Pathogenesis-driven treatment of primary pulmonary alveolar proteinosis

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Shareable abstract (@ERSpublications)
PAP is an ultra-rare syndrome characterised by the accumulation of alveolar surfactant, leading to respiratory failure. WLL is still the standard of care while emerging therapies address GM-CSF augmentation and cholesterol efflux with promising results. https://bit.ly/3yUxY2Y

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Abstract

Pulmonary alveolar proteinosis (PAP) is a syndrome that results from the accumulation of lipoproteinaceous material in the alveolar space. According to the underlying pathogenetic mechanisms, three different forms have been identified, namely primary, secondary and congenital. Primary PAP is caused by disruption of granulocyte–macrophage colony-stimulating factor (GM-CSF) signalling due to the presence of neutralising autoantibodies (autoimmune PAP) or GM-CSF receptor genetic defects (hereditary PAP), which results in dysfunctional alveolar macrophages with reduced phagocytic clearance of particles, cholesterol and surfactant. The serum level of GM-CSF autoantibody is the only disease-specific biomarker of autoimmune PAP, although it does not correlate with disease severity. In PAP patients with normal serum GM-CSF autoantibody levels, elevated serum GM-CSF levels is highly suspicious for hereditary PAP. Several biomarkers have been correlated with disease severity, although they are not specific for PAP. These include lactate dehydrogenase, cytokeratin 19 fragment 21.1, carcinoembryonic antigen, neuron-specific enolase, surfactant proteins, Krebs von Lungen 6, chitinase-3-like protein 1 and monocyte chemotactic proteins. Finally, increased awareness of the disease mechanisms has led to the development of pathogenesis-based treatments, such as GM-CSF augmentation and cholesterol-targeting therapies.

Introduction

Pulmonary alveolar proteinosis (PAP) is an ultra-rare syndrome with an estimated prevalence of about seven cases per million [1]. It is characterised by progressive accumulation in the alveoli of periodic acid–Schiff (PAS)-positive eosinophilic acellular material, consisting of surfactant proteins (SPs) and phospholipids with a small quantity of cell debris. The alveolar filling results in a variable impairment of the respiratory function until hypoxaemic respiratory failure and an increased risk of secondary infections [2, 3].

PAP classification

First described in 1958 as a single disease [4], PAP is now recognised as a syndrome comprising different diseases, classified according to the underlying pathogenetic mechanisms. Primary PAP is due to the disruption of granulocyte–macrophage colony-stimulating factor (GM-CSF) signalling, leading to an impaired surfactant clearance. It can be autoimmune, caused by high levels of neutralising GM-CSF autoantibodies, or hereditary, due to mutations in colony stimulating factor 2 receptor (CSF2R) subunit alpha (CSF2RA) and CSF2R subunit beta (CSF2RB) genes, encoding for the GM-CSF receptor subunits. Secondary PAP is associated with conditions causing a reduction of the number and/or function of alveolar macrophages (AMs), such as haematological disorders, immune deficiency syndromes, chronic inflammatory syndromes, chronic infections and environmental exposure to pneumotoxic agents. Congenital PAP is caused by mutations in genes involved in surfactant production or lung development,

particularly SP-B, SP-C, ATP-binding cassette subfamily A (ABCA) 3 and NK2 homeobox 1. In rare cases, the aetiology of PAP is undefinable; these cases constitute unclassified PAP [5, 6].

The classification of PAP syndrome is summarised in table 1.

TABLE 1 Current classification of pulmonary alveolar proteinosis (PAP) syndrome

Clinical type	Pathogenesis
Primary PAP	Disruption of GM-CSF signalling
Autoimmune	GM-CSF autoantibodies [7, 8]
Hereditary	GM-CSF receptor α/β chain encoding gene mutations [9]
Secondary PAP	Reduced alveolar macrophage functions and/or numbers
Haematological disorders	Myelodysplastic syndrome [10] Acute lymphatic leukaemia [11] Acute myeloid leukaemia [12, 13] Chronic myeloid leukaemia [14–16] Hairy cell leukaemia [17, 18] Hodgkin's disease [19] Non-Hodgkin's lymphoma [20] Multiple myeloma [20, 21] Essential thrombocythaemia [22] Polycythaemia vera [20] Amyloidosis [23] Fanconi's anaemia [24, 25] GATA 2 deficiency [26, 27]
Other malignancies	Adenocarcinoma [28–30] Glioblastoma [25] Melanoma [31]
Immune deficiency syndromes	Monoclonal gammopathy [32] Selective IgA deficiency [33] Severe combined immunodeficiency [34] OPAID [35, 36]
Chronic inflammatory and autoimmune syndromes	Inflammatory bowel disease [37, 38] Systemic lupus erythematosus [39] Polymyositis and dermatomyositis [40, 41] Rheumatoid arthritis [42] Multiple sclerosis [3] Autoimmune haemolytic anaemia [43] ANCA-associated vasculitis [44] Others: sarcoidosis, hypersensitivity pneumonitis [45, 46]
Toxic inhalation syndromes	Inorganic dusts: aluminium, cement, silica, titanium, indium, tin [47–52] Organic dust: sawdust, fertiliser/agricultural dust, bakery flour [53–55] Fumes: synthetic plastic, gasoline [55] Others: varnish, chlorine, petroleum, cleaning products [56, 57]
Metabolic disorders	Lysinuric protein intolerance [58–60]
Miscellaneous	IgA nephropathy [61] Coeliac disease [62] Lung transplantation [63]
Congenital PAP	Impaired surfactant production:
SP-B and SP-C mutations	SP-B and SP-C deficiency, abnormal surfactant [64, 65]
ABCA3 mutations	Disrupted surfactant homeostasis [66–68]
NKX2-1 mutations	Abnormal lung development [69, 70]
Niemann–Pick disease type C	Progressive lysosomal disorder [71, 72]
FARSB mutation	Impaired tRNA aminoacylation and metabolism of proteins [73]
MARS mutation	Loss-of-function defect in a member of the cytosolic multisynthetase complex [74]

