Clinical and laboratory signs of haemophagocytic lymphohistiocytosis associated with pandemic influenza A(H1N1) infection in patients needing extracorporeal membrane oxygenation

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BACKGROUND Severe pandemic influenza has been associated with the hyperinflammatory condition secondary haemophagocytic lymphohistiocytosis (HLH).

OBJECTIVES To determine the frequency, degree, character and possible cause of influenza-associated HLH in critically ill patients with severe acute respiratory distress syndrome due to influenza A(H1N1) infection requiring extracorporeal membrane oxygenation (ECMO) support at our hospital.

DESIGN A retrospective observational study.

PATIENTS AND SETTING Medical data were retrieved retrospectively from 11 consenting patients of thirteen adults infected with pandemic influenza A(H1N1) 2009 requiring ECMO between July 2009 and January 2010 at the ECMO Centre of Karolinska University Hospital, Stockholm, Sweden. All patients were evaluated for HLH using HLH-2004 criteria and HScore.

RESULTS Eleven patients (median age 31 years) were included in the study and all survived. All patients showed signs of multiple organ dysfunction and pronounced inflammation, more severe in the four patients with HLH who had significantly higher peak serum concentrations of ferritin ($P = 0.024$), alkaline phosphatase ($P = 0.012$) and gamma-glutamyl transferase ($P = 0.024$), lower concentration of albumin ($P = 0.0086$) and more frequently hepatomegaly ($P = 0.048$). Abnormal lymphocyte cytotoxicity (lytic units < 10) and a low proportion of natural killer (NK) cells were observed in three of four patients with HLH. Notably, we found a significant inverse correlation between serum ferritin concentration and NK cell and cytotoxic T lymphocyte percentages ($r_s = -0.74$, $P = 0.0013$ and $r_s = -0.79$, $P = 0.0025$, respectively). One HLH patient received HLH-directed cytotoxic therapy, another intravenous immunoglobulin and the other two no specific HLH-directed therapy.

CONCLUSION Critically ill patients, including healthy young adults, with pandemic influenza may develop HLH and should be monitored for signs of hyperinflammation and increasing organ dysfunction, and evaluated promptly for HLH because HLH-directed therapy may then be beneficial. The association of low NK percentages with hyperferritinaemia may suggest a role for reduced NK cell numbers, possibly also cytotoxic T lymphocytes, and subsequently reduced lymphocyte cytotoxicity, in the pathogenesis of hyperinflammation and secondary HLH.

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Introduction

The 1918 to 1919 ‘Spanish’ influenza virus caused the worst pandemic in recorded history and resulted in approximately 50 million deaths worldwide.\(^1\) The case fatality rates were more than 2.5% (<0.1% in other pandemics) with a ‘W-shaped’ (tri-modal) age-specific mortality curve featuring an unexplained peak in young adults.\(^1,2,13\) Similar trends were observed in infections with influenza A(H5N1) virus.\(^4\) In contrast, the 2009 pandemic influenza A(H1N1) was milder, with an estimated case fatality of one to 10 per 100,000 infections, but a persistent two to four times increased risk of worse outcome in young adults compared with seasonal influenza.\(^5,6\) Globally two to four times increased risk of worse outcome in young adults compared with seasonal influenza.\(^5,6\) Pandemic influenza constitutes a major medical risk, even in young adults, with a need for better treatments.

Severe pandemic influenza A(H1N1) infection may develop many similarities to the highly fatal hyperinflammatory condition secondary haemophagocytic lymphohistiocytosis (sHLH).\(^3\) Fatal influenza has been associated with haemophagocytosis, a prominent feature in HLH, and high levels of circulating inflammatory cytokines similar to the massive hypercytokinaemia associated with HLH.\(^8-10\) Patients with HLH often die in a sepsis-like hyperinflammatory condition with multiple organ failure, also commonly seen in patients with severe influenza A(H1N1).\(^11,12\) HLH can be triggered by viral infections, predominantly Epstein–Barr virus, which along with other forms of virus-associated HLH can be successfully treated with HLH-directed therapy, including corticosteroids and cytotoxic therapy when necessary.\(^13,14\) In 2010, we reported, to our knowledge, the first successful treatment of severe influenza A(H1N1) with HLH-directed cytotoxic therapy.\(^15\) Beutel et al.\(^16\) later reported sHLH in nine (36%) of 25 critically ill patients with confirmed 2009 influenza A(H1N1) infection, of whom eight (89%) died, some despite modified HLH-directed therapy. Furthermore, mutations in HLH-causing genes have been reported in fatal cases of influenza A(H1N1).\(^17\)

