Submitted 27-03-23, Revised October 2023

Elevated ferritin, mediated by IL-18 is associated with systemic inflammation and mortality in acute respiratory distress syndrome (ARDS)

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Submitted 27-03-23, Revised October 2023

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Article Type: Original Research

Title: 17/20 words

Abstract: 225/250 words

Total: 3687 /3500 words

Number of Figures: 3

Number of Tables: 2

Supplementary Figures: 6

Supplementary Tables: 5

Supplementary Appendices: 3

References: 38 /40

Submitted 27-03-23, Revised October 2023

Keywords:

ARDS, ferritin, HARP-2, ROSE, IL-18 immunomodulation

Abstract (225/250 words)

Background: Inflammatory subphenotypes have been identified in ARDS. Hyperferritinaemia in sepsis is associated with hyperinflammation, worse clinical outcomes, and may predict benefit with immunomodulation. Our aim was to determine if raised ferritin identified a subphenotype in patients with ARDS.

Methods: Baseline plasma ferritin concentrations were measured in patients with ARDS from two randomised controlled trials of simvastatin (HARP-2; discovery cohort, UK) and neuromuscular blockade (ROSE; validation cohort, USA). Results were analysed using a logistic regression model with restricted cubic splines, to determine the ferritin threshold associated with 28-day mortality.

Results: Ferritin was measured in 511 patients from HARP-2 (95% of patients enrolled) and 847 patients (84% of patients enrolled) from ROSE. Ferritin was consistently associated with 28-day mortality in both studies and following a meta-analysis, a log fold increase in ferritin was associated with an OR 1.71 [95%CI 1.01-2.90] for 28-day mortality. Patients with ferritin >1380 ng/ml (HARP-2 28%, ROSE 24%) had a significantly higher 28-day mortality and fewer ventilator free days in both studies. Mediation analysis, including confounders (APACHE-II score and ARDS aetiology) demonstrated a statistically significant contribution of IL-18 as an intermediate pathway between ferritin and mortality.

Conclusions: Ferritin is a clinically useful biomarker in ARDS and is associated with worse patient outcomes. These results provide support for prospective interventional trials of immunomodulatory agents targeting IL-18 in this hyperferritinaemic subgroup of patients with ARDS.

Submitted 27-03-23, Revised October 2023

Thorax Summary Key Points:

What is already known on this topic

Randomised controlled trials (RCTs) of pharmacotherapies in ARDS have shown no benefit to patients, which has been attributed to clinical and biological heterogeneity within the patient population. Analysis of clinical and biomarker data from previous RCTs in ARDS have identified hypo- and hyper-inflammatory phenotypes of ARDS. However, prospective patient stratification using this method is challenging as it requires measurement of biomarkers that are not routinely available.

What this study adds

In this post-hoc analysis of two RCTs (HARP2 and ROSE) in ARDS, patients with a baseline ferritin >1380 ng/ml had a significantly higher 28-day mortality and fewer ventilator-free days. In HARP-2 patients with ferritin >1380 ng/mL had longer ICU (3 days, 95% CI 0.01-5) and hospital stays (8 days, 95% CI 2-15). This appears to be partly dependent on IL-18-driven (and hence inflammasome-dependent) inflammation.

How this study might affect research, practice or policy

High ferritin, a routinely available marker, identifies a cohort of patients with ARDS at risk of worse outcome. Additionally, these results provide support for prospective trials targeting IL-18 pathway in the hyperferritinaemic subgroup of patients with ARDS.

Submitted 27-03-23, Revised October 2023

Introduction:

Acute respiratory distress syndrome (ARDS) is characterised by lung injury that results in respiratory failure with hypoxemia, decreased lung compliance, and bilateral alveolar opacities on chest imaging[1]. ARDS carries a mortality of approximately 42-50% despite supportive care.[2] In ARDS, respiratory failure and alveolar damage result from severe dysregulated inflammation, with endothelial activation, disruption of the alveolar-capillary barrier and exudation of protein-rich fluid into the alveolar space.[3]

There are no approved pharmacological treatments for ARDS, and the management is supportive. Randomised controlled trials (RCTs) of pharmacotherapies in ARDS have shown no benefit to patients, which has been attributed to clinical and biological heterogeneity within the patient population[4]. Latent class analysis (LCA) of clinical and biomarker data from five randomised controlled trials (RCTs) identified hypo- and hyper-inflammatory phenotypes of ARDS exhibiting divergent biological characteristics, clinical features and outcomes.[4, 5] Prospective patient stratification using this method is difficult as it requires measurement of biomarkers such as soluble tumour necrosis factor receptor 1 (sTNFR1) and interleukin (IL-) -6 and -8 that are not routinely available.[6]

