Pulmonary Alveolar Proteinosis Management: Current and Future Therapeutic Strategies

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Abstract

Pulmonary alveolar proteinosis is a rare disease first described in 1958 by Samuel H Rosen and characterised by the accumulation in the alveolar space of lipoproteinaceous materials due to insufficient catabolism of surfactant lipoproteins by alveolar macrophages. As a result, gas exchanges between the alveolar space and the blood are impaired, and affected patients develop progressive dyspnoea and pulmonary infections. In the mid-1990s the observation of a pulmonary alveolar proteinosis-like disease in GM-CSF deficient mice led to the discovery of GM-CSF signalling impairments in patients with pulmonary alveolar proteinosis. Additional studies showed that GM-CSF signalling disruption was responsible for incomplete maturation of alveolar macrophages both in mouse and human. Two main types of GM-CSF signalling disruption were observed, autoimmune pulmonary alveolar proteinosis, due to the presence in the blood and bronchoalveolar lavage fluid of neutralising anti-GM-CSF autoantibodies, and hereditary pulmonary alveolar proteinosis due to genetic mutations affecting the expression or the functionality of the GM-CSF receptor. A third type of the disease named secondary pulmonary alveolar proteinosis has also been described in patients suffering from hematologic malignancies, primary immunodeficiency or exposed to various toxic dust. Since its description in 1963 by Dr Jose Ramirez-Rivera, whole lung lavage has been a central therapeutic procedure for pulmonary alveolar proteinosis management. In this article, we will review the critical distinctions between the different forms of pulmonary alveolar proteinosis, as well as the efficacy of current, and prospective therapies for autoimmune and hereditary pulmonary alveolar proteinosis.

Keywords: *Pulmonary Alveolar Proteinosis; Whole Lung Lavage; Nebulized GM-CSF Replacement Therapy; Rituximab; Macrophages Pulmonary Transplantation Therapy; iPSC; Stem Cells; Gene Therapy*

Abbreviations

[A-a]DO$_2$: Oxygen Alveolar-Arterial Gradient; ABCA3: ATP Binding Cassette Subfamily A Member 3; ABCA3: ATP Binding Cassette Subfamily A Member 3; AEC2: Type II Alveolar Epithelial Cells; AM: Alveolar Macrophages; aPAP: Autoimmune Pulmonary Alveolar Proteinosis; b.i.b: Bis in Die (Twice a day); BALF: Bronchoalveolar Lavage Fluid; BMDM: Bone-Marrow-Derived Macrophages; CD: Cluster of Differentiation; CLL: Chronic Lymphocytic Leukaemia; CSF2R: Colony Stimulating Factor 2 Receptor; DLCO% Pred: Predicted Normal Diffusing Lung Capacity for Carbon Monoxide; FEV1.0: Forced Expiratory Volume; GATA2: GATA-Binding Factor 2; GM-CSF: Granulocyte Macrophage Colony Stimulating Factor; GVHD: Graft vs Host Disease; hPAP: Hereditary Pulmonary Alveolar Proteinosis; HSCT: Hematopoietic Stem Cell Transplantation; IL-2RG: Interleukin-2 Receptor Subunit Gamma; iPSC: Inducible Pluripotent Stem Cells; KO: Knock-Out; NKX2-1: NK2 Homeobox 1; PaO$_2$: Partial Pressure of Oxygen; PAP: Pulmonary Alveolar Proteinosis; PMT: Pulmonary Macrophage Transplantation; RAG2: Recombination Activating 2; rhGM-CSF: Recombinant Human Granulocyte Macrophage-Colony Stimulating Factor; SFCT: Surfactant; SLC7A7: Solute Carrier Family 7 Member 7; sPAP: Secondary Pulmonary Alveolar Proteinosis; STAT5: Signal Transducer and Activator of Transcription 5; TALENs: Transcription Activator-Like Effector Nucleases; WLL: Whole Lung Lavage; WT: Wild Type

Introduction

Pulmonary Alveolar Proteinosis (PAP), is a rare disease first described in 1958 by Samuel H Rosen., *et al.* and characterised by the accumulation of surfactant (SFCT) inside alveolar macrophages (AM) and the alveolar space [1]. Depending on the countries, PAP prevalence varies between 3.7 and 40 cases per million inhabitants, and its incidence is estimated to be around 0.2 cases per million per year [2].

SFCT are lipid-protein complex produced by type II alveolar epithelial cells (AEC2), whose function is to prevent alveolar collapsing and protect the lung from potentially harmful environmental particles [2,3]. As a consequence of SFCT lipid-protein complex accumulation, gas exchanges between the alveolar space and the blood are impaired, leading to breathlessness, infections and ultimately for some patients, to respiratory failure [4].