Based on the intriguing association between HLH and severe pandemic influenza, we aimed to study retrospectively the clinical characteristics, laboratory findings, treatment and outcome in a well defined cohort of critically ill patients with influenza A(H1N1) who required extracorporeal membrane oxygenation (ECMO) for severe hypoxaemic respiratory failure, particularly to estimate the frequency, degree and character of HLH development. Moreover, since we have previously shown a correlation between hyperferritinaemia/hyperinflammation and low cytotoxic function, the cause of primary HLH, lymphocyte cytotoxicity and HLH-associated genes were studied in patients whose samples were available.\(^18\)

Methods

The study was approved (2009/248-31/4) by the Ethics Committee at Karolinska Institutet, Stockholm, Sweden on 4 March 2009. In an earlier prospective study on hyperferritinaemia in critically ill ECMO-treated patients with pandemic A(H1N1) infections were excluded. Subsequently, we retrospectively studied these patients using the same ethical approval. We included patients who had influenza A(H1N1) infection confirmed by quantitative real-time reverse transcriptase PCR assay in bronchoalveolar lavage (BAL), who required ECMO support, and who gave informed consent. All patients were treated at the ECMO Centre, Karolinska University Hospital, Stockholm, Sweden, from July 2009 until February 2010. Criteria for ECMO support were respiratory failure, with or without circulatory and/or multiple organ failure, related to confirmed influenza A(H1N1) infection, as previously described.\(^19\) Clinical data, such as demographics, predefined comorbidities (obesity, cardiovascular or pulmonary disease, chronic renal insufficiency, immunosuppression, diabetes mellitus, liver disease, malignancy and pregnancy), radiology, clinical and laboratory parameters, and treatments were retrieved from medical records. Collected laboratory parameters included routine blood tests, ferritin and soluble CD25 (sCD25), as well as microbiology results from routine weekly viral, bacterial and fungal surveillance, and cultures and PCR assays performed on clinical indication.

Haemophagocytic lymphohistiocytosis criteria

Evaluation and diagnosis of HLH during ECMO care were based solely on clinical suspicion by the treating physician. Bone marrow examination in patients 1 and 2 were performed on suspicion of HLH, but in patients 3 and 4 on other haematological indications and without evaluation for HLH at the time. For this study, all patients were retrospectively evaluated for HLH using adapted HLH-2004 criteria, and additionally evaluated with an HLH probability score validated in adults (HScore) (Table 1) with best cut-off value for HScore 169 (sensitivity 93% and specificity 86% with accurate classification of 90% of the patients).\(^20,21\) Fever and haemoglobin (Hb) concentration were excluded as HLH-2004 criteria because ECMO regulates body temperature and regular blood transfusions were given to maintain Hb concentration above 100 g l\(^{-1}\) to improve oxygenation. Consequently, patients were evaluated for HLH on seven HLH-2004 criteria, with bicarbonate evaluated on platelet and neutrophil values. A patient was classified as having HLH when at least four of seven HLH-2004 criteria were fulfilled over two or more consecutive days. If daily samples were missing, blood test results ±48 h from sampling were used, and abdominal radiology, bone marrow and lymphocyte cytotoxicity results ±7 days from examination (otherwise evaluated as missing); HLH was evaluated daily from available concurrent results.

Lymphocyte cytotoxicity assays and genetics

Lymphocyte cytotoxicity functional analyses were performed in Ficoll-isolated peripheral blood mononuclear cells from heparinised whole blood in patients with
available samples. Percentages of natural killer (NK) cells (CD3-CD56+ cells) and CD8+ cytotoxic T lymphocytes (CTLs) were counted. NK cell percentages less than 5% of peripheral lymphocytes were considered low (normal reference 5 to 15%). NK cell cytotoxicity was assessed by standard chromium-release assay as previously described, and considered abnormal if lytic units were less than 10. NK cell degranulation was assessed via detection of surface CD107a after K562 cell stimulation, as previously described, and degranulation less than 5% was considered defective and 5 to 10% abnormal.