Ferritin is an acute phase protein, which is induced by pro-inflammatory cytokines including -IL-1β and tumour necrosis factor (TNF).[6] Ferritin is a readily available biomarker in clinical practice and is in integrated in several classification systems or risk models for hyperinflammatory disorders. These include familial haemophagocytic lymphohistiocytosis (HLH) (ferritin ≥500 ng/mL),[7] macrophage activation syndrome associated with systemic juvenile idiopathic inflammatory arthritis (ferritin ≥684 ng/mL),[8] macrophage activation-like syndrome associated with sepsis (ferritin ≥4420 ng/mL)[9] and more recently coronavirus disease 2019 (COVID-19)-associated hyperinflammation (ferritin ≥700 or ≥1500 ng/mL in different models).[10, 11] The precise role of, whether as a bystander or direct contributer to hyperinflammation, in these syndromes is poorly understood. A recent study suggested that ferritin promotes neutrophil activation and the formation of neutrophil extracellular traps (NETs), contributing to the pathogenesis of hyperinflammation in adult onset Still's disease.[12] Interestingly, *in vitro* studies have shownferritin activate nucleotide-binding oligomerization domain, leucine-rich repeat receptor and pyrin-domain containing-protein 3 (NLRP3) inflammasomes.[13] Inflammasomes are protein signalling complexes that regulate production and activation of IL-1β

and IL-18, and also cause pyroptosis, a form of lytic cell death.[14] High plasma IL-18, a surrogate of inflammasome activation, is reported in patients with ARDS,[15] and is associated with high mortality in patients with ARDS.[15-17]

Although prognostic value of ferritin has been described in sepsis, its role is in ARDS is unknown.. In a secondary analysis of a RCT in patients with severe sepsis, patients with hyperinflammation (using coagulopathy and hepatobiliary dysfunction as proxy indicators), had improved outcomes with anakinra, a recombinant IL-1 receptor antagonist.[18] A ferritin concentration ≥4420 ng/mL (found in ~4% of patients with sepsis) was associated with increased mortality and a pro-inflammatory cytokine signature[9]. In patients at-risk of ARDS, ferritin can predict the subsequent development of ARDS and multi-organ failure in the context of trauma[19, 20] and COVID-19.[21] Theferritin threshold for predicting ARDS in COVID-19 was ≥950 ng/mL.[21] In a small single-centre study of patients with ARDS (n=48), the mean serum ferritin concentrations (<24 hours of hospitalisation) was higher in non-survivors compared with survivors.[22]

We hypothesised that ferritin was associated with poor clinical outcomes in ARDS and aimed to evaluate ferritin as a prognostic biomarker in patients with ARDS, using data from two RCTs.

Methods:

Study design:

Secondary analysis of two multi-centre, randomised controlled trials of patient with ARDS to describe the association between baseline plasma ferritin concentrations and 28-day mortality using each RCT as a discovery/validation cohort.

Outcomes:

The primary outcome was to establish whether ferritin was consistently associated with 28-day mortality in patients with ARDS. The secondary outcomes were to determine: a ferritin threshold associated with 28-day mortality for potential stratification of patients in future studies; if there was a treatment effect in each RCT for patients in the high ferritin subgroup; whether there was an association between ferritin and other biomarkers associated with poor

outcomes in patients with ARDS and if the association between ferritin and mortality was mediated by inflammasome activation. However, given the complexity, it is uncertain that increased baseline plasma IL-18 represents increased inflammasome activation[23].

Populations:

For the discovery population, we undertook a secondary analysis of the Hydroxymethylglutaryl-CoA Reductase Inhibition with Simvastatin in Acute Lung Injury to Reduce Pulmonary Dysfunction-2 (HARP-2) Study, a multicentre RCT conducted in the U.K. and Ireland comparing daily simvastatin to placebo in 540 mechanically-ventilated patients with ARDS from any cause[24]. For the validation cohort, we performed a secondary analysis of a USA multicentre RCT in mechanically ventilated patients with ARDS from any cause, of a 48-hour continuous infusion of cisatracurium with deep sedation compared with usual-care without routine neuromuscular blockade and lighter sedation(ROSE)[25]. Both RCTs had similar inclusion/exclusion criteria (supplementary materials), however there were some important differences: the enrolment PaO_2:FiO_2 threshold was lower in ROSE (<150 mm Hg) compared with HARP-2 (<300 mm Hg) and patients who had been mechanically ventilated for more than five days were excluded from enrolment in ROSE.

Plasma biomarker measurement

Plasma was collected from patients within 48 hours of onset of ARDS and prior to randomisation in the discovery and validation cohorts and stored at –80°C. IL-6, IL-18, angiopoietin-2 (Ang-2), soluble receptor for advanced glycation end products (sRAGE), surfactant protein-D (SP-D) and soluble tumour necrosis factor receptor-1 (sTNFr-1) were previously measured in plasma samples from HARP-2 patients, using commercially available ELISAs for IL-6, IL-18, Ang2, RAGE and SP-D using Duoset ELISA kits (R&D Systems, MN, USA), Quantikine kits, (R&D Systems) for sTNFR-1. These data were not available from the ROSE cohort). Free IL-18, which is the biologically active form of IL-18, was calculated using total IL-18 and IL-18BP concentrations, given that a single IL-18BP molecule binds a single IL-18 molecule[26].

Ferritin was measured in plasma from both HARP-2 and ROSE patients using ferritin (FTL) ELISA kit (Abcam, UK). For values above the upper limit of detection of the assay, the highest limit of detection corrected for dilution was assigned.

