Krebs von den Lungen-6 (KL-6) is a pathophysiological biomarker of early-stage acute hypersensitivity pneumonitis among pigeon fanciers

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Abstract

Background: Identifying early stages of hypersensitivity pneumonitis (HP) is hampered by variable presentation, heterogeneous or undetected causal antigens and lack of gold-standard biomarkers. Krebs von den Lungen (KL)-6 is pathophysiological biomarker of alveolar epithelial damage. Pigeon fanciers, susceptible to HP, provide a model to investigate early HP.

Objective: To test the hypothesis that plasma concentrations of KL-6 are increased in early-stage acute HP.

Methods: Clinical history, spirometry and blood samples were obtained from pigeon fanciers, 20 with intermittent acute symptoms indicative of developing HP, 27 with no symptoms and 10 healthy subjects with no avian exposure. Plasma KL-6 (units/mL) and pigeon antigen-specific IgG antibody were quantified by enzyme immunoassay. Blood lymphocytes were quantified by flow cytometry and antigen specificity by in vitro cytokine production.

Results: KL-6 was higher in fanciers than controls, median (IQR) 452 (244, 632) vs 274 (151, 377), \( P = .01 \). Although fanciers with symptoms had similar antigen exposure and lung function, they had higher KL-6 than those without, 632 (468, 1314) vs 320 (200, 480), \( P < .001 \). KL-6 correlated with IgG antibody titre in those with symptoms, \( r = .591 \), \( P = .006 \). High KL-6, irrespective of symptom category, was associated with higher antibody (\( P = .006 \)) and lymphocyte proliferation (\( P = .041 \)), and lower CD4+ T lymphocyte proportion (\( P = .032 \)).

Conclusion and Clinical Relevance: Raised KL-6 is associated with acute symptoms of early-stage HP, and its correlation with antibody may support therapeutic strategies when HP is suspected. KL-6 may act as a mechanistic biomarker of early...
INTRODUCTION

Early detection of hypersensitivity pneumonitis (HP) is crucial for prompt intervention. This can be compromised by heterogeneous clinical presentation and delay in identifying often cryptic causal antigens; meanwhile, HP can become insidious and progress to treatment-refractory chronic HP. Objective tests to assess the pathophysiological significance of early mild symptoms would provide timely biomarkers for subjects at-risk of progression. This knowledge gap in the pathogenesis of early HP may be addressed by investigating HP among pigeon fanciers. This population is at known risk for HP, where the causal exposure to antigenic dust from pigeons and the immune hypersensitivity responses can be measured, along with clinical responses. These include interstitial inflammation and restrictive lung dysfunction causing shortness of breath, and acute symptoms described as influenza-like with pyrexia and myalgia suggest systemic inflammation. The determinants of these outcomes are unresolved, for example persistently raised serum IgG antibody against pigeon antigens can occur in asymptomatic fanciers suggesting that antibody may be necessary but not sufficient for progression. Additional biomarkers indicative of a pathophysiological response may help explain early progression.

Krebs von den Lungen-6 (KL-6) is a mucinous extracellular high-molecular-weight (~200kDa) sialylated glycoprotein fragment of the transmembrane mucin MUC-1 (CD227), classified as cluster 9 of lung tumour and differentiation antigens. It is expressed on the apical surface of type 2 alveolar epithelial cells (AEC2). KL-6 is cleaved at the cysteine bond near the epithelial membrane surface, released into lung lining fluid and is measurable in BAL fluid. KL-6 diffuses into the circulation providing evidence of increased lung epithelial/endothelial permeability. AEC2 replicate homeostatically to replace alveolar epithelial cells and proliferate to repair damaged alveolar epithelium, for example in pulmonary alveolar proteinosis and following oxidative stress and epithelial apoptosis in ILD including IPF and cHP. The density of AEC2 reflects the severity of recent alveolar damage; therefore, the associated increased KL-6 production measured in plasma may be a sensitive indicator of alveolitis. In ILD, plasma KL-6 is a particularly useful molecular biomarker, with concentrations reflecting disease activity, severity and the effectiveness of corticosteroid therapy conferring value as a diagnostic and prognostic biomarker of progression or survival. In a study of ILD, HP had the highest BALF KL-6 and the most prominent alveolitis compared with IPF and sarcoidosis, and the serum KL-6 correlated with BALF KL-6, albumin and lymphocyte counts, particularly cytotoxic CD8 T cells.