Genomic DNA, isolated from peripheral blood according to standard procedures, was sequenced for mutations in known HLH causing genes in patients with DNA available. Exons and exon/intron boundaries of PRF1, STX11, STXBP2, UNC13D, and exon/intron boundaries of THOC8, PRF1, STX11, UNC13C, and PRF1 were sequenced for mutations in known HLH causing genes. Genomic DNA, isolated from peripheral blood according to standard procedures, was sequenced for mutations in known HLH causing genes in patients with DNA available. Exons and exon/intron boundaries of PRF1, STX11, STXBP2, UNC13D, and exon/intron boundaries of THOC8, PRF1, STX11, UNC13C, and PRF1 were sequenced for mutations in known HLH causing genes. Genomic DNA, isolated from peripheral blood according to standard procedures, was sequenced for mutations in known HLH causing genes in patients with DNA available. Exons and exon/intron boundaries of PRF1, STX11, STXBP2, UNC13D, and exon/intron boundaries of THOC8, PRF1, STX11, UNC13C, and PRF1 were sequenced for mutations in known HLH causing genes. Genomic DNA, isolated from peripheral blood according to standard procedures, was sequenced for mutations in known HLH causing genes in patients with DNA available. Exons and exon/intron boundaries of PRF1, STX11, STXBP2, UNC13D, and exon/intron boundaries of THOC8, PRF1, STX11, UNC13C, and PRF1 were sequenced for mutations in known HLH causing genes. Genomic DNA, isolated from peripheral blood according to standard procedures, was sequenced for mutations in known HLH causing genes in patients with DNA available. Exons and exon/intron boundaries of PRF1, STX11, STXBP2, UNC13D, and exon/intron boundaries of THOC8, PRF1, STX11, UNC13C, and PRF1 were sequenced for mutations in known HLH causing genes. Genomic DNA, isolated from peripheral blood according to standard procedures, was sequenced for mutations in known HLH causing genes in patients with DNA available. Exons and exon/intron boundaries of PRF1, STX11, STXBP2, UNC13D, and exon/intron boundaries of THOC8, PRF1, STX11, UNC13C, and PRF1 were sequenced for mutations in known HLH causing genes.

### Statistical analysis
Statistical data analysis was performed using IBM SPSS Statistics version 22 (IBM Corp. Released 2013, Armonk, NY) and R version 3.2.0 (R Core Team (2015), R Foundation for Statistical Computing, Vienna, Austria). For descriptive analysis frequencies, simple percentage values, medians and minimum–maximum range were used. Patients with and without HLH were compared with Fisher’s exact test for categorical variables and Mann–Whitney U test for continuous variables.

### Results
Eleven of 13 patients with PCR-confirmed 2009 influenza A(H1N1) infection requiring ECMO support at our ECMO Centre during the influenza season 2009 to 2010 were eligible (Fig. 1). The study population had a male predominance (n=7), a young median [range] age of 31 years [22 to 55] and 5/11 patients were previously healthy without comorbidities. Identified comorbidities and main characteristics for each patient are outlined in Table 2. Median duration on ECMO was 16 days [3 to 51]. All patients required vasopressor support (noradrenaline and continuous renal replacement therapy due to circulatory instability, renal failure and/or fluid retention. All patients were on broad spectrum antibacterial treatment and antifungal prophylaxis or treatment. Overall, 3/11 patients had a possibly clinically relevant nosocomial bacterial or fungal infection prior to the highest HLH score. All 11 patients survived. All patients had fever (>38.4 °C) prior to initiation of ECMO support. Of the nine radiologically examined patients, four had splenomegaly and five hepatomegaly, of whom three had both. Cytopenias varied with leukocyte and neutrophil ranges of 0.7 to 102.2 × 10⁹ l⁻¹ (median 11.2) and 0.1 to 38.3 × 10⁹ l⁻¹ (median 8.3), respectively. However, all values were more than 1.5 × 10⁹ l⁻¹ if we exclude etoposide-induced low values in patient 11 and 1-day neutropenia (0.5 × 10⁹ l⁻¹) in patient 9 who then quickly developed leucocytosis. The
The median platelet count on admission was $122 \times 10^9\text{l}^{-1}$ [19 to 351] compared with $61 \times 10^9\text{l}^{-1}$ [19 to 138] at the nadir. No patient fulfilled the HLH-2004 criteria of bicytopaenia, but 5/11 patients had bicytopaenia according to the HScore (Table 2). The median albumin nadir was low at 18 g l$^{-1}$ [14 to 22]. Median peak values observed for liver and kidney function are shown in Table 3. Signs of inflammation were observed with median peak concentrations of C-reactive protein (CRP) of 448 mg l$^{-1}$ [178 to 500], procalcitonin 13 μg l$^{-1}$ [0.98 to 334] and ferritin 1742 μg l$^{-1}$ [686 to 24 301].