Biomarker analysis on stored samples from the HARP-2 trial[24] was approved by Queen's University of Belfast Faculty of Medicine Research Ethics Committee (reference PREC18.08). The institutional review board for the ROSE trial approved plasma collection and biomarker analysis.[25]

Statistical Analysis:

Descriptive data are presented as median with interquartile ranges and counts with percentages. Statistical comparisons of baseline characteristics and protein biomarker concentrations between ferritin groups used the Wilcoxon rank sum test for continuous variables and chi-squared test for categorical variables.

Primary outcome in discovery and validation cohorts

To model the non-linear, continuous association between ferritin and mortality we used logistic regression with restricted cubic splines.[27, 28] To account for patient heterogeneity we made adjustments for baseline physiological features and age (accounted for by APACHE scores), and aetiology of ARDS. The APACHE scoring methods were differed between the two cohorts, with APACHE-II used in the discovery cohort and APACHE-III in the validation cohort. We imputed missing APACHE scores using chained equations and adjusted models were fitted using ten pooled sets.

After determining the effect size of ferritin in the discovery cohort, we performed a power calculation to estimate the number of baseline samples required to observe a similar effect with 90% power in the validation cohort. We also accounted for a 10% assay failure rate due to the use of stored samples.

As these analyses were performed *post hoc* on patients recruited to RCTs, we included treatment allocation as an interaction term in our models (see Supplementary Materials). A meta-analysis with random effects was performed using the study-specific weights to inform a pooled estimate of the association between ferritin and 28-day mortality. The DerSimonian-Laird estimator was used for heterogeneity.

To assess the performance of ferritin as a prognostic biomarker we used the area under the receiver operator characteristic (AUROC) and decision curve analysis (DCA). In DCA, we compared the net-benefit of ferritin for predicting 28-day mortality with APACHE scores. Additional model validation methods

included examination of calibration curves and calculation of Brier's scores. More detailed information relating to these specific methods are described in the Supplementary Materials.

High ferritin threshold calculation

To facilitate descriptive analysis of a subgroup with elevated ferritin levels and to offer clinical utility for prospective measurement of ferritin, we determined a threshold value using the Youden index. This method calculates a statistical trade-off between sensitivity and 1-specificity. Although dichotomisation of continuous variables is generally not recommended, we decided to proceed with this analysis, given that the relationship between ferritin and ARDS patients has not been previously defined and is non-linear, to explore the characteristics of patients with high ferritin and identify potential confounding features.

Mediation analysis

We developed a directed acyclic graph (Figure S1) to delineate the causal relationship between ferritin and 28-day mortality, whilst integrating confounders such as the APACHE-II score and aetiology of ARDS. Both of these factors are have shown to influence outcomes in patients with ARDS.[29, 30]

To describe the contribution of inflammasome activation in outcome we incorporated IL-18 as an intermediary variable in our model. Mediation analysis quantifies the effect on an intermediate variable within a causal sequence and simultaneously accounts for confounders that could influence the primary cause, proposed mediator and outcome. Additional background information on statistical mediation analysis can be found in the Supplementary Material. A flexible Bayesian approach using Baysian regression modelling strategies (brms) was required to account for ARDS risk factors (Figure S2) [31]. As free IL-18 is the biologically active form of IL-18, we carried out our mediation analysis with total IL-18 and calculated free IL-18 separately.

Results from models are presented as odds ratios with 95% confidence limits. Analysis was conducted using R (version 4.0.2, the R Core Team, Vienna) or STATA (version 17.0, STATACorp, College Station, TX, USA) with a significance level of p <0.05.

Results:

Plasma ferritin concentrations were measured using samples obtained on the day of randomisation in 511 of the 540 patients in the discovery cohort (HARP-2). We found no differences in the standardised means of baseline features between subjects that had samples available for ferritin measurement in the discovery cohort (n = 511) and in those whose samples were unavailable (n=29) (Table S1). Using our power we calculation we estimated that 847 samples would be required to replicate our results. We measured the ferritin concentration in 847 of the 1006 patients in the validation cohort (ROSE). Baseline characteristics of the included patients from both cohorts are presented in Table 1 and Table S2.

In the discovery cohort median ferritin concentration was 610 ng/mL (IQR 284-2963). Ferritin concentration was significantly higher in non-survivors (median 882 ng/mL, IQR 363-2809) compared with survivors (median 583 ng/mL, IQR 263-1274 ng/mL; p=0.003). In the validation cohort, median ferritin concentration was 406 ng/mL (IQR 158-1308). Of note the usual reference range in clinical laboratories for ferritin is 41 – 400 ng/mL. Ferritin concentrations were significantly higher in non-survivors (median 740 ng/mL, IQR 271-2209) compared with survivors (median 271 ng/mL, IQR 127-736 ng/mL); p<0.0001.