High concentrations of serum KL-6 have been reported in diagnosed HP associated with domestic and occupational environments. KL-6 is higher in acute than chronic HP suggesting that alveolitis may already be prominent early in the disease trajectory of HP. We hypothesized that KL-6 may be an early biomarker of HP and tested this among undiagnosed pigeon fanciers with intermittent acute symptoms associated with antigen exposure. We identified an endotype of increased KL-6 associated with IgG antibody and lymphocyte profile, linking lung pathophysiology with immunity that was indicative of early HP in this at-risk population.

METHODS

2.1 Participants

Participants were recruited at a community-based cross-sectional study of early-stage acute HP at a national convention of pigeon fanciers where our clinical research team provided advice about pigeon fancier's lung, colloquial for hypersensitivity pneumonitis. Those interested to participate were fully informed of the detail and purpose of the study and provided signed informed consent. All information collected was anonymized and linked by a unique study number for each person. Information collected included details of age, brief relevant medical history, cigarette smoking history and cumulative exposure to pigeons (years keeping pigeons, numbers of pigeons kept and the average number of hours per week in contact). Ethics application for this study titled Data Collection in Extrinsic Allergic Alveolitis (Hypersensitivity Pneumonitis) was approved on 13 March 2001 (Approval Number 00/44) by the Research Ethics Committee, Stobhill NHS Trust, Glasgow G21 3UW. Each participant completed a structured questionnaire supervised by respiratory physicians with expertise in HP and familiarity with the culture of pigeon fanciers. They recorded their recollection of any of the following symptoms: dyspnoea, influenza-like symptoms, chest tightness, cough, polymyalgia, fever, fatigue, diaphoresis and wheeze, occurring 4-12 hours after exposure to undue amounts of pigeon dust (eg cleaning-out pigeon loft and/or during the moulting when feathers are shed copiously), that resolved usually by the next day. A history of at least one respiratory symptom simultaneously with at least one systemic symptom on at least three occasions in the last year was recorded. Although this was insufficient for a diagnosis of HP, for the purposes of this study this symptom profile was used to categorize a group with "early-stage acute" HP who reported recurrent episodes of acute symptoms after contact with pigeons, that resolved quickly, but who had not (yet) had an established diagnosis of...
HP by a hospital specialist or general practitioner. We excluded those who did not currently keep pigeons, anyone with symptoms suggestive of a recent respiratory tract infection or any current symptoms, those with a history of any respiratory disease or co-morbid conditions (such as heart disease), those who were taking anti-inflammatory medications, those with a history of a dusty occupation and current and recent ex-smokers (within the last 10 years). We also excluded those with an established diagnosis of HP after medical investigations as we wished to focus on those with early stage or subclinical disease. Of 155 interviewed, 47 pigeon fanciers fulfilled study criteria and were enrolled; 20 had a history of acute symptoms indicative of early-stage acute HP, and 27 subjects had a history of no symptoms. These subjects performed pulmonary function spirometry and donated a 7 mL heparin blood sample. A control group of 10 volunteers from the research study team who fulfilled the same exclusion criteria but without any significant avian exposure donated a 7 mL blood sample.