**Haemophagocytic lymphohistiocytosis vs. nonhaemophagocytic lymphohistiocytosis**

Four of 11 patients, all males, were evaluated as having developed HLH (denominated 'HLH') and are compared with patients who did not have (denominated 'non-HLH') in Table 3. Patients with HLH had a maximum HScore of 213 to 240 (median 226), compared with a maximum HScore of 19 to 136 (median 86) in non-HLH patients ($P = 0.006$) (Table 3). If full scoring for missing HScore parameters was added to the current highest HScore in non-HLH patients none of them would achieve an HScore of more than 142 (i.e. a probability of HLH below 18%), except patient 5 who would achieve 171 (55% probability of HLH).

HLH patients did not have more comorbidities than non-HLH patients. Three of four HLH patients required venoarterial ECMO on admission ($n=1$) or during ECMO treatment ($n=2$) due to circulatory failure. Patients who developed HLH seemed more severely ill on admission and required longer ECMO support compared with non-HLH patients (Table 3). Hepatomegaly was significantly more common in HLH patients ($P = 0.048$). HLH patients also had a higher median peak ferritin concentration of 10 735 μg l$^{-1}$ compared with 1204 μg l$^{-1}$ in non-HLH patients ($P = 0.024$). Only one patient without HLH, but with chronic leukaemia and invasive candidiasis (patient 5), had a ferritin concentration above 2000 μg l$^{-1}$ ($P = 0.015$). Median peak sCD25 levels were nonsignificantly higher in HLH patients than non-HLH patients (median peak 7323 and 4604 U ml$^{-1}$, respectively, $P = 0.37$), but many in the latter group had missing values ($n=5$). Furthermore, HLH patients displayed a significantly lower median albumin nadir, 15.5 g l$^{-1}$, compared with non-HLH patients, 18 g l$^{-1}$ ($P = 0.0087$).

Patients who developed HLH had more affected liver function values on admission, with significantly more elevated alanine aminotransferase ($P = 0.047$) and gamma-glutamyl transferase (GGT) ($P = 0.012$), compared with non-HLH patients. Subsequently, HLH patients developed more marked signs of obstructive hepatitis with significantly higher alkaline phosphatase (ALP) ($P = 0.012$) and GGT ($P = 0.024$), and nonsignificantly higher bilirubin (Table 3). Bone marrow examination results were only available for the four patients who developed HLH and all showed haemophagocytosis.

**Haemophagocytic lymphohistiocytosis-directed treatment**

Two (patients 1 and 2) of four retrospectively diagnosed HLH patients were diagnosed with HLH while on...
Table 2 Descriptive data and clinical and laboratory findings in A(H1N1)-positive patients on extracorporeal membrane oxygenation