ABCA3: ATP-binding cassette subfamily A member 3; ANCA: antineutrophilic cytoplasmic antibody; FARSB: phenylalanine-transfer RNA synthetase, β-subunit; GM-CSF: granulocyte–macrophage colony-stimulating factor; MARS: methionyl aminoacyl-transfer RNA synthetase; NKX2-1: NK2 homeobox 1; OPAID: OAS1-associated polymorphic autoinflammatory immunodeficiency disorder; SP: surfactant protein.

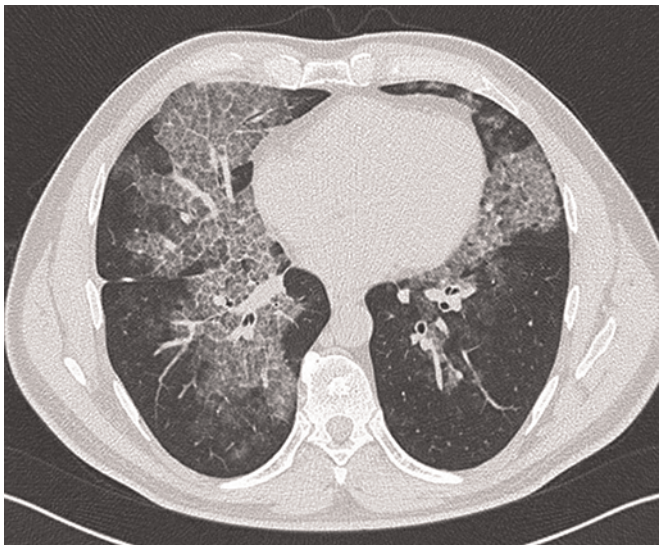

FIGURE 1 Typical high-resolution computed tomography appearance of pulmonary alveolar proteinosis, characterised by diffuse and geographically distributed ground glass opacifications with thickening of the septa (crazy paving aspect).

Clinical presentation and principles of diagnosis

The age of onset of primary autoimmune PAP (which accounts for ~90% of cases of PAP) typically ranges between 40 and 50 years old [54, 55], but it can vary in cases of hereditary PAP according to the underlying GM-CSF receptor mutation. The majority of patients (70–90%) present with slowly increasing dyspnoea on exertion and cough. Less frequently, they may experience fever, weight loss or chest pain. Lung fibrosis can affect up to 20% of PAP patients, independent of the specific form, but it generally occurs during the late stages of the disease [75].

Diagnosis of PAP syndrome typically relies on high-resolution computed tomography (HRCT) (ground glass opacities with geographical distribution and crazy paving) (figure 1) and bronchoalveolar lavage (BAL) findings (macroscopically milky aspect; on light microscopy, cell debris, foamy macrophages and PAS-positive staining) (figure 2). In the vast majority of patients, a confirmatory biopsy is not necessary. Serum GM-CSF autoantibody detection is mandatory to confirm autoimmune PAP, whereas GM-CSF signalling and genetic testing are indicated if hereditary PAP is suspected.

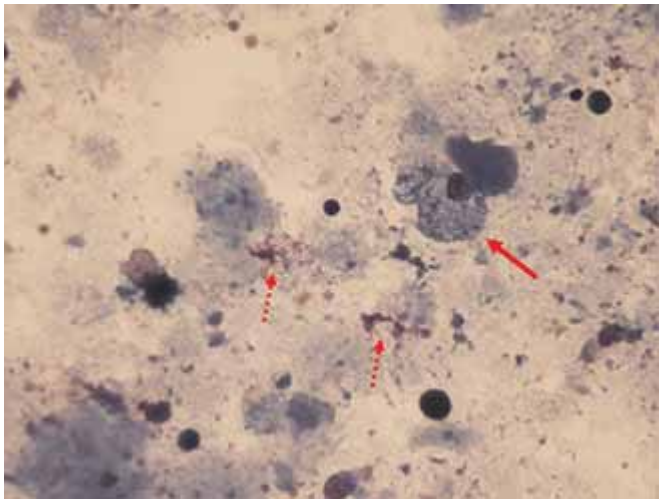

FIGURE 2 Periodic acid–Schiff stain. The optical microscopy evaluation of bronchoalveolar lavage fluid reveals foamy macrophages (solid arrow), cell debris and diffuse periodic acid–Schiff-positive material (dashed arrow). Magnification: ×100.

Pathogenesis of primary PAP

Primary PAP pathogenesis is associated with impaired GM-CSF signalling, which results in impaired AM function, which, in turn, leads to poor surfactant clearance and altered surfactant homoeostasis in the lung. Indeed, surfactant production remains unaltered. Other critical GM-CSF-dependent macrophage functions, such as antigen presentation, phagocytosis and Toll-like receptor signalling, are also affected [76]. The pathogenesis of primary PAP is summarised in figure 3.

GM-CSF is a 23 kDa glycoprotein immunomodulatory cytokine produced by multiple cell types, including macrophages, lymphocytes, endothelial cells, fibroblasts and alveolar epithelial cells (AECs). GM-CSF promotes monocytic and granulocytic progenitor cell growth, differentiation and activation [77].