2.2 | Measurement of pigeon antigen-specific IgG antibody

The predominant species-specific antigen in the respirable dust in pigeon lofts is pigeon serum gamma-globulin, and the subjects’ antibody activity against this antigen was measured by indirect enzyme immunoassay (EIA). Briefly, 96-well polystyrene microtitre EIA plates (Dynatech Ltd, UK) were coated with purified antigen (donated by Dr P. Lynch); 5 μg/mL in bicarbonate buffer (0.02 mol/L, pH 9.6), 100 μL/well 24 hour at 4°C. Plate wells were washed three times in detergent buffer (phosphate-buffered saline, 0.02 mol/L, pH 7.4, containing 0.05% Tween-20, PBS-T). Plasma samples were diluted optimally at 1:200 with PBS-T and incubated in duplicate at 100 μL/well for 1 hour at room temperature, after which the plates were washed as above. Bound antibody was quantified using alkaline phosphatase conjugated anti-human IgG at 1:10 000 dilution in PBS-T (Sigma, UK). After washing as before, the plate wells were incubated with the colourless substrate p-nitrophenyl phosphate (Sigma, UK) at 1 mg/mL in 10% diethanolamine, pH 10. This is converted by enzyme activity to a yellow product with an absorbance at 405 nm. After approximately 30 minutes, the optical density was measured by spectrophotometer (Dynatech Ltd UK). If all other reagents are in excess, the E405 nm was proportional to the antibody activity and this was quantified by interpolation using an optical density standard curve from serial dilutions of a standard serum with known antibody concentration previously titrated by quantitative precipitation.

2.3 | Blood leucocyte cytology and lymphocyte culture

Total and differential white blood cell counts were determined by Coulter counter (CBCS, Coulter Electronics) and by flow cytometry (FACScan, BD Biosciences, UK) using FITC-anti-CD45 and PE-anti-CD14 (Sigma, UK). The major T lymphocyte subsets were counted by FACS using FITC-anti-CD3 with either PE-anti-CD4 or PE-anti-CD8 (Sigma). The cell counts and proportions are listed in Supplementary Table S1.

Antigen-specific lymphocyte responses were measured by whole blood assay. Briefly, each blood bottle was resuspended by inversion, and 20 μL was added to 80 μL DMEM containing penicillin and streptomycin (Gibco, UK) per well in 96-well cell culture plates (Fisher Scientific, UK). Each blood was tested in quadruplicate, with and without 5 μg/mL of pigeon serum gamma-globulin antigen. Plates were incubated at 37°C in a humidified 5% CO₂ atmosphere. After 5 days, each well was pulsed with 10 μL (0.015 MBq) tritiated thymidine (TRA-120, GE Healthcare, Amersham, UK) and incubated for a further 6 hours. Thymidine incorporation into DNA, indicative of lymphocyte proliferation, was measured as counts per minute (cpm) by beta-counter (MicroBeta Trilux, PerkinElmer) of cells harvested onto a glass-fibre filter mat (MicroBeta Filtermate Harvester, PerkinElmer, Bucks, UK). Antigen-specific lymphocyte proliferation was measured by subtracting the background count, obtained from each sample well without antigen, from the count from the corresponding sample well cultured with antigen. The average of the four replicate counts per person was corrected for the number of lymphocytes in the culture well. These experiments were performed using Institutional Health and Safety guidelines.

2.4 | Plasma KL-6

Plasma KL-6 was measured by sandwich enzyme immunoassay (Eitest KL-6 kit, Sekisui Medical Co. Ltd. Japan) according to the manufacturer’s instructions. Reference levels are listed in Table S2.

2.5 | Statistical analysis

The primary aim was to test the working hypothesis that the plasma KL-6 concentration was increased in pigeon fanciers with a symptom history indicative of risk of progression to hypersensitivity pneumonitis. Secondary aims included investigating linear relationships between KL-6 with antigen exposure, lung function and immune reactivity. Variables were summarized as median and interquartile range; between-group comparisons were tested by Mann-Whitney U test. The linear relationships were tested by Spearman’s rho. Analysis was performed using Minitab software (Minitab Inc, State College, PA). Secondary aims were considered exploratory, and there was no correction for multiple comparisons. P < .05 was considered statistically significant.

3 | RESULTS

3.1 | Categorization of pigeon fanciers according to symptom history

Of 155 interviewed, 20 pigeon fanciers described a history of intermittent acute symptoms following exposure to pigeon dust (Methods), and 27 had a history of no symptoms (Table 1). There
was no difference between the “early-stage acute” and “asymptomatic” categories for age, pigeon antigen exposure or spirometric lung function; therefore, these groups were considered suitable for a comparative investigation for biomarkers of risk of progression to HP.