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---------|---|---|---|---|---|---|---|---|---|----|----|
| HLH diagnosis | HLH | HLH | HLH | No HLH | No HLH | No HLH | No HLH | No HLH | No HLH | No HLH | No HLH |
| Sex (M/F) | M | M | M | M | F | F | F | M | M | M | M |
| Age (years) | 31 | 23 | 22 | 55 | 53 | 47 | 28 | 27 | 22 | 44 | 54 |
| Comorbidities | None | None | None | ibd, ps, ob, dm, cll | None | ob, dm, cll | None | ob, ps, pg | pg | None | ob, hpt, dm, hpt |
| Onset of A(H1N1) symptoms to ECMO admission (days) | 19 | 14 | 14 | 7 | 7 | 5 | 0 | 7 | 2 | 6 | 5 |
| ECMO admission to HLH (days) | 31 | 21 | 15 | 11 | 12 | 36 | 18 | 22 | 6 | 2 |
| HLH to off ECMO (days) | – | – | – | – | – | – | – | – | – | – |
| Duration on ECMO support (days) | 44 | 30 | 11 | 2 | – | – | – | – | – | – |
| Onset of A(H1N1)-PCR before HLH (days) | Positive (34) | Positive (17) | Positive (4) | Positive (7) | – | – | – | – | – | – |
| Bacterial infection (days) | CoNS BAL(0) | No | No | Candida blood(0) | Candida (BAL) | Candida (BAL) | Candida (BAL) | Candida (BAL) | Candida (BAL) | Candida (BAL) | Candida (BAL) |
| Fungal infection | No | No | Candida blood(0) | No | No | No | No | No | No | No | No |
| Fever (<38.4°C) | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| Sphenomegaly | Yes | Yes | Yes | No | Yes | No | No | No | No | ND | ND |
| Hepatomegaly | Yes | Yes | Yes | No | Yes | No | No | No | No | ND | ND |
| Comorbidities | HLH-2004 criteria | Yes | Yes | No | Yes | No | No | No | No | ND | ND |
| Platelet nadir (<50 x 10^9/l) | 61 | 72 | 67 | 35 | 28 | 106 | 138 |
| Leukocyte nadir (<5 x 10^9/l) | 11 820 | 24 301 | 2300 | 7829 | 12 933 | 1359 | 1742 |
| Peak ferritin concentration (<200 mg/l) and/or fibrinogen (<1.5 g/l) | 2460 | 4440 | 2352 | 1392 | 1170 | 1080 | 1704 |
| Peak ALT (U/l) | 283 | 497 | 125 | 35 | 277 | 277 | 145 | 23 | 25 | 42 | 13 |
| Peak LDH (U/l) | 1572 | 1668 | 204 | 456 | 78 | 132 | 282 |
| Peak GGT (U/l) | 3240 | 1614 | 234 | 336 | 25 | 120 | 510 |
| Lowest albumin (g/l) | 388 | 500 | 448 | 449 | 471 | 487 | 307 | 399 | 494 | 390 | 178 |
| Haemophagocytosis | Yes, BM | Yes, BM | Yes, BM | Yes, BM | Yes, BM | Yes, BM | Yes, BM | Yes, BM | Yes, BM | Yes, BM | Yes, BM |
| sCD25 > 2400 U/ml | Yes (7500) | ND | Yes (7146) | Yes (7500) | ND | Yes (7500) | Yes (7500) | Yes (7500) | Yes (7500) | Yes (7500) | Yes (7500) |
| Abnormal NK-cell activity | No | Yes | No | Yes | No | ND | ND | ND | ND | ND |
| Peak AKP (U/l) | 2460 | 4440 | 2352 | 1392 | 1170 | 1080 | 1704 | 1560 | 912 | 636 |
| Peak bilirubin (mg/dl) | 388 | 500 | 448 | 449 | 471 | 487 | 307 | 399 | 494 | 390 | 178 |
| Peak ALP (U/l) | 3240 | 1614 | 234 | 336 | 25 | 120 | 510 |
| Peak GGT (U/l) | 3240 | 1614 | 234 | 336 | 25 | 120 | 510 |
| Highest score | 240 | 230 | 221 | 213 | 213 | 107 | 93 | 86 | 83 | 63 | 19 |