In the lungs, GM-CSF is produced by type II AECs and binds to the GM-CSF receptor (GM-CSFR) expressed on type II AECs and AMs. The GM-CSFR is a multimeric complex constituted by a specific low-affinity ligand-binding α subunit (GM-CSFRα, also known as CDw116) and a signal-transducing β subunit (GM-CSFRβ, also known as CD131), the latter shared with interleukin (IL) 3 and IL-5 receptors.

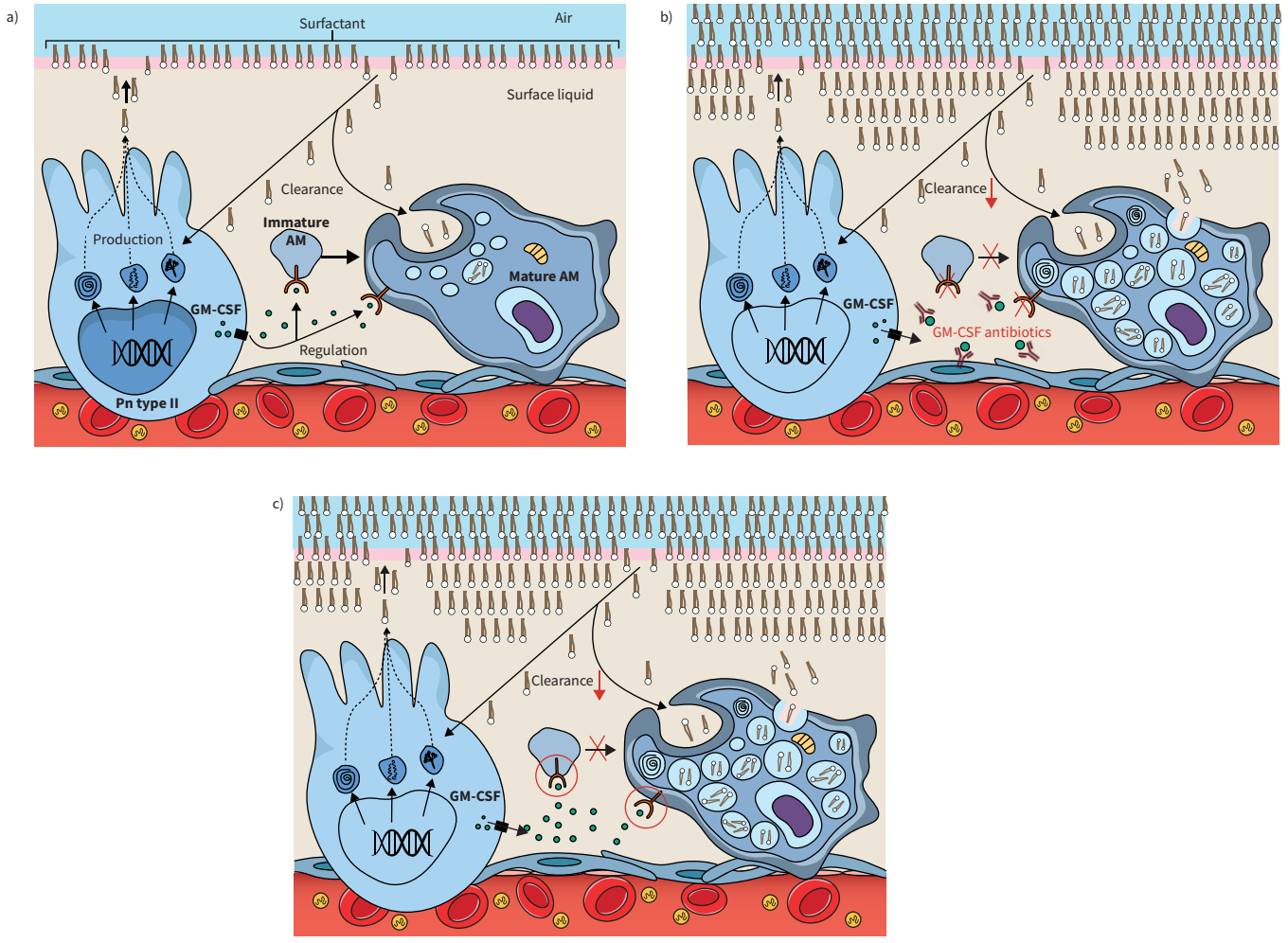

FIGURE 3 a) During normal homeostasis, pulmonary surfactant is synthesised, packaged and secreted by type II pneumocytes, then migrates to the air–liquid interface. Granulocyte–macrophage colony-stimulating factor (GM-CSF) secreted from type II pneumocytes binds to GM-CSF receptors on immature alveolar macrophages (AMs) promoting maturation, as well as regulating phagocytosis, immune and other nonimmune functions in mature AMs. Clearance of surfactant occurs *via* uptake and recycling in type II pneumocytes (Pn) and *via* phagocytosis and catabolism in AMs. b) In autoimmune pulmonary alveolar proteinosis (PAP), anti-GM-CSF antibodies disrupt maturation and activation of AMs, leading to intracellular build-up of lipid, impaired clearance and accumulation of surfactant in the alveolus. c) Conversely, in hereditary PAP, mutations in the α and β chains of the GM-CSF receptor results in conformational changes and reduced receptor function or cell surface expression, again leading to impaired AM function and accumulation of surfactant in the alveolus.