### 3.2 Blood leucocytes

Compared with control subjects (Table S1), the pigeon fanciers had lower total blood leucocyte counts ($P = .041$), and lower relative proportions ($P = .003$) and absolute counts ($P = .01$) of CD8 lymphocytes. Within the categories of pigeon fanciers (Table 1), those with early-stage acute HP had a lower proportion of CD4 lymphocytes. Within the categories of pigeon fanciers (Table 1), those with early-stage acute HP had a lower proportion of CD4 lymphocytes.

#### TABLE 1 Median (IQR) describing age, indices of cumulative exposure to antigenic dust from pigeons (number of pigeons kept, number of years exposure and the average number of hours per week in contact with pigeons), spirometric measures of lung function, and blood total leukocyte counts and relative proportions in pigeon fanciers comparing those with and without a history of acute symptoms indicative of subclinical HP

| Variable                      | Symptoms indicative of early-stage HP | $P^*$  |
|-------------------------------|--------------------------------------|-------|
| Age years                     | No ($n = 27$)                        | 53 (43, 61) |
| Antigen exposure              | Yes ($n = 20$)                       | 48 (41, 60)   | .584 |
| Number                        |                                      | 47 (40, 65)   | .281 |
| Years                         |                                      | 20 (19, 39)   | .45  |
| Hours/week                    |                                      | 16 (7, 20)    | .38  |
| Spirometry                    |                                      |               |      |
| FEV$_1$                       |                                      | 94.0 (78.0, 104) | .375 |
| FVC                           |                                      | 98.5 (79.0, 114) | .309 |
| FEV$_1$/FVC                   |                                      | 94.1 (84.1, 103) | .716 |
| FEF$_{25-75}$                 |                                      | 72.0 (50.5, 95.2) | .808 |
| Blood cytology                |                                      |               |      |
| Leucocytes                    |                                      | 7.21 (6.79, 8.3) | .613 |
| Neutrophils                   |                                      | 4.59 (3.83, 5.16) | .624 |
| Monocytes                     |                                      | 0.44 (0.4, 0.52) | .181 |
| Lymphocytes                   |                                      | 2.58 (2.07, 3.01) | .739 |
| CD4                           |                                      | 1.02 (0.93, 1.34) | .355 |
| CD8                           |                                      | 0.39 (0.29, 0.5)  | .138 |
| % neutrophils                 |                                      | 59.0 (54.0, 68.0) | .712 |
| % monocytes                   |                                      | 6.0 (4.0, 7.0)   | .208 |
| % lymphocytes                 |                                      | 34.0 (26.0, 39.0) | .966 |
| CD4%                          |                                      | 45.0 (37.0, 51.0) | .045 |
| CD8%                          |                                      | 17.0 (13.0, 20.0) | .02  |

*Percent-predicted forced expiratory volume in one second (FEV$_1$), forced vital capacity (FVC), FEV$_1$/FVC ratio and forced expiratory flow at 25-75% of FVC (FEF$_{25-75}$).

*Mann-Whitney U test, $P < .05$ considered statistically significant.

(P = .045) and a higher proportion of CD8+ lymphocytes ($P = .02$) than their asymptomatic counterparts.

### 3.3 Immune reactivity against pigeon antigens and symptom category

Pigeon fanciers had high IgG antibody (Figure 1A) and modest in vitro lymphocyte proliferative responses (Figure 1B) against pigeon antigens, that were negligible among control subjects with no significant avian exposure. There was no correlation between the IgG antibody concentration or the lymphocyte proliferative response with age, antigen exposure, lung function or blood cytology in all pigeon fanciers and in the “early-stage acute” and “asymptomatic” categories (Table S3). There was a significant correlation between the humoral (IgG antibody titre) and the cellular (lymphocyte proliferation) responses, $r = .429$, $P = .003$ (Figure S1).

Fanciers with early-stage acute HP had higher IgG antibody ($\mu$g/mL): 61.0 (41.0, 83.5) vs 44.0 (9.0, 59.0), $P = .011$, and a trend towards greater lymphocyte proliferation (in vitro proliferation, cpm corrected for lymphocyte count); 3.7 (2.0, 6.5) vs 2.5 (1.7, 3.8), $P = .064$, compared with asymptomatic fanciers. However, there were many asymptomatic subjects with evidence of immune reactivity, and several symptomatic subjects with relatively low immune reactivity and this overlap suggested that the immune response profile was insufficient to cleanly differentiate asymptomatic from early-stage acute HP; therefore, we investigated KL-6 as a pathophysiological biomarker between these categories.