All patients were PCR positive for influenza A(H1N1) on admission, negative for aspergillosis and all patients survived. Empirical treatment = broad spectrum antibiotics and antifungal therapy/prophylaxis. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAL, broncho-alveolar lavage; BM, bone marrow; CoNS, coagulase-negative staphylococci; CRP, C-reactive protein; CS, corticosteroids; F, female; GGT, gamma-glutamyl transferase; IVIG, intravenous immunoglobulins; LDH, lactate dehydrogenase; M, male; NA, not applicable; ND, not done; NK, natural killer; sCD25, soluble CD25; T, triglycerides; VP16, etoposide. a Comorbidities: prg, pregnancy; ob, obesity; hpt, hypertension; dm, diabetes mellitus; cll, chronic lymphocytic leukaemia (untreated and stable); ibd, inflammatory bowel disease (stable on azathioprine); pd, pulmonary disease; is, immunosuppression. b Remaining treatment days were in V-V ECMO. c Clinically relevant bacterial and fungal infections [i.e. positive culture (not colonisation), clinical deterioration or increased CRP] and howlong (days) before HLH/highest score. d ECMO regulates body temperature; fever excluded as a criterion. All patients had fever (>38.4°C) on admission prior to ECMO. ePlatelets less than 100 x 10^9/l and neutrophils less than 1.0 x 10^9/l; haemoglobin not applicable. fLeucocytes less than 5.0 x 10^9/l and platelets less than 100 x 10^9/l. gPrior to cytotoxic therapy. hHLH-2004 criteria as described by Henter et al. i, out of 7 criteria, fever excluded. In brackets, number of criteria with available data for evaluation. jHScore as described by Fardet et al. k, fever excluded.
ECMO support. Treatment of all four HLH patients therefore varied significantly (Table 2). Patient 1 was diagnosed with HLH at day 32 on ECMO support with persistent elevated inflammatory parameters and multiple organ failure without evident nosocomial infections until possibly relevant coagulase-negative staphylococci (CoNS) in BAL on day 32. He received aggressive supportive care without additional HLH-directed therapy, but had a long recovery time (29 days) from HLH diagnosis to ECMO discontinuation. Nonetheless, at the time of HLH (retrospectively diagnosed), patient 4 received 5 days of IVIGs when he deteriorated with already elevated oscillating inflammatory markers, left-shift and haemophagocytosis in bone marrow, and a possible *Candida albicans* and CoNS sepsis; ECMO was discontinued 2 days.** In contrast, patient 2, also with persistent inflammation without evident nosocomial infection, recovered from HLH with optimised antimicrobial treatment and aggressive supportive care without additional HLH-directed therapy, but had a long recovery time (29 days) from HLH diagnosis to ECMO discontinuation. Patients 3 and 4 were not evaluated for HLH during ECMO care. Nonetheless, at the time of HLH (retrospectively diagnosed), patient 4 received 5 days of IVIGs when he deteriorated with already elevated oscillating inflammatory markers, left-shift and haemophagocytosis in bone marrow, and a possible *Candida albicans* and CoNS sepsis; ECMO was discontinued 2 days.**

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days later. Patient 3 did not receive any HLH-directed therapy.

**Lymphocyte cytotoxicity and genetics**

Lymphocyte cytotoxic function was analysed in six patients, four with HLH and two without HLH. Abnormal NK cell cytotoxicity (lytic units <10) was noted in four patients, of whom three (75%) had HLH and also low NK cell [0.6 to 2.1%] and CTL [1.6 to 4.1%] percentages. A significant negative correlation was found between serum ferritin concentration and NK cell and CTL percentages (Spearman’s correlation coefficient $r_s = -0.741$, $P = 0.0013$ and $r_s = -0.79$, $P = 0.0025$, respectively). No other significant correlations with other lymphocyte cytotoxicity results were observed in this small subgroup. Patient 9, initially suspected of having HLH but not fulfilling the criteria, was observed to have abnormal lytic units and degranulation (normal percentages NK cells and CTL), which had normalised after recovery; genetic sequencing was normal.

Genetic sequencing was performed in five of these six patients. Patient 2, with both low lytic units (1 LU) and low NK cell (0.6%) and CTL (2.3%) percentages, was found to have a rare heterozygous variant in **UNC13D** (rs118049905, c.2896C>T p.Arg966Trp); MAF (0.004, according to gnomAD https://gnomad.broadinstitute.org/variant/6-144508563-G-A?dataset=gnomad_r2_1/Polyphen2=0.996/SIFT=0). Lymphocyte cytotoxic function analysis after recovery showed normalised values (lytic units 417, NK cells 12% and CTL 20%). One other rare heterozygous variant (c.799G>A p.Val267-Met) of unknown significance (MAF = 0.005, according to gnomAD https://gnomad.broadinstitute.org/variant/6-144508563-G-A?dataset=gnomad_r2_1) was found in **STX11** in a patient without HLH (patient 7); there were no available cytotoxicity data.