GM-CSF binding causes the phosphorylation of the GM-CSFRβ subunit, which leads to the activation of downstream signalling pathways involving Janus kinase (JAK) 2/signal transducer and activator of transcription 5, and extracellular signal-regulated kinase (ERK), with ERK activity linked to GM-CSF enhancement of human monocyte survival *in vivo* [78]. Moreover, JAK2 promotes the activation of transcription factor PU.1 (encoded by SPI1) [79] and peroxisome proliferator-activated receptor-γ (PPARγ) signalling pathways [77]. This complex sequence of reactions ultimately leads to the terminal differentiation and upregulation of the myeloid progenitor.

Results from murine models showed that transcription factor PU.1 was markedly reduced in AMs of GM-CSF knockout mice *in vivo* and was restored by pulmonary administration of GM-CSF. Furthermore, the addition of exogenous GM-CSF to AMs *in vitro* leads to the upregulation of PU.1-dependent markers CD32 (FCγII), mannose receptor and macrophage colony-stimulating factor receptor, which, in turn, induce improved surfactant catabolism [79].

BAL cells from PAP patients demonstrate a modest but significant decrease in PU.1 mRNA expression and PU.1-dependent terminal differentiation marker, consistent with the GM-CSF knockout mouse [80].

The intricate interaction between GM-CSF and PU.1 suggests that GM-CSF is required for healthy lung homeostasis by regulating PU.1 expression and thus myeloid and B-cell lineage proliferation and differentiation. Furthermore, *via* PU.1, GM-CSF enhances the phagocytic activity of AMs. For these reasons, GM-CSF-deficient mice showed increased susceptibility to a wide range of microbial pathogens, including bacteria, viruses, fungi and parasites [81–84].

In particular, GM-CSF seems to play a pivotal role in preventing *Nocardia* species infection and the blockade of GM-CSF by autoantibodies may permit dissemination of infection [85].

Data from a murine model suggest that the absence of GM-CSF, by targeted ablation of the GM-CSF gene or its receptor, induces functional impairments in multiple cell types, including macrophages and neutrophils. In particular, GM-CSF signalling interruption results in the development of large foamy, surfactant-laden macrophages, with reduced phagocytic clearance of particles, cholesterol and surfactant. Furthermore, GM-CSF knockout mice show an impaired chemotaxis of AMs. The functional impairment of AMs may increase susceptibility to infections and superimposed pulmonary opportunistic infections that are detectable in ∼13% of cases and can be present at disease onset. Moreover, systemic or pulmonary infections can complicate PAP and sometimes can be (18–20% of cases) responsible of deaths related to PAP. Opportunistic pathogens often include *Aspergillus*, *Nocardia*, *Mycobacterium* or fungal species and are associated with a poor prognosis [6].

GM-CSF enhances macrophage function by increasing their capacity for antigen presentation and boosting antibody-mediated phagocytosis through a complementary process. It also enhances the microbicidal capacity of macrophages, as well as leukocyte chemotaxis and adhesion. In addition, GM-CSF induces the production of several cytokines, including IL-6, IL-12p70, IL-23 and tumour necrosis factor-α. Furthermore, recent data on $Csf2rb^{-/-}$ mice demonstrated that GM-CSF regulates multiple metabolic pathways required for macrophage proliferation, including mitochondrial turnover, functions and integrity, as well as mitochondrial fatty acid oxidation and cellular ATP production [86].

Autoimmune PAP

Studies in a GM-CSF knockout murine model found that mice developed a syndrome similar to human PAP, with alveoli containing granular eosinophilic material and lamellar bodies, consistent with surfactant accumulation and functional impairment of AMs and neutrophils, with an increased risk of opportunistic bacterial and fungal infections [87].

In 1999, GM-CSF-neutralising antibodies (GMAbs) were first identified in serum and bronchoalveolar lavage fluid (BALF) samples of patients with idiopathic PAP (later classified as autoimmune PAP) [7, 8].

GMAbs are polyclonal, IgG1–2 subclass antibodies with low levels of IgG3–4 and are present in high titre in serum and BALF of PAP patients [24]. In the serum (but not in lavage fluid) of 80% of PAP patients, IgM antibodies of lower affinity and neutralising capacity for GM-CSF were also identified; however, their concentration was lower than IgG and they are not considered pathogenic for PAP [88].

Adoptive transfer of GMAbs has demonstrated the pathogenic role of GM-CSF autoantibodies. When nonhuman primates were injected with GMAbs derived from a PAP patient, the pulmonary features of

PAP were reproduced. Moreover, GMAbs from passively immunised primates neutralised the GM-CSF activity on leukocytes. Together, these results demonstrated that GMAbs are the disease-causing agents of autoimmune PAP [89, 90].

Serum titre of GM-CSF autoantibodies does not correlate with disease severity in patients with autoimmune PAP, as evaluated by a severity score, based on symptoms and degree of hypoxaemia [2]. Low levels of GM-CSF autoantibodies are ubiquitously present in healthy subjects, where they likely serve as normal regulators of the cytokine activity [91]. Nevertheless, above a certain threshold of concentration, GMAbs can be neutralising and a serum GMAb concentration of 5 $\mu g \cdot mL^{-1}$ has been identified as the minimum required to block GM-CSF signalling. Higher concentrations are strongly suggestive for a diagnosis of autoimmune PAP [92, 93].