### 3.4 Plasma KL-6 as a pathophysiological biomarker of symptoms indicative of early-stage acute HP

There was no linear relationship between plasma KL-6 concentration and cumulative exposure to pigeon antigens, spirometric lung function or blood cytology (Table S3). The median (IQR) units/mL plasma KL-6 (Figure 2) was higher in the pigeon fanciers than in the control subjects with no significant avian exposure, 452 (244, 632) vs 274 (151, 377), $P = .01$, and the pigeon fanciers with early-stage acute HP had higher KL-6 than those without, 632 (468-1314) vs 320 (200-480), $P < .001$. There was no difference in KL-6 between fanciers with no symptoms and control subjects, 320 (200, 480) vs 274 (151, 377), respectively, $P = .137$.

The plasma concentration of KL-6 correlated with the IgG antibody titre $r = .494$, $P < .001$ (Figure S2). This linear relationship was stronger among pigeon fanciers with a history of acute symptoms indicative of early-stage HP, $r = .591$, $P = .006$ (Figure 3), but did not reach statistical significance among those without a history of acute symptoms ($r = .180$, $P = .369$). The KL-6 concentration did not correlate with the lymphocyte proliferative response to pigeon antigens among all pigeon fanciers $r = .250$, $P = .09$ (Figure S3), nor in either
category of “early-stage acute” HP ($r = .146, P = .539$) or “asymptomatic” fanciers ($r = .152, P = .451$).

### 3.5 | Comparison between objective categories of normal and high plasma KL-6 concentrations

A 95th centile of normality for KL-6 was calculated from control subjects. This generated an upper cut-off normal limit at 452 µ/mL which was consistent with published levels (Table S2). We used this to as an objective discriminator of “normal” and “high” categories of KL-6 and used this, rather than subjective symptom-recall, to compare lung function, antigen exposure and immune response among the pigeon fanciers (Table 2). There was no difference in age, antigen exposure and lung function, but those with “high” KL-6 had higher antigen-specific IgG antibody titre, 59 (44, 85) vs 28.5 (11.2, 51.7) $P = .006$, and greater lymphocyte proliferation, 3.5 (2.1, 6.6) vs 2.2 (1.5, 3.9) $P = .041$, and lower proportions of blood CD4 T lymphocytes, 41 (32, 45) vs 45 (36.2, 51) $P = .032$.

### 3.6 | Evaluation of serum KL-6 and IgG antibody in factory workers exposed a contaminated humidification system

For comparison, we conducted a serological study of KL-6 and IgG antibody activity among undiagnosed factory workers, some with acute HP symptoms associated with exposure to recognized but uncharacterized antigens aerosolized from the water sump of a

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**FIGURE 1** The pigeon antigen-specific IgG antibody (A) and in vitro lymphocyte proliferative response (B) in healthy control subjects with no significant avian exposure, and in pigeon fanciers categorized according to history of acute symptoms indicative of subclinical HP. Box-and-whisker plot showing median, IQR and range. Mann-Whitney U test comparing pigeon fanciers with and without a history of symptoms indicative of subclinical acute HP, for IgG $P = .011$ and for proliferation (cpm corrected for lymphocyte number), $P = .065$

**FIGURE 2** The plasma concentration of KL-6 in healthy control subjects with no significant avian exposure generated an upper 95 percentile of normality at 452 units/mL. Box-and-whisker plot showing median, IQR and range. Mann-Whitney U test demonstrated a higher KL-6 in pigeon fanciers with a history of acute symptoms indicative of subclinical HP compared with asymptomatic counterparts ($P < .001$). The KL-6 values are indicated on a log10 scale for clearer representation. The mean values are superimposed as bold bars at 268, 359 and 1121 units/mL for control healthy donors, pigeon fanciers with no symptoms and pigeon fanciers with a history of symptoms indicative of subclinical acute HP, respectively