**Discussion**

Pandemic influenza A(H1N1) has been reported to affect a younger population among whom healthy young adults and pregnant women were high-risk groups, and more likely to suffer severe complications requiring intensive care and rescue therapies, including ECMO support, compared with seasonal influenza.\(^5\,^{12}\,^{24}\) These previous findings are in line with our 11 predominantly young, previously healthy adults with severe influenza A(H1N1) infection requiring ECMO support, and perhaps are even more marked in our patients with HLH [median age 27 years (44 years in non-HLH patients) and 3/4 without comorbidities (2/7 in non-HLH patients)]. The biology behind this globally observed trend in influenza pandemics remains poorly understood. An overexuberant inflammatory and immunological response of a robust and H1N1-naïve immune system to a very virulent H1N1 virus has been proposed.\(^3\,^{25}\) HLH has been reported in severe and fatal influenza A(H1N1), including patients requiring ECMO support.\(^9\,^{16}\,^{17}\) Four of 11 (36%) patients in our study with influenza A(H1N1) infection on ECMO support developed HLH according to adapted HLH-2004 criteria and with a maximum HScore more than 212 (median 226), evaluated as more than 93% probability of HLH (far beyond the defined best cut-off value for HLH of 169), compared with a maximum HScore less than 137, evaluated as less than 16% probability of HLH (median 86) ($P = 0.006$), and only one to three fulfilled HLH-2004 criteria in patients who did not develop HLH.\(^20\,^{23}\) Our patients portray the incremental gradient of hyperinflammation, that is some with manifest hyperinflammation, others very limited, and some (e.g. patient 5) in between.

Our results support the proposal that HLH may develop secondary to influenza, and even on ECMO support these patients can be distinguished from other critically ill patients with current diagnostic tools. In line with the study of Beutel et al.,\(^{16}\) patients with HLH showed more extensive signs of inflammation and severe illness compared with non-HLH patients, with significantly higher peak ferritin concentration ($P = 0.024$), lower albumin concentration ($P = 0.008$) and hepatomegaly ($P = 0.048$), and a trend to more frequent splenomegaly and higher lactate dehydrogenase concentrations, the latter also observed with ECMO treatment. Ferritin is a well known marker of inflammation and a current diagnostic criterion of HLH.\(^{20}\,^{26}\) Although not pathognomonic of hyperinflammation, extreme hyperferritinaemia is characteristic of HLH.\(^{11}\,^{27}\) Machowicz et al.\(^{28}\) suggest that highly elevated ferritin concentration and splenomegaly support the diagnosis of HLH rather than sepsis. The cholestatic hepatitis in our HLH patients, with hepatomegaly and significantly higher peak ALP and GGT concentrations ($P = 0.012$ and 0.024, respectively), may be caused by inflammatory periportal infiltration.\(^{29}\) These findings could, however, also be caused by congestive hepatopathy due to right ventricular failure (RVF) from severe acute respiratory distress syndrome, particularly in the three HLH patients who required veno-arterial ECMO due to circulatory failure.\(^{30}\) Of note, splenomegaly is generally absent in RVF-associated congestive hepatopathy.\(^{30}\)

Haemophagocytosis in the bone marrow, a hallmark of HLH, but also reported in other critically ill patients, was found in all four HLH patients but could not be compared with patients without HLH.\(^{31}\) Whether ECMO-related systemic inflammatory response syndrome, an inflammatory response observed at initiation of ECMO, may contribute to the development of hyperferritinaemia and HLH remains unclear.\(^{32}\) However, of our seven patients without HLH, only one had ferritin concentrations more than 2000 μg/l, and similar to Beutel et al,\(^{16}\) we observed a delay from onset of influenza A(H1N1) symptoms to the development of HLH later in the course.
of ECMO care, supporting the concept that they are separate hyperinflammatory entities.\textsuperscript{16}

Impaired NK and T-cell cytotoxic function, such as in genetic primary HLH, resulting in a defective down-regulation of the immune response and an innate over-activation with hyperferritinaemia, is associated with excessive, life-threatening cytokine-driven inflammatory responses.\textsuperscript{10,33} NK cell cytotoxicity acts to reduce tissue infiltration by inflammatory macrophages and to limit hyperactivation of CTLs (HLH manifestations).\textsuperscript{34} Lymphopaenia with low NK cell, CD4 and CD8 T-cell counts and delayed or excessive CTL activation in patients with severe influenza A(H1N1) has been described previously.\textsuperscript{35} Furthermore, A(H1N1)-virus may also directly infect and induce apoptosis in human NK cells.\textsuperscript{36} Reduced NK cell numbers and cytotoxicity have been reported in paediatric sepsis and in critically ill septic adults also in association with hyperferritinaemia, as well as suggested in the pathophysiology of other systemic hyperinflammatory and autoimmune conditions.\textsuperscript{18,37–39}