Two mechanisms can explain how GMAbs exceeding the pathogenic threshold inhibit the biological activity of GM-CSF, as follows: 1) they irreversibly sequester GM-CSF and prevent its interaction with the receptor and 2) they induce Fcγ receptor-dependent clearance of GM-CSF. While healthy donors may have serum anti-GM-CSF antibodies at low concentrations (thus unable to promote GM-CSF sequestration and degradation), PAP patients produce high levels of GM-CSF autoantibodies that form immune complexes. The subsequent recognition by Fcγ receptors mediates the sequestration and degradation of the cytokine [94].

Hereditary PAP

Disruption of GM-CSF signalling by CSF2RA or CSF2RB mutations results in reduced protein expression on the cell surface and causes a hereditary form of PAP. This results in clinical manifestations similar to those in patients with autoimmune PAP, characterised by surfactant accumulation affecting gas exchange, but occurring at a younger age [9, 95].

CSF2RA and CSF2RB encode the α and β chains of the GM-CSF receptor and are located in the pseudo-autosomal region of chromosome X and in chromosome 22, respectively. The identification of their role in hereditary PAP came from animal studies. Mice with mutated or deleted GM-CSF receptor subunits developed a lung phenotype similar to GM-CSF-deficient mice [96, 97]. Furthermore, mice with homozygous disruption of the Csf2rb gene (encoding the murine homologue of the human CSF2RB gene) develop a lung disease identical to hereditary PAP in patients with recessive CSF2RB mutations [9, 98]. Likewise, the homozygous disruption of the Csf2ra gene in mice leads to a disease identical to hereditary PAP caused by recessive or compound heterozygous mutations of CSF2RA gene. This confirms that hereditary PAP pathogenesis occurs in AMs and targets their GM-CSF receptors as the molecular site [99].

A variability in disease severity across family members with identical GM-CSF receptor mutations has been reported, thus suggesting the potential role of other determinants of surfactant production or catabolism [9].

Circulating biomarkers in PAP

An increased serum titre of GM-CSF autoantibodies is the only disease-specific biomarker of autoimmune PAP. A diagnostic test by enzyme-linked immunosorbent assay has been standardised using a patient-derived polyclonal, neutralising GM-CSF autoantibody as the reference standard. The reported sensitivity and specificity for autoimmune PAP is 100% [93]. Although low detectable concentrations of GM-CSF autoantibodies (usually <1 $\mu g \cdot ml^{-1}$) can be evaluable in serum from healthy people [91] and patients with malignancies [39], inflammatory conditions [100] or secondary alveolar proteinosis due to dust exposure [101], higher levels (>9 $\mu g \cdot ml^{-1}$) of GM-CSF autoantibodies are only detectable in patients with autoimmune PAP [5]. PAP patients with a normal serum GM-CSF autoantibody level and no underlying disease or condition known to cause secondary PAP are suspected of hereditary PAP, where the presence of dysfunctional receptors leads to an increase in serum GM-CSF levels owing to reduced receptor-mediated clearance [102]. GM-CSF autoantibodies are thus helpful in establishing a diagnosis of autoimmune PAP. Currently, GM-CSF antibody measurements are not widely available or standardised. The test is still under development and validation, and different centres may use different methods [93, 103, 104]. This lack of standardisation makes it challenging to compare results from different centres.

Although neither specific nor diagnostic of PAP, several biomarkers have been correlated with impaired metabolism through the dysfunction of AM and consequently with the severity of disease (table 2). GM-CSF antibody measurements are not yet implemented in clinical practice and remain in the early stages of investigations aimed at gathering information and generating hypotheses [6].

The serum level of lactate dehydrogenase (LDH) is increased in 82% of PAP patients [3]. The cellular turnover of AMs is likely one source of the raised LDH level; furthermore, LDH was correlated with serum SP-B levels, suggesting that it may be an indirect indicator of total lung surfactant accumulation. Serum levels of LDH are also associated with conventional measures of lung function, including alveolar–arterial oxygen tension difference (P_{A-aO_2}) and vital capacity (VC), and this suggests a role in monitoring disease progression and response to treatment [105].

The serum levels of some tumour markers are also elevated in patients with autoimmune PAP. Epithelial damage and increased production in the epithelium lead to an overexpression of cytokeratin 19 fragment 21.1 (CYFRA21-1). Moreover, serum levels of carcinoembryonic antigen (CEA) and neuron-specific enolase and serum LDH values demonstrated a significant association [106]. CEA and CYFRA21-1 levels have been related to partial pressure of oxygen (P_{aO_2}), forced expiratory volume in 1 s (FEV_1), HRCT score and diffusing capacity of the lung for carbon monoxide (D_{LCO}). Interestingly, serum CYFRA21-1 levels have proven to be more sensitive than LDH and CEA in revealing the severity of autoimmune PAP among mild to moderate forms of disease [107].

Krebs von Lungen 6 (KL-6), a mucin-like glycoprotein coded by the MUC1 gene and involved in morphogenetic signal transduction, is a sensitive biomarker for various interstitial lung diseases (ILDs) and has proven to be a predictive serum biomarker for the outcome of PAP. It is mainly secreted by proliferating, stimulated or damaged alveolar type II epithelial cells and then likely transferred into the bloodstream *via* lymphatic vessels. It is significantly correlated with lung function (P_{A-aO_2}, D_{LCO} and VC), total lung capacity and blood gas analyses (P_{aO_2}) [2, 108, 109]. Of importance, the change over time of serum KL-6 correlated with the change over time of D_{LCO} [108], even if a single nucleotide polymorphism (rs4072037) inside exon 2 of the MUC1 gene is associated with the inter-individual variability of serum