Ferritin as a prognostic biomarker in ARDS

The relationship between ferritin concentration and 28-day mortality in the discovery cohort was non-linear (Figure 1), and better characterised by a spline model compared to a linear model (likelihood ratio test p<0.001).. A log-fold increase in ferritin was associated with an increased 28-day mortality in both the discovery (OR 2.61 [95% CI 1.27-5.36, p = 0.009])and in the validation cohorts (OR 1.59 [95% CI 1.36-1.86, p<0.001]). Following adjustment for APACHE scores, aetiology and treatment allocation, this association remained significant in both cohorts (discovery OR 2.57, 95% CI 1.20-5.51; p=0.02; validation OR 1.43, 95% CI 1.13-1.81; p <0.001). There was no statistical significance associated with treatment group interaction term on mortality in either the discovery or validation cohorts (Table S3a). In a sub-group analysis the association between ferritin and 28-day mortality was abrogated in the simvastatin arm of the

discovery cohort ((OR 0.94, 95%CI 0.44-2.00; Table S3b), but this was not observed in the ciatracurium arm of the validation cohort where the association between ferritin and mortality remained unchanged (Table S3b).

In a random-effects meta-analysis the pooled odds ratio for the association between ferritin (on a log scale) and 28-day mortality was 1.71 [95% CI 1.01-2.90, p=0.048] (Figure 2). Heterogeneity in this meta-analysis was moderately high but not statistically significant (I^2=52.1%, τ^2=0.09, p=0.15). Univariate meta-analysis results are shown in Figure S3.

The AUROC for ferritin was 0.59 (95% CI 0.53-0.65) in the discovery cohort and 0.66 (95% CI 0.63-0.70) in the validation cohort. In a pooled analysis the AUROC was 0.62 (95% CI 0.59-0.65), which was comparable to APACHE-II (discovery AUROC 0.62; 95%CI 0.55-70) and higher than the Berlin definition severity criteria (discovery AUROC 0.56; 95% CI 0.49-0.64). Decision curve analysis showed ferritin had a comparable net benefit compared to APACHE-II (discovery) and APACHE-III (validation) scores for discriminating patients at higher risk of 28-day mortality at a given threshold risk (Figure S4). The upper limit of quantification in the validation cohort was 3968 ng/ml and 82 samples (9.7%) had ferritin values greater than this value. Given the greater range of ferritin values in the discovery cohort we fitted our model in the validation cohort and checked for calibration in the subset of discovery cohort patients with ferritin values < 3968 ng/ml (Figure S5). The Brier score for this calibration curve was 0.216.

The association between ferritin and other inflammatory mediators and ARDS biomarkers

In the discovery cohort, ferritin was significantly positively correlated with some plasma biomarkers of systemic inflammation (IL-18, which is downstream of inflammasome activation, and sTNFR1) but interestingly, not IL-6 (Figure 3). Ferritin correlated with markers of endothelial cellular injury (Angiopoietin-2 (Ang-2)) and type 1 alveolar epithelial cellular injury (plasma soluble receptor for advanced glycation end products (sRAGE)) (Figure 3).

We did not observe a significant association between ferritin and the type II epithelial cell marker surfactant protein-D (SP-D). We found that free IL-18, the biological active form of IL-18, was also significantly correlated with ferritin (Figure S6).

A causal mediated effect of IL-18

The parameter estimates for the casual paths between ferritin, IL-18, confounders and outcomes are shown in the DAG in Figure S1. The average causal mediated effect of IL-18 on mortality was 0.06 (credible interval = 0.0003-0.13) (Table S4a). Similar causal mediated effect sizes were obtained with free IL-18 (effect = 0.05, credible interval = 0.01 – 0.10) (Table S4b).

Calculation of a high ferritin threshold and features of these patients

The Youden index calculated 1380ng/mL as the threshold for a high ferritin subgroup in the discovery cohort. Mortality was 36.8% in patients with a ferritin >1380ng/mL, compared with 20.9% in patients with ferritin <1380 ng/mL ($p<0.01$ Chi-squared test). Patients with high ferritin (>1380 ng/mL) had significantly higher non-pulmonary SOFA scores at baseline. There were no significant differences in PaO_2:FiO_2 ratio between ferritin subgroups. These patients had fewer VFDs (median difference 16 days; $p<0.001$), longer ICU stays (median difference 3 days, 95% CI 5-0.01 days; $p < 0.02$]) and longer hospital stays (median difference 8 days, 95% CI 15-2 days; $p<0.006$]) (Table 2).

In the validation cohort 204 out of 847 patients (24.1%) had a ferritin concentration >1380 ng/mL, which conferred a mortality of 60%, compared with 35% in patients with ferritin <1380 ng/mL ($p<0.0001$ Chi-squared test) (Table 2). At this threshold the sensitivity for 28-day mortality was 42.7% (discovery) and 36.4% (validation), whilst the specificity was 76.5% (discovery) and 83.7% (validation). Similar to the discovery cohort, high ferritin patients in the validation cohort also had fewer ventilator-free days (median difference 14 days; $p <0.001$). There was no evidence of change in outcomes for patients with high ferritin allocated to the treatment arms of either trial (Table S3). In the validation cohort, the aetiology of ARDS differed significantly between patients in the high and low ferritin subgroups ($p<0.001$). Patients in the validation cohort with high ferritin were younger in age with a higher frequency of non-pulmonary sepsis and lower frequency of gastric aspiration (Table 1).