**FIGURE 3** Linear relationship between the plasma concentrations of IgG antibody against pigeon antigen and KL-6 in pigeon fanciers with a history of symptoms indicative of subclinical acute HP. Spearman’s rho = 0.591, $P < .001$
TABLE 2  Pigeon fanciers categorized according to normal (≤452 U/mL) or high (>452 U/mL) serum KL-6

|                      | KL-6 normal | KL-6 high | \( \epsilon \) |
|----------------------|-------------|-----------|---------------|
| Age years            | 51.5 (9.9)  | 51.5 (11) | .982          |
| Antigen exposure     |             |           |               |
| Number               | 50 (40, 70) | 60 (42.5, 80) | .226          |
| Years                | 20.0 (12.7, 39.7) | 20 (12.5, 34) | .913          |
| Hour/week            | 14 (7, 25)  | 14 (7.5, 24) | .967          |
| Spirometry          |             |           |               |
| FEV\(_1\)           | 94 (78, 107) | 81 (66, 97.5) | .178          |
| FVC                  | 96 (79.2, 115) | 92 (77.5, 108) | .402          |
| FEV\(_1\)/FVC        | 99.1 (91.7, 103) | 89.6 (77.3, 102) | .168          |
| FEF\(_{25-75}\)      | 85 (34, 101) | 57 (34, 101) | .206          |
| Immunology           |             |           |               |
| IgG antibody         | 28.5 (11.2, 51.7) | 59.0 (44.0, 85.0) | .006          |
| Lymphocyte proliferation | 2.2 (1.5, 3.9) | 3.5 (2.1, 6.6) | .041          |
| Blood cytology       |             |           |               |
| Leucocytes           | 7.15 (6.72, 8.52) | 7.40 (6.80, 8.40) | .459          |
| Neutrophils          | 4.49 (3.63, 5.17) | 4.33 (3.44, 5.32) | .947          |
| Monocytes            | 0.44 (0.35, 0.55) | 0.49 (0.43, 0.58) | .239          |
| Lymphocytes          | 2.65 (2.2, 3.0) | 2.28 (1.95, 2.94) | .45           |
| CD4                  | 1.09 (0.89, 1.37) | 0.93 (0.68, 1.35) | .187          |
| CD8                  | 0.42 (0.32, 0.59) | 0.44 (0.28, 0.65) | .915          |
| % neutrophils        | 59 (54, 62.5) | 59.5 (54, 70.2) | .785          |
| % monocytes          | 6 (4.2, 7.7) | 6.5 (5.7, 7.7) | .684          |
| % lymphocytes        | 35 (27.7, 39) | 33 (24, 37) | .494          |
| CD4%                 | 45 (36.2, 51) | 41 (32, 45) | .032          |
| CD8%                 | 18 (13, 21.5) | 19 (15, 24) | .21           |

Note: Comparison of age, indices of exposure to pigeon antigens, lung function, immune responses measured by pigeon antigen-specific IgG antibody concentration and in vitro lymphocyte proliferation, and blood total and differential absolute counts (10\(^6\)/mL) and relative proportions. Percent-predicted forced expiratory volume in one second (FEV\(_1\)), forced vital capacity (FVC), FEV\(_1\)/FVC ratio and forced expiratory flow at 25-75% of FVC (FEF\(_{25-75}\)).

\( P < .05 \) considered statistically significant.

factory workers with significantly high titres (>596 µ/mL) was linearly related to the IgG antibody titre, Spearman’s rho (95% CI) = 0.381 (0.095, 0.608).

4 | DISCUSSION

4.1 | Main findings

To address the search for a biomarker of pathogenesis of post-exposure symptoms and potential risk of progression to HP among pigeon fanciers, this study identified 20 candidates with an indicative symptom history, and 27 with no symptoms; with equivalent age, spirometry and pigeon antigen exposure. The pigeon fanciers with early-stage acute symptoms had raised plasma KL-6 commensurate with IgG antibody. Raised KL-6 is an objective biomarker of alveolar remodelling and IgG represents immune hypersensitivity, together demonstrating pathophysiological changes in those with early-stage acute HP. These observations suggest that high KL-6 has merit to identify at-risk pre-clinical individuals and prioritize those for further investigation and follow-up, and might additionally serve as an interim surrogate for antibody detection in HP of unrecognized cause.