We observed reduced NK cell and CTL percentages and lymphocyte cytotoxicity in three of four HLH patients and, furthermore, to our knowledge not previously described, a statistically significant negative correlation between both NK cell and CTL percentages and ferritin concentration ($P = 0.0013$ and 0.0025, respectively). We suggest that a reduced number of cytotoxic cells and consequently deficient lymphocyte cytotoxicity, may generate an impaired regulation of the immune system that promotes the hyperinflammation seen in our patients with influenza-associated HLH. The role of low CTL proportions is more unclear but reduced numbers have been reported in acute sepsis and severe influenza A(H1N1).\textsuperscript{35,40} In addition, we showed normalised NK numbers and cytotoxicity after clinical recovery in an HLH patient, supporting a temporary infection-associated reduction.\textsuperscript{35,39} The identified rare heterozygous variants are of unclear significance but possibly contribute to hyperinflammation in an ‘add-on’ effect to finally develop HLH.\textsuperscript{41}

Delayed viral clearance has been associated with lymphopaenia and impaired lymphocyte response, and with disease severity, hypercytokinaemia and organ dysfunction.\textsuperscript{35,42–44} This may explain why some patients with influenza A(H1N1) infection develop hyperinflammation/HLH, as perhaps in patients 3 and 4 with positive A(H1N1)-PCR within a week of HLH diagnosis. However, it does not explain the pathobiology of all secondary HLH. Patients 1 and 2, with negative A(H1N1)-PCR prior to HLH diagnosis, had sustained marked inflammatory responses from A(H1N1) infection on admission until HLH diagnosis, with persistently elevated CRP up to 309 and 500 mg l\(^{-1}\), respectively, and clinical deterioration in the absence of prior nosocomial infection. The exaggerated inflammation may be sustained by a persistent impaired immune response to the initial influenza A(H1N1) infection. Studies with sequential viral loads describing their course in severe A(H1N1) influenza patients who develop HLH are rare, as are studies of viral titres in plasma, which were not analysed in any of our patients with negative A(H1N1)-PCR in BAL. However, bacterial infections, also an important trigger of sHLH, cannot be excluded as a possible add-on trigger in patients 1 and 4, contributing to the development of HLH in an already raised inflammatory state.\textsuperscript{35}

All 11 patients survived compared with reported fatality rates in ECMO patients with influenza A(H1N1) ranging from 8 to 65\%.\textsuperscript{46} All four patients with HLH survived with aggressive supportive care and varying extents of HLH-directed therapy, in line with current literature stating that the severity of symptoms should dictate the need for and intensity of HLH-directed therapy, which may include immunomodulants and cytotoxic drugs such as etoposide when necessary.\textsuperscript{15,29,45,47–49}

The value of corticosteroids in the treatment of virus-associated hyperinflammation has been questioned. Significantly, dexamethasone was reported recently to reduce deaths by one-third in ventilated patients with COVID-19 (rate ratio 0.65; $P = 0.0003$).\textsuperscript{50} The drastic improvement in our severely ill HLH patient after steroids and a single reduced dose of etoposide also illustrates (possible) benefits of timely and individualised etoposide treatment in selected cases of A(H1N1)-associated HLH.\textsuperscript{15}

The current study has several limitations such as a naturally small study population in a rare condition, and the retrospective evaluation and diagnosis of HLH. Although small, our study series is fairly representative (69\%) of the 16 patients in total with severe pandemic influenza A(H1N1) 2009 infection requiring ECMO treatment that season in Sweden.\textsuperscript{19} ECMO treatment makes certain HLH criteria difficult to interpret. Not all HLH-2004 criteria parameters were evaluated in all patients, which could imply HLH being underdiagnosed. However, despite adding on full score for missing HScore parameters in non-HLH patients, our four HLH patients still remained distinctly identifiable.

**Conclusion**

Patients, including healthy young adults, with severe pandemic influenza may develop HLH. They can be distinguished from other critically ill patients even on ECMO support and, therefore, should be monitored for signs of hyperinflammation and increasing organ dysfunction to be evaluated promptly for HLH using current diagnostic tools, because HLH-directed therapy may be beneficial. The association of a low proportion of NK cells with hyperferritinaemia supports a role for deficient lymphocyte cytotoxicity, possibly also low CTLs, in the pathogenesis of hyperinflammation and secondary HLH.
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