Discussion:

In this secondary analysis of two large RCTs of patients with ARDS, higher plasma ferritin concentration was associated with higher 28-day mortality and fewer VFDs in both study populations. In the discovery cohort, patients with ferritin concentration >1380 ng/mL had higher non-pulmonary organ failure scores, and longer duration of ICU and hospital stay. Notebly, both cohorts had similar degrees of hypoxaemia irrespective of ferritin subgroup.

In the discovery cohort ferritin concentration was strongly associated with plasma biomarkers of systemic inflammation (IL-18 and sTNFR1), suggesting that poor outcomes may be driven by immune dysregulation. However, ferritin was not significantly associated with higher IL-6 concentrations, suggesting that different inflammatory processes and pathways may be implicated in this subgroup of patients with ARDS. The stronger association between ferritin and Ang-2 compared with sRAGE, worse non-pulmonary organ failure, and lack of an association with severity of hypoxaemia in these patients further support the hypothesis of a dysregulated inflamamtion. Interestingly, in the discovery cohort, patients treated with simvastatin had no significant association between ferritin level and 28-day mortality (OR 0.94, 95%CI 0.44-2.00; Table S3b). However when we dichotomised patients in the treatment arms from both RCTs, into low and high ferritin groups, there were no differences in the 28-day mortality, irrespective of low/high ferritin status (Table S5)

Biological heterogeneity has likely accounted for an absence of effective pharmacotherapies in ARDS. Unlike previous approaches to identify sub-groups,[32] our study demonstrates that a routinely available marker (ferritin), may delineate patients with ARDS at risk of worse outcomes. Furthermore, mediation analysis (accounting for confounders; APACHE-II score and ARDS aetiology) provides evidence for a biologically-plausible mechanism – given a statistically significant contribution of IL-18 in the intermediate pathway between ferritin and mortality in a causal model (Figure S1). The relatively small effect size with wide confidence limits of the effect of IL-18 mediating outcome (0.06, 95%CI 0.003-0.13; Table S4A) is likely to related to the small measured quantities of IL-18 in the the stored samples and suggested that the precise roles of ferritin and IL-18 in patients with ARDS require further exploration with experimental models. The mediated effect size of free IL-18, the biologically active form, was greater with narrower confidence limits (0.05, 95%CI 0.009 – 0.10; Table S4B) further supporting our proposed mechanism. It is generally considered that ferritin is not only a marker of

inflammation, but is also a pro-inflammatory mediator critical to disease pathomechanisms.[33] IL-18 is considered a surrogate marker of inflammasome activation, and is readily measured in the systemic compartment in ARDS, unlike IL-1β which has rapid clearance and a short half-life in plasma which does not reflect tissue levels.[34] Previous studies have shown that IL-1β can induce ferritin transcription, creating a feedback loop to propagate inflammation.[35, 36] Our findings support the emerging evidence implicating IL-18 in the aetiopathogenesis in a subgroup of patients with ARDS[23] and support the need for investigation of immunomodulatory therapies that target IL-18- (and hence inflammasome)-dependendent inflammation in hyperferritinaemic ARDS.

The strengths of our study include that our findings are consistent in two independent, large RCTs and the mediation analysis supportsa biologically plausible mechanism for the effect of ferritin. Inclusion of patients with ARDS from all-causes in both studies enhances the generalisability of our findings. Our findings are partially consistent with previously described hyper-inflammatory phenomena in patients with ARDS, which have been linked to worse outcomes.[5, 37] Although we observed abrogation of the effect of ferritin on mortality in patients treated with simvastatin in the discovery cohort, this finding is likely to be spurious given the lack of observable difference in outcomes between the high and low ferritin groups in patients treated with simvastatin (Table S5).

The AUROC for ferritin was 0.59 (95% CI 0.53-0.65) in the discovery cohort and 0.66 (95% CI 0.63-0.70) in the validation cohort. In a pooled analysis the AUROC was 0.62 (95% CI 0.59-0.65), which was comparable to APACHE-II (discovery AUROC 0.62; 95%CI 0.55-70) and higher than the Berlin definition severity criteria (discovery AUROC 0.56; 95% CI 0.49-0.64).

The AUROC for ferritin predicting 28-day mortality was 0.59 (95% CI 0.53-0.65) in the discovery cohort and 0.66 (95% CI 0.63-0.70) in the validation cohort. This is comparable to the Berlin definition for ARDS 0.577 (95% CI, 0.561-0.593).[38] Noteably, in the discovery cohort, ferritin was a better determinant of 28-day mortality compared to the Berlin severity criteria. In both cohorts, the correlation between elevated ferritin and mortality was consistent and the high ferritin sub-groups were a similar proportion. Ferritin performance mirrored APACHE scores in both cohorts, as demonstrated by decision curve analysis (Figure S3). Furthermore, our mediation model supported the hypothesis that hyper-inflammatory processes may be driving the impact on patient outcomes.