TABLE 2 Circulating biomarkers and their potential utility in pulmonary alveolar proteinosis (PAP)

Biomarker	Biologic function/origin	Association in PAP
		Diagnosis
GM-CSF autoantibody	Neutralise circulating GM-CSF/present also in healthy patients at low titre (<1 $\mu g \cdot ml^{-1}$)	Diagnostic for autoimmune PAP [93]
GM-CSF	Growth factor for granulocytes–macrophages/AM	Increased serum level in hereditary PAP, in presence of normal serum GM-CSF autoantibody level [102]
		Disease activity, outcome and response to treatment
LDH	Marker of cellular death/all cell types	P_{A-aO_2} and VC [3, 105, 106]
CYFRA21-1	Tumour tissue antigen/mostly epithelial cells	P_{aO_2}, FEV_1, D_{LCO} and HRCT score [106, 107]
CEA	Tumour tissue antigen/mostly epithelial cells	P_{aO_2}, FEV_1, D_{LCO} and HRCT score [106, 107]
NSE	Tumour tissue antigen/neuroendocrine cells	LDH, disease activity [106, 107]
KL-6	Mucin 1, host defence/type II pneumocytes	P_{A-aO_2}, D_{LCO}, VC, TLC and P_{aO_2}, response to treatment (WLL) [2, 108–110]
SP-A	Surfactant protein, host defence of the lung/type II pneumocytes	Alveolar type II cell hyperplasia [111]
SP-D	Surfactant protein, host defence of the lung/type II pneumocytes	Alveolar type II cell hyperplasia [111]
YKL-40	Chitinase-like protein/AM	D_{LCO}, disease outcome [113, 114]
MCP	Monocyte chemotactic proteins/mostly epithelial cells	Mononuclear chemoattractant activity and CCR2 receptor suppression on macrophages [115]

AM: alveolar macrophages; CEA: carcinoembryonic antigen; CCR2: C-C chemokine receptor type 2; CYFRA: cytokeratin-fragment; D_{LCO}: diffusing capacity of the lung for carbon monoxide; FEV_1: forced expiratory volume in 1 s; GM-CSF: granulocyte–macrophage colony-stimulating factor; HRCT: high-resolution computed tomography; KL-6: Krebs von den Lungen 6; LDH: lactate dehydrogenase; MCP: monocyte chemoattractant protein-1; NSE: neuron-specific enolase; P_{aO_2}: arterial oxygen tension; P_{A-aO_2}: alveolar–arterial oxygen tension difference; SP: surfactant protein; TLC: total lung capacity; VC: vital capacity; WLL: Whole lung lavage; YKL-40: chitinase 3-like 1 protein.

KL-6 expression level. In particular, the A/A genotype seems to be associated with more severe pulmonary impairment and a higher rate of disease progression in PAP patients [110].

SP-A and SP-D belong to the collectin family and play pivotal roles in host defence of the lung. They act in keeping the lung in a relatively uninflamed state. It has been shown that SP-A and SP-D increase by at least more than 10-fold in the BALF of PAP patients. This increase is likely attributable to alveolar type II cell hyperplasia with overproduction of SPs and leakage into the bloodstream due to epithelium, endothelium and/or basement membrane damage [111]. In fact, without proper surfactant function, the alveoli are more prone to collapsing repeatedly, putting stress on the basement membrane [112].

The serum levels of chitinase-3-like protein 1 (YKL-40), a glycoprotein belonging to the chitinase family, were also elevated in patients with ILD and were correlated with lung function impairment and disease outcome (stable/improved or progressed) in PAP patients [113]. It stimulates fibroblast growth and is known to be upregulated in inflammatory conditions, where it may contribute to tissue inflammation and remodelling [114].

The monocyte chemotactic proteins (MCPs) are small chemoattractant proteins crucial for the initiation of inflammation in the lung by the specific recruitment and activation of monocytes and lymphocytes. MCP-1, MCP-2 and MCP-3 share C-C chemokine receptor type 2 (CCR2) and are all elevated in the lungs of patients with PAP, where they exert chemoattractant activity for mononuclear cells and suppress CCR2. Interestingly, while circulating PAP monocytes demonstrated normal levels of CCR2, the reduction of CCR2 on circulating PAP lymphocytes limits their entry to the lung, thus affecting their activity within this tissue [115].

Standard treatment

Whole lung lavage (WLL) is a bronchoscopic procedure in which excessive surfactant is mechanically removed from the alveoli. It is considered as the standard treatment for PAP syndromes [1]. When PAP is associated with other diseases, the treatment should target the underlying condition. The management of congenital PAP depends on the patient's age at presentation, severity of symptoms and anticipated disease course [2]. When disease progression has resulted in severe respiratory failure, lung transplantation has also been attempted [116].

Pathogenesis-driven treatments

Targeting GM-CSF augmentation

GM-CSF augmentation has been investigated over the last two decades. GM-CSF is available in two recombinant forms, namely sargramostim (produced in *Saccharomyces cerevisiae*) and molgramostim (produced in *Escherichia coli*), which differ in terms of amino acid sequence and rate of glycosylation [117, 118].

Human studies showed that the administration of high doses of GM-CSF allows the saturation of anti-GM-CSF antibodies, overcoming their neutralising action, and the binding of GM-CSF receptors on the macrophage surface, restoring their function in surfactant catabolism and elimination of infectious pathogens [80, 85, 119].