4.2 | Comparison with other studies

The published studies of KL-6 in HP generally report diagnosed cases. Our findings extend and complement these by investigating KL-6 in “pre-clinical” or undiagnosed early-stage acute HP. Our 95th centile of normality for KL-6 calculated at 453 µ/mL compared well with others (eg 458 µ/mL, 465 µ/mL, 500 µ/mL Table S2). Among the pigeon fanciers with no symptoms, the KL-6 at 320 (200, 480) µ/mL was not different from normal. In our pigeon fanciers with early-stage acute HP, the KL-6 at 632 (468, 1314) µ/mL was higher than normal but less than levels reported in cases of diagnosed HP 2996 ± 2016 µ/mL\(^23\) and 1263 ± 288 µ/mL\(^25\). In a comparative study with different ILDs,\(^24\) median KL-6 levels were higher in acute HP (2710 µ/mL) than chronic HP (1500 µ/mL), and both higher than in IPF, CVD-IP and sarcoidosis. In chronic HP, increasing KL-6 concentration might usefully discriminate progressive from stable disease,\(^31\) whereas in acute HP, the KL-6 might also reflect antigen exposure. For example, different seasonal peaks of serum KL-6 reflect different seasonal exposures to either domestic or avian antigen exposures,\(^32\) and antigen avoidance for more than one month among bird fanciers was associated with reducing KL-6 and improved lung function.\(^32,33\) An index case of acute pigeon fanciers HP demonstrated that raised KL-6 levels returned to normal after 8 months of antigen avoidance\(^17\) and was indicative of long-term clinical improvement. Our study in pigeon fanciers with no diagnosis of HP suggests that raised KL-6 is associated with acute symptoms from antigen exposure and alveolitis occur early in the disease trajectory of HP and before diagnosis.
4.3 | What can KL-6 reveal about the pathogenesis of early-stage acute HP

The pathogenic determinants of onset and progression of HP are unresolved. Antigen-specific antibody and cellular immune responses appear necessary but not sufficient for the development of symptoms. Our findings highlight KL-6 as a useful candidate biomarker for early progression. KL-6 produced by regenerating type-2 alveolar epithelial cells (AEC2) is increased with the proliferation required to repair apoptotic epithelial cells associated with alveolar damage in HP.32,34 IHC of HP lung tissue show apoptotic markers (Fas, Fas-L, p53, p21) on epithelial cells 23,34 and apoptotic epithelial cells can stimulate induction of experimental HP by enhancing maturation of dendritic cells and lung adaptive immunity.34,35 In the present study, the fanciers had robust IgG responses to pigeon antigens but only those with raised KL-6 had symptoms indicative of early-stage acute HP. This suggests that asymptomatic pigeon fanciers, even with considerable cumulative exposure to pigeon antigens and a robust immune response, can have normal plasma KL-6 levels. Raised IgG antibody, commensurate with exposure to pigeon antigens and a robust immune response, can have normal plasma KL-6 levels. Raised IgG antibody, commensurate with increased KL-6 we suggest is indicative of early pathophysiological changes and risk of clinically significant HP.

Elevated plasma KL-6 is indicative of lung remodelling. KL-6 is a large ~200kDa fragment shed from Muc-1 on AEC2 and raised plasma levels indicate increased airway epithelial permeability. We37 and others38 have demonstrated increased lung permeability in sero-positive asymptomatic pigeon fanciers, and sero-positive farmers without HP and increased lung permeability and raised serum KL-6.22 In ILD, raised KL-6 correlated with: chest Ga-67 scintigraphy,39 degree of computed tomography,40 A-a O2 tension difference,41 severity of symptoms and chest X-ray findings, and inversely with PaO2 and DLco42,43 and symptoms, opacities on chest radiograph, and arterial blood gases.13 Together these biomarkers suggest that increased serum KL-6 concentration is indicative of interstitial inflammation and pathological changes, and levels serve as diagnostic, and prognostic biomarker of progression or survival.20

In pulmonary sarcoidosis, the serum KL-6 correlated with serum ACE44 and with BAL fluid albumin and lymphocyte proportion.45 In chronic HP, serum KL-6 levels correlated with increased proportion of BAL fluid T cells,22 and in the present study increasing KL-6 levels trend with a decrease of circulating CD4+ T cells (r = -.279, P = .058), perhaps reflecting lymphocyte traffic from blood into inflamed lung. These observations strongly suggest that KL-6 is indicative of considerable disturbances in lung pathophysiology with the prospect that similar disturbances might occur perhaps at lower levels commensurate with KL-6 levels in early-stage acute HP.