Limitations include that these are secondary *post hoc* analyses, which were not prespecified in the clinical trial protocols and therefore should be regarded as hypothesis generating, requiring prospective confirmation. There were some differences observed between the two study cohorts; in the discovery cohort, patients with high and low ferritin did not significantly differ by age or aetiology. However, in the validation cohort, patients with high ferritin were younger (a statistically, but unlikely to be clinically significant difference), with a higher incidence of non-pulmonary sepsis and lower incidence of gastric aspiration (Table 1). In the validation cohort, 84 patients had a ferritin value above the upper limit of detection of the assay, and were assigned the highest limit of detection corrected for dilution, whereas for the discovery cohort no imputation was necessary. This may explain why the relationship between ferritin and mortality appears linear at higher ferritin values in the validation cohort (Figure 1B) and why our models were poorly calibrated (Figure S4).An additional limitation is the moderate heterogeneity determined in the meta-analysis (I^2 = 52.7%). This could be attributed to a greater variance in ferritin values and smaller sample size in the discovery cohort, alongside variation in ARDS aetiology and clinical uncertainties (e.g. sepsis, gastric aspiration), and genetic/ancestral differences between the two trial populations and different eligibility criteria for enrolment, e.g. the enrolment PaO_2:FiO_2 threshold was lower in ROSE (<150 mm Hg) compared with HARP-2 (<300 mm Hg). Additionally, our analyses are based on ferritin concentrations measured at a single, early (<48 hours of ARDS diagnosis) time-point, indicating the prognostic utility of early ferritin measurement and precluding the evaluation of longitudinal ferritin trends and their association with outcomes.

In summary, our secondary analyses of the HARP-2 and ROSE RCTs, indicate that plasma ferritin, a readily available biomarker, can serve as valuable prognostic tool in ARDS. A ferritin concentration of >1380 ng/mL isassociated with an increased 28-day mortality, potentially due to IL-18 dependent inflammation. This study strengthens the case for targeting IL-18-dependent inflammation in the hyperferritinaemic cohort.

Tables

	Discovery (HARP-2) n=511			*Validation (ROSE) n=847*		
	Ferritin < 1380 ng/mL	**Ferritin > 1380 ng/mL**	***p***	**Ferritin < 1380 ng/mL**	**Ferritin > 1380 ng/mL**	***p***
n	367 (71.8%)	144 (28.2%)	-	643 (75.9%)	204 (24.1%)	-
Sex (male)	200 (54.5%)	90 (62.5%)	*0.12*	347 (54.0%)	121 (59.3%)	*0.18*
Age (mean (SD))	53.3 (16.7)	55.5 (15.5)	*0.14*	56.5 (15.3)	53.6 (14.3)	*0.016*
BMI (mean (SD))	27.4 [7.1]	26.8 [6]	*0.42*	31.5 (9.0)	30.7 (8.4)	*0.27*
APACHE II score (median [IQR])	17 [14-23]	21 [16-24]	*0.003*	-	-	-
APACHE-III score (median [IQR]	-	-	-	97 (78-118)	119 (97-142)	*< 0.001*
SOFA score (median [IQR])	8 [6-10]	9 [7-12]	*0.003*	-	-	-
Non-pulmonary SOFA score (median [IQR])	5 [3-7]	6 [4-9]	*0.002*	-	-	-
PaO_2:FiO_2 (mmHg) (median [IQR])	114 [85-161]	118 [90-155]	*0.67*	95 [76-123]	100 [77-124]	*0.51*
Oxygenation Index (median [IQR])	14 [8.7 -20.6]	14.1 [8.9-20]	*0.95*	-	-	-
Simvastatin treatment	190 (51.8%)	57 (39.6%)	*0.02*			
Cisatracurium for neuromuscular blockade	-	-	-	320 (49.8%)	107 (52.5%)	*0.50*
ARDS aetiology			*0.06*			*< 0.001*
Pneumonia	202 (55%)	78 (54.2%)	-	283 (44.0%)	99 (48.5%)	-
Sepsis (non-pulmonary)	61 (16.6%)	32 (22.2%)	-	73 (11.4%)	44 (21.6%)	-
Gastric Aspiration	38 (10.4%)	10 (6.9%)	-	125 (19.4%)	17 (8.3%)	-
Trauma	26 (7.1%)	5 (3.5%)		28 (4.4%)	4 (2.0%)	-
Pancreatitis	7 (1.9%)	9 (2.1%)	-	7 (1.1%)	3 (1.5%)	-
Smoke / toxin inhalation	2 (0.5%)	0	-	1 (0.2%)	1 (0.5%)	-
Other	27 (6.5%)	8 (6.9%)	-	126 (19.6%)	36 (17.6%)	-

Table 1. Baseline characteristics of patients at randomisation, in whom ferritin levels were measured, from the HARP-2 (n=511) and ROSE (n=847) randomised controlled trials, with ferritin concentrations above and below 1380 ng/mL, the threshold associated with adverse patient outcomes. Data from ROSE are shown, where available.Data are presented as mean (SD), median [interquartile range] or n (percentage).

BMI body mass index. APACHE acute physiology and chronic health evaluation. SOFA sequential organ failure assessment.