The first approach to GM-CSF as a therapy for PAP was attempted by subcutaneous administration. Three prospective studies using daily injections of GM-CSF showed a significant improvement in symptoms and arterial oxygenation in ∼50% of cases, with variable therapeutic responses among patients, likely dependent on dose and treatment duration. Adverse effects included local reactions at the site of injection, fever, chills, nausea, vomiting, malaise, headache, fatigue, arthralgia and dyspnoea [3, 120, 121].

Inhaled administration allows direct delivery of the drug to the lungs, thereby increasing the concentration at the local site of disease and minimises adverse effects. Moreover, the inhaled route showed more efficacy than subcutaneous one in a meta-analysis [122].

After the promising results of several case reports and small studies [123–126], two large randomised clinical trials assessed the efficacy and safety of aerosolised GM-CSF. The PAGE trial (NCT02835742) was a double-blind, placebo-controlled trial which evaluated inhaled recombinant human GM-CSF (sargramostim, 125 μg twice a day, for 7 days, every other week for 24 weeks, or placebo) in 64 autoimmune PAP patients. The primary end-point was the change in the P_{A-aO_2} between baseline and week 25. The change in the P_{A-aO_2} and computed tomography involvement was significantly better in the GM-CSF group than the placebo group. Serious adverse events were reported in six patients of the

GM-CSF group and in three patients of the placebo group [127]. The IMPALA trial (NCT02702180) was a double-blind, placebo-controlled, three-group trial assessing the efficacy of inhaled recombinant GM-CSF (300 μg once daily, molgramostim), either continuously or intermittently, compared with placebo, administered for 24 weeks in 138 autoimmune PAP patients. The primary end-point was the change from baseline in the P_{A-aO_2} at week 24. Even if it was not significantly different between the continuous molgramostim group and the placebo group when analysed using the entire analysis set, after replacing the invalid data from patients who received supplemental oxygen during blood gas measurement, the change was greater among patients who received continuous molgramostim than among those who received the placebo. Furthermore, continuous treatment with molgramostim was associated with greater improvement than placebo in measures of pulmonary gas transfer (D_{LCO}), functional health status (expressed by Saint George Respiratory Questionnaire score) and pathologic features (ground-glass opacification score and serum biomarker levels). The treatment was well tolerated and the unique adverse effect that was more frequent in the molgramostim group compared with the placebo one was chest pain [128].

However, these studies were not of sufficient duration to permit determination of the effects of inhaled GM-CSF on the requirement for WLL therapy, for which the median time between procedures is 15 months. A long-term Italian clinical trial (NCT00901511), sponsored by the Italian Medicine Agency, was conducted in patients with severe autoimmune PAP requiring WLL and defined by a resting P_{aO_2} of less than 60 mmHg or if more than 60 mmHg then a resting peripheral blood oxygen saturation of less than 90% or a 5% or greater decline during exercise [129]. 18 autoimmune PAP patients were randomised to receive WLL (WLL group, n=9) or WLL followed by inhaled GM-CSF (sargramostim, 250 μg daily every other week for 12 weeks then two of every 14 days for 6 months (GM-CSF group, n=9); all were then followed for at least 30 months. The primary end-point was the time to first rescue WLL and the study demonstrated that inhaled GM-CSF was able to reduce the requirement for further WLL. Seven control patients and only one GM-CSF-treated patient required rescue WLL; moreover, the time to first rescue WLL was longer in the GM-CSF-treated patients than in the controls (30 *versus* 18 months, p=0.0078). Inhaled GM-CSF was also able to improve lung function and was safe. The GM-CSF group showed a greater improvement in mean (95% confidence interval) D_{LCO} (11.6 (1.9–21.3) % pred), P_{aO_2} (9.5 (5.7–13.3) mmHg), P_{A-aO_2} (−10.1 (−14.8–−5.4) mmHg). Adverse events were similar in both groups.

The IMPALA-2 study is currently ongoing (ClinicalTrials.gov identifier: NCT04544293). This is a multicentre, randomised, placebo-controlled trial evaluating the efficacy of 300 μg of inhaled molgramostim per day *versus* placebo, in adult subjects who are diagnosed with autoimmune PAP. The primary end-point is change from baseline in percentage predicted D_{LCO} to week 24.

As we wait to see the published results of this latest trial, the possibility of using inhaled GM-CSF as the standard of care for the treatment of autoimmune PAP and leaving WLL as a rescue option is becoming more and more concrete [130].

Targeting the reconstitution of GM-CSF receptor expression

GM-CSFR is constituted by α and β subunits, encoded respectively by the CSF2RA and CSF2RB genes. Correction of PAP has been experimented in $Csf2rb^{-/-}$ and $Csf2ra^{-/-}$ mice by pulmonary macrophage transplantation [131, 132]. Direct intrapulmonary instillation of wild-type bone marrow derived or gene-corrected macrophages restores GM-CSF signalling and surfactant homeostasis, further demonstrating the key role of AMs in the pathogenesis of PAP.

In 2023, a phase I and II clinical trial (identifier: NCT05761899) was initiated to evaluate the safety and efficacy of lentiviral-mediated CSF2RA gene transfer/pulmonary macrophage transplantation therapy for hereditary PAP patients. NCT05761899 is the first trial testing this therapy in humans. The trial will assess both the safety and effectiveness of this potentially curative treatment for hereditary PAP patients.

