx), where FTH and FTL levels were not significantly different in SS versus CS (G). Paired analysis of FTH and FTL mRNA expression in matching BAL and TBBx revealed that BAL cells are the main ferritin source in the lung (H, I). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, one-way non-parametric analysis of variance (Kruskal–Wallis test) or paired *t*-test was conducted where appropriate, data represent mean ± SEM

regulatory protein (IRP-1), where their binding is stimulated by multiple cellular signals including cellular iron levels and oxidative stress.[23] Consistent with our clinical observations, silica complexed with iron generates greater production of FTL in human alveolar macrophages compared to silica alone under in vitro conditions.[24] Hence, we propose that dysfunctional lung macrophages produce excessive levels of ferritin in response to iron-loaded silica exposure, where ferritin spills over into the lung vasculature to increase serum ferritin levels.

Excess iron and ferritin may also directly contribute to fibrotic remodelling through modulation of pathogenic macrophages. Macrophages exhibit distinct phenotypic and functional states in response to environmental cues, which give rise to a range of effector functions including tissue repair, regeneration and fibrosis.[25,26] There may be distinct macrophage phenotypes in silicosis, which differentially respond to silica nano- and micro-particle uptake and toxicity.[27,28] In a murine model of silicosis, Zhao et al. demonstrated that polarization of pulmonary macrophages was dysregulated during silicosis development, where pro-inflammatory macrophages were induced in early disease and anti-inflammatory macrophages were found to promote dysfunctional repair and fibrosis in late disease.[29] Emerging evidence demonstrates that iron has the potential to directly influence macrophage polarization.[19,30]

Iron, in the form of ferric ammonium citrate (FAC), can drive the differentiation of THP-1 macrophages towards a wound-healing phenotype. Additionally, FAC induced a significant increase in ferritin expression.[30] Of significance, co-culture of FAC pre-treated macrophages with human dermal fibroblasts (HDFs) led to the upregulation of type-1 and -3 collagen mRNA expression in HDFs.[30] FTL may also directly promote fibrosis, where FTL knockdown can dramatically repress endothelial-to-mesenchymal transition and migration by regulating AKT/GSK3β/β-catenin signalling.[31] Further studies are needed to establish whether FTL can exhibit similar effects in the context of chronic lung diseases including silicosis.

Our study has a number of limitations. Whilst we did observe a differential increase in serum ferritin levels in patients with simple silicosis, the sample size is small. Additionally, the present study represents data from a single time-point. Future studies should ideally focus on longitudinally tracking ferritin levels in the serum and BAL to determine whether ferritin levels are predictive of patients who progress to complicated silicosis as determined by HRCT. Such studies may delineate whether ferritin is valuable as a prognostic biomarker. Moreover, BAL and serum longitudinal analysis of silica-exposed workers who are yet to develop radiological disease may be of great insight into the development and progression of silicosis. Whilst the NALP3 inflammasome pathway can acutely stimulate the production of inflammatory and acute-phase mediators following RCS exposure, we found that systemic inflammation did not persist in patients with simple or complicated silicosis. Potentially of greater significance to the development of silicosis is the iron and ferritin status, where iron-loaded RCS particles stimulate a phenotypic shift in BAL macrophages, which develop a foam cell morphology and chronically overexpress FTL. Excess iron and FTL overexpressing macrophages have the potential to contribute to lung fibrosis via promoting the accumulation of lipid-loaded foam macrophages that stimulate collagen deposition and promoting endothelial-to-mesenchymal transition through β-catenin signalling. Future work is needed to investigate the significance of excess iron and FTL in the development of silica-induced lung fibrosis.

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CONFLICT OF INTEREST

This study was supported by a research grant from Multiplex Australia.

AUTHOR CONTRIBUTION

Christian Anthony Aloe: Data curation (equal); formal analysis (equal); methodology (equal); project administration (equal); writing – original draft (supporting). **Tracy Li-Tsein Leong:** Data curation (equal); formal analysis (equal); investigation (equal); writing – review and editing (equal). **Hari Wimaleswaran:** Formal analysis (supporting); investigation (supporting); writing – review and editing (supporting). **Paris Clarice Papagianis:** Formal analysis (equal); investigation (equal); writing – review and editing (equal). **Jonathan Luke McQualter:** Formal analysis (equal); investigation (equal); project administration (equal); writing – review and editing (equal). **Christine Faye McDonald:** Formal analysis (equal); supervision (equal); writing – review and editing (equal). **Yet Hong Khor:** Formal analysis (equal); validation (equal); writing – review and editing (equal). **Ryan Francis Hoy:** Writing – review and editing (equal). **Aviraj Ingle:** Formal analysis (equal); investigation (equal); methodology (equal); writing – review and editing (equal). **Vipul Bansal:** Formal analysis (equal); methodology (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Nicole Soo Leng Goh:** Formal analysis (equal); investigation (equal); writing – original draft (equal). **Steven Bozinovski:** Conceptualization (lead); formal analysis (equal); investigation (equal); project administration (lead); writing – original draft (lead).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

HUMAN ETHICS APPROVAL DECLARATION

This study was performed in accordance with the Declaration of Helsinki and was approved by the Austin Health

Human Research Ethics Committee – approval: 54116/2019. All adult participants provided written informed consent to participate in this study.

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