Table 2 Outcomes for patients in the discovery (HARP-2) and validation (ROSE) populations, stratified by ferritin above and below 1380 ng/mL. VFD Ventilator free days. Outcomes for ROSE are shown, where available.

		Discovery (HARP-2)					***Validation (ROSE)***		
		Ferritin < 1380 ng/mL		**Ferritin > 1380 ng/mL**	***p***		**Ferritin < 1380 ng/mL**		**Ferritin > 1380 ng/mL**	***p***
n		367		144	-		639		208	
ICU mortality		66 (18%)		53 (36.8%)	*<0.001*		-		-	-
28-day mortality		71 (20.9%)		52 (36.8%)	*<0.001*		224 (35.1%)		125 (60.1%)	*< 0.001*
VFD score (median [IQR])		16 [0-22]		0 [0-19]	*<0.001*		13 (0-22)		0 (0-12.5)	*< 0.001*
Duration of ICU Stay (days)* (median days [IQR])		10.5 [6-18]		14.5 [6-27]	*0.02*		-		-	-
Duration of hospital stay* (median days [IQR])		25 [14-46]		36 [19-58]	*0.006*		-		-	-

** for survivors*

Figure Legends:

Figure 1 Restricted cubic spline curves demonstrating the varying 28-day mortatlity for ferritin in the discovery (HARP-2) (A) and validation (ROSE) (B) populations. The solid lines show the estimated odds ratios and 95% confidence bounds are shown by the shaded regions. The Youden index, which balances sensitivity and specificity at different value, calculated a threshold value for ferritin equal to 1380 ng/ml.

Figure 2 Meta-analysis results for the effect of ferritin on 28-day mortality following adjustment for variables described in the causal model in Figure S1, accounting for treatment group allocation in each trial and random effects.

Figure 3 Scatter plots demonstrating the correlation between ferritin and key ARDS-associated protein biomarkers in the discovery cohort. Ferritin was siginificantly positivel correlated with IL-18, sTNFR1 and weakly with sRAGE and Ang-2. There was no significant association between IL-6 and ferritin which suggested that they may have been associated with different inflammatory processes in patients with ARDS.

sTNFR-1: soluble tumour necrosis factor receptor-1, Ang-2: angiopoietin-2, sRAGE: soluble receptor for advanced glycation end products, SP-D: surfactant protein-D, r: Pearson's correlation coefficient.

Figure S1 Directed acyclic graph describing the causal paths between ferritin in patients with ARDS and 28-day mortality. Arrows show possible causal paths between identified features of patients and their outcomes. The aetiology of ARDS and APACHE scores are all established associations with mortality in patients with ARDS but may also have contributed to the release of ferritin and IL-18 in these patients. Adjusting for these variables blocks the paths through them to the outcome which permits the calculation of the primary causal path between ferritin and mortality. The proposed indirect path (mediated path, shown in blue) through IL-18 estimates the contribution of IL-18 on the outcome for these patients. Mediation analysis calculated that the indirect path from ferritin, through IL-18, was significantly associated with 28-day mortality.

Figure S2 Random effects metanalysis results for the unadjusted effect of ferritin on 28-day mortality in the discovery and validation cohorts.

Figure S3 Decision curve analysis demonstrating the that ferritin conveys useful information for estimating the risk of 28-day mortality, similar to that provided by APACHE-II/III scores for patients in the discovery and validation cohorts. The "Treat All" line refers to estimation of 28-day moratlity if no information was used to guide risk estimation. The "Treat None" does not apply in this context. Lines that project above the Treat All line (higher net benefit) at a given threshold probablity can be considered to provide additional information which improves the calculation of estimated risk of mortality in these patients. This could be used to guide a therapeutic option if one was available.

Figure S4 Calibration curves for the ferritin logistic regression model of 28-day mortality, fitted on the validation cohort and tested in the discovery cohort. 1 and 0 values on the rug plot below the curve denote "Died" and "Alive" outcomes in the discovery cohort. The Brier's score for the model was 0.216.

Figure S5 Scatter plot between calculated free IL-18 and ferritin in baseline samples from patients in the discovery cohort. Free IL-18 concentrations were estimated from combined measurements of IL-18 and IL-18 binding protein. Free IL-18 was significantly correlated with ferritin in these patients (r = 0.29, $p < 0.0001$).

Figure S6 Diagnostic plots for mediation model of IL-18 on outcome. A flexible Bayesian framework that was able to account for 1. logistic link between outcome and predictors, 2. gaussian relationship between IL-18 and ferritin and 3. categorical data for ARDS risk factors was fitted using Bayesian regression modelling strategies (brms). The above plots show the posterior predictive checks between the densities of samples and the actual data (left panels) and leave one out probability integral transform (LOO-PIT) to check how well calibrated the model is (right panels).