In 2017, a paediatric case, diagnosed with hereditary PAP due the loss of both CSF2RA alleles, who underwent a haematopoietic stem cell transplantation (HSCT) with a 10/10 human leucocyte antigen (HLA)-matched unrelated male donor was reported. Despite a life-threatening graft-*versus*-host disease (GVHD) at 6 months after HSCT, improvement in chest HRCT and respiratory status was documented in the patient, suggestive for a full recovery of donor-derived haematopoietic function, leading to AM replacement. At 14 months after HSCT, the patient experienced severe respiratory impairment and obliterative bronchiolitis was suspected, but the patient slowly recovered over 9 months and, at last follow-up, 40 months after HSCT, pulmonary function tests were stable [133].

Another successful HSCT in PAP was reported in 2022 [134] in a male patient, diagnosed at the age of 35 months with hereditary PAP due to complete deficiency of GM-CSF receptor α subunit. The patient underwent multiple sequential WLLs, which started when he was 3 years old. However, his pulmonary status gradually worsened, with the development of lung fibrosis and at the age of 18 he was referred for bilateral lung transplantation and subsequent allogeneic HSCT from an HLA-identical unrelated donor.

Therefore, HSCT could be a therapeutic approach to reconstitute GM-CSF receptor expression in patients with hereditary PAP affected by GM-CSF receptor gene mutations but the success of this strategy is limited by a high risk of infection, GVHD and drug toxicity, due to the long-lasting immunosuppression [135].

Targeting lipid metabolism

Disruption of GM-CSF signalling is also associated with a downregulation of genes required for fatty acid metabolism, namely carnitine palmitoyltransferase 1 (CPT-1α), CPT-2 and fatty acid binding protein-1, indicating a putative role of GM-CSF in fatty acid metabolism in macrophages [86].

AMs from PAP patients and GM-CSF knockout mice show a deficit of PPARγ and ATP-binding cassette (ABC) lipid transporter ABCG1, both involved in cholesterol efflux from macrophages [64], suggesting that the disruption of GM-CSF signalling affects lipid homeostasis in macrophages through the suppression of PPARγ signalling that regulates ABCG1 expression [136, 137].

In PAP patients, their pulmonary surfactant showed increased cholesterol content and a higher cholesterol to phospholipid ratio. Cytological analysis of BALF from PAP patients reveals the presence of foamy PAS-positive macrophages that contain large amounts of cholesterol, indicative of impaired cholesterol clearance. This suggests the possibility of considering novel therapeutic approaches that target cholesterol homeostasis.

In particular, statins are extensively used for the treatment of hypercholesterolaemia and hyperlipoproteinaemia [138]. The administration of statins reduces the cholesterol content in PAP-patient-derived *ex vivo* macrophages by increasing the expression of sterol regulatory element-binding protein-2 and facilitating cholesterol efflux [139]. Statins have been administered in autoimmune PAP patients with significant improvement of lung disease, as demonstrated by HRCT densitometry amelioration, functional improvement and symptom relief. These results were confirmed both in patients with and without hypercolesterolaemia [139, 140]. Furthermore, oral atorvastatin (20 mg daily) therapy was prospectively evaluated in 47 PAP patients without hypercholesterolaemia, demonstrating improvements in arterial blood gas, pulmonary function and radiographic assessment [141]. Considering the safety, low cost and availability of statins, the feasibility of this treatment should be determined in larger samples of patients.

Since PPARγ promotes activation of ABCA1 and ABCG1, key molecules in cholesterol efflux by macrophages, and PPARγ expression is reduced in PAP human and murine macrophages, PPARγ agonists were tested *in vitro* and *in vivo* [137, 142]. The results supported the initiation of the first human trial with oral pioglitazone (commercially available for diabetes) for autoimmune PAP patients (Pioglitazone Therapy of Autoimmune Pulmonary Alveolar Proteinosis Autoimmune Pulmonary Alveolar Proteinosis (PioPAP), NCT03231033). Pioglitazone was also used as a compassionate treatment in a patient with autoimmune PAP refractory to WLL, inhaled GM-CSF and rituximab. Improvements in dyspnoea and oxygenation and a reduction in the extent of crazy paving opacities was achieved in 6 months and maintained after 1 year of therapy [143]. Another case report reported limited benefits from pioglitazone use, but the treatment was administered at an advanced stage of disease when significant fibrosis was evaluable. This timing may have minimised the potential therapeutic effects [144].

Finally, liver receptor X (LXRα), which is able to increase the expression of ABCA1 and ABCG1, should be another molecular target of the cholesterol pathway [137, 145]. However, an LXRα agonist of proven clinical safety for humans is not yet available.

Conclusion

Pathogenesis of PAP syndrome is complex and involves several pathways resulting in surfactant metabolism disruption. The most frequent form is related to GM-CSF impaired signal. Pathogenesis-driven treatments are emerging and nebulised recombinant GM-CSF is a promising novel approach. In hereditary forms, related to CSFR mutations, haematopoietic stem-cell transplantation can reverse disease. Clinical trials with statins and other promising compounds are ongoing and results will have the potential to revolutionise the treatment landscape of this neglected syndrome.

Points for clinical practice

- PAP is a rare syndrome caused by progressive accumulation of surfactant within pulmonary alveoli leading to variable gas exchange impairment.
- PAP includes different diseases currently classified as primary, secondary or congenital according to the pathogenesis.
- Diagnosis of PAP should be highly suspected in the presence of a compatible clinical picture, typical chest HRCT findings and a milky BALF appearance.
- Autoantibodies targeting GM-CSF are pathognomonic for autoimmune PAP, which accounts for 90% of cases of PAP.
- WLL remains the gold standard therapy, even if pathogenesis-driven treatments are emerging and nebulised recombinant GM-CSF is a promising novel pathogenesis-based approach.

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