Although KL-6 is associated with changes in lung pathophysiology, it might directly cause pathogenic remodelling. KL-6 is chemotactic for human fibroblasts in vitro and may contribute to the intra-alveolar fibrosis in HP.46 Speculating on this observation, resolution or increase of KL-6 might usefully discriminate stable or progressive cHP phenotypes.31 HP likely has clinical phenotypes beyond the inadequate classification of acute, subacute and chronic.57 These phenotypes could be determined by combinations of immunological, for example antigen-specific antibody and lymphocyte activities, and remodelling mediators, for example KL-6. A combination of these might reflect an endotype of lower-lung pathology that could guide appropriate anti-inflammatory or anti-fibrotic therapies. Treatment and monitoring of HP that is chronic with fibrosis is difficult; therefore, therapeutic targeting KL-6, for example with corticosteroids early in the disease trajectory, would have optimum impact to forestall fibrosis before disease becomes refractory and plasma KL-6 levels might identify and stratify HP patients for relevant therapies or trials.

4.4 | Strengths and limitations

**Strength** Classification of HP based on symptoms into acute, subacute or chronic categories is inadequate, with subacute HP particularly difficult to define.47,48 The excellent engagement with pigeon fanciers as a homogeneous study group at known risk of HP, and their familiarity with symptom patterns of “pigeon lung” helped provide well-categorized groups based on post-exposure symptoms which underpinned this study.

**Limitations** a) The relatively low number of subjects with early-stage acute HP reflects the reality of identifying this category of HP. However, it would be important to validate the findings of raised plasma KL-6 in an extended early-stage acute HP group with normal spirometry along with high-resolution CT and physiological markers of parenchymal changes such as transfer factor and oscillometry. b) Longitudinal measurement of KL-6, lung function, high-resolution CT and emerging symptoms from baseline KL-6 and post-exposure symptoms would validate KL-6 as a prognostic marker of HP. A useful prototype would be the study that identified newly diagnosed ILD in systemic sclerosis in a follow-up of those with elevated baseline KL-6.49 c) MUC1 rs4072037 single-nucleotide polymorphisms affect serum concentration of KL-6 and severity of ILD.50 Correcting serum KL-6 level for the MUC1 haplotypes may increase its value as a prognostic marker. SNP genotype analysis will be included in a follow-up study to address these limitations.

5 | CONCLUSION

This study demonstrated that early-stage acute HP can be identified by raised plasma KL-6, and commensurate increased antibody is evidence of an underlying immunological diathesis. These findings should stimulate further research to facilitate and hasten the identification of HP and improve the clinical management and monitoring of individuals at-risk of HP, for example screening agricultural workers, and in antigen-occult HP, for example humidifier fever. Immunological and pathophysiological biomarkers add endotype evidence for dynamic clinical phenotypes. Among these, early-stage acute HP implicates AEC2 as an axis of homeostatic or aberrant remodelling and therefore a potential target for early therapeutic intervention in hypersensitivity pneumonitis.
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CONFLICT OF INTEREST

Nobuoki Kohno holds a patent for KL-6. The remaining authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Funding acquisition: PPL, GB, KB, CMS Project administration: KB, GB Concept and design: CMS, SJB, GB, PPL Engagement with the pigeon fanciers: PPL, GB, SJB, MS, LVW, CMS Collecting and processing of specimens: ID, YJ, MC, SJB, CMS Measurement of biomarkers: ID, YJ, MC, NK, CMS Statistical analysis: CMS, YJ, MC Interpretation of data: CMS, MS, LVW, SJB, MC, YJ, NK. All authors participated in critical revision and approved the final version.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.