Contributorship:

Conceived and designed study: PM, RJS, CMO and DFM. Analysed data from the ROSE cohort: KDW. Drafted manuscript: PM, RJS, RC Coll, DFM and CMO. Data collection, analysis and interpretation: PM, RJS, KDW, RCC, TW, PGM, AJB, JC, MS-H, AR, CSC, MAM, CS, RCC, DFM, CMO to. Revision of the manuscript and approved the final version: PM, RJS, KDW, RCC, TW, PGM, AJB, JC, MS-H, AR, CSC, MAM, CS, RCC, DFM, CMO .

Funding Statement:UK Efficacy and Mechanism Evaluation Programme

Declarations of interest: PM and RJS are/were clinical training fellows within the Experimental Medicine Initiative to Explore New Therapies (EMINENT) network and receive project funding unrelated to this article from the Medical Research Council and GlaxoSmithKline. PM also receives co-funding by the National Institute for Health Research (NIHR) University College London Hospitals Biomedical Research Centre. RJS receives co-funding by the Cambridge NIHR Biomedical Research Centre. PM reports consultancy fees from SOBI, UCB, Lilly, Abbvie, Boehringer and EUSA Pharma outside the submitted work. KDW reports grants from National Institutes of Health, during the conduct of the study. TJW reports PhD studentship funding from the Department for the Economy Northern Ireland. RC Coll reports grants from the Royal Society and the Academy of Medical Sciences during the conduct of the study; personal fees from BioAge Labs, personal fees from ImmVention Therapeutix, outside the submitted work; In addition, Dr. Coll has a patent US 10,538,487 licensed to Inflazome Ltd, a patent PCT/IB2017/053059 licensed to Inflazome Ltd, and a patent PCT/EP2017/053498 licensed to Inflazome Ltd. AJB reports received funding from the Northern Ireland Health and Social Care Research and Development office for research outside of the submitted work. MSH reports grants and other from National Institute for Health Research, during the conduct of the study. MS-H is supported by the NIHR Clinician Scientist Award (CS-2016-16-011). AR reports other from Merck, outside the submitted work. CSC reports grants from NIH R35-HL140026, during the conduct of the study; grants and personal fees from Roche/Genentech, grants and personal fees from Bayer, personal fees from Quark Pharmaceuticals, personal fees from Gen1e Life Sciences, personal fees from Vasomune, grants from Quantum Leap Healthcare Collaborative, outside the submitted work. MM reports grants from Roche Genentec, personal fees from Novartis, personal fees from Johnson and Johnson, personal fees from Gilead Pharmaceuticals, personal fees from Pliant Therapeutics, outside the submitted work; .CS is the Director of the EMINENT research training scheme supporting RJS and PM, and reports research funding to her institution from the Wellcome Trust, the Medical Research Council, the NIHR, GlaxoSmithKline, AstraZeneca and the Cambridge NIHR Biomedical Research Centre, all unrelated to this work. CS also reports personal fees for consultancy from Roche, GlaxoSmithKline and Abbvie. RCC reports grants from UKRI-MRC, NIHR-UCLH BRC and GSK, outside the submitted work. DFM chairs the NIHR and Medical Research Council funding committee for COVID-19 for therapeutics and vaccines. DFM reports personal fees from consultancy for ARDS for GlaxoSmithKline, Boehringer Ingelheim, and Bayer; in addition, his institution has received funds from grants from the UK NIHR, Wellcome Trust, Innovate UK, and others, all unrelated to this Correspondence. DFM also has a patent issued to his institution for a treatment for ARDS. DFM is a Director of Research for the Intensive Care Society and

NIHR Efficacy and Mechanism Evaluation Programme Director. CO reports grant funding from the Wellcome Trust, MRC, Innovate UK and NIHSC R&D for unrelated work. CO reports fees for consultancy from Insmed and for reviews from the California Institute of Regenerative Medicine (CIRM) unrelated to this work. CO's spouse has received consultancy fees from BI, Bayer and GSK unrelated to this work. All other authors declare no competing interests.

The views expressed in this manuscript are those of the authors and not necessarily those of the Medical Research Council (MRC), National Health Service, NIHR, or Department of Health and Social Care. This study was supported by the UK EME Programme, an MRC and NIHR partnership (08/99/08 and 16/33/01). The EME Programme is funded by the MRC and NIHR, with contributions from the Chief Scientist Office in Scotland, the National Institute for Social Care and Health Research in Wales, and the Health and Social Care (HSC) Research and Development Division, Public Health Agency for Northern Ireland. TJW is supported by a PhD studentship from the Department for the Economy Northern Ireland. RC Coll is supported by an Academy of Medical Sciences Springboard Award (SBF005\1104), a Royal Society Research Grant (RGS\R1\201127), and institutional funding from the Wellcome-Wolfson Institute for Experimental Medicine and Queen's University Belfast.

Ethics Approval: Biomarker analysis on stored samples from the HARP-2 trial[24] was approved by Queen's University of Belfast Faculty of Medicine Research Ethics Committee (reference PREC18.08). The institutional review board for the ROSE trial approved plasma collection and biomarker analysis.[25]

Data Sharing: Data will be available upon reasonable request

Acknowledgments: Jason Abbott and Chayse Jones performed ferritin concentration measurements in the ROSE cohort. Kevin Delucchi reviewed the statistical analysis plan in the ROSE cohort.

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