Patients affected by PAP usually present with dyspnoea, cough with or without expectoration, fever and weight loss [5]. Chest radiography of PAP patients present a bilateral alveolar opacities in a peri-hilar and basilar distribution without air-bronchograms referred to as "batwing distribution" [2]. High resolution computed tomography scans on the other hand often shows intralobular thickening and diffuse ground-glass opacities referred to as "Crazy paving" pattern [2]. Although suggestive of PAP, these patterns are not specific to the disease. The diagnosis of PAP is confirmed in 75% of cases by bronchoscopy with bronchoalveolar lavage. The bronchoalveolar lavage fluid (BALF) of PAP patients unlike those of normal patients have a milky and opaque appearance due to the presence of an excess of SFCT lipid-protein complexes [2]. The diagnostic can also be reinforced by testing the patients for circulating biomarkers such as SFCT protein A, B, and D, cytokeratin 19, serum carcinoembryonic antigen, serum lactate dehydrogenase, and Krebs von den Lungen-6 [6].

PAP Physiopathology

To understand the cause of SFCT protein accumulation in the lung of PAP patients, researchers studied the principal actors of its homeostasis. As said earlier, SFCT lipoproteins are produced by AEC2; however, studies carried out in adult rabbit suggest that 70 - 80% of the SFCT complexes present in the alveolar space are taken up by AEC2 and recycled, the remaining 30 - 20% being taken up and catabolised by alveolar macrophages [3,7,8]. Early studies aimed at identifying the possible cause of SFCT lipid-protein complex accumulation in PAP, pointed to the dysfunction (e.g. impaired phagocytosis) of AM from PAP patients [9,10]. The involvement of haematopoietic derived cells such as AM and other factors external to the lung in the pathology was further supported by the re-appearance of the disease in patients having undergone bilateral lung transplantation [11]. In 1994 the observation of a PAP-like disease in Granulocyte Macrophages-Colony Stimulating Factor (GM-CSF) knock-out mice led to a breakthrough discovery into the pathogenesis of PAP [12]. GM-CSF is a hematopoietic cytokine binding to and signalling through a heterodimeric cell-surface receptors comprised of two chains [13]. The ligand-binding α chain (CD116) is encoded by the gene CSF2RA located in the pseudoautosomal region 1 of chromosome X and Y [14], whereas the affinity-enhancing β chain (CD131) shared with the IL-3 and IL-5 receptors are located on chromosome 22 [13,15]. GM-CSF is a pleiotropic cytokine controlling myeloid cells survival, proliferation, differentiation, and functional activation [16]. The unexpected relationship between GM-CSF and SFCT homeostasis in the lung was further confirmed by the analysis of GM-CSF function in PAP patients. In 1997 a study by Dirksen., *et al.* reported a CSF2RB deficiency in 3 paediatric cases of human PAP [17].

Moreover, the eosinophilia usually induced by GM-CSF administration (5 μg/kg/d) in normal individuals was strongly reduced in PAP patients suggesting its dysfunction in human PAP as well [18]. It was later discovered that, GM-CSF, by inducing in AM progenitors the expression of the transcription factors PU.1 and PPARγ play a critical role in their terminal differentiation and functionality, especially by promoting the expression of scavenger receptors, lysosomal enzymes and the ATP Binding Cassette transporter ABCG1 required for SFCT protein-lipid complex catabolism and homeostasis [3,19]. Thus in PAP, SFCT accumulation is often due to a decrease in its clearance rather than an increase in its production [2,3]. On top of promoting the expression of genes involved in the housekeeping function of AM, GM-CSF through PU.1 also regulates the expression by AM of genes involved in process critical to their immune function (e.g. Chemotaxis, Cytokines secretions, bacterial killing, phagocytosis), offering an explanation for the increased susceptibility of PAP patients to opportunistic infections [7].

Interestingly, the restoration of GM-CSF production by the pulmonary epithelium in GM-CSF KO mice (i.e. GM-CSF KO mice expressing the GM-CSF gene under the promoter of SFCT protein C also known as SFC-GM$^{+/+}$/GM$^{-/-}$), was able to cure PAP in this model, highlighting further the role played by GM-CSF signalling disruption in PAP development [20]. The same way, GM-CSF inhalation by GM-CSF KO mice has been shown to restore SFCT homeostasis and cured PAP in this model [21]. Although AEC2, express the GM-CSF receptors, the analysis of GM-CSF KO mice did not reveal any change in their production and secretion of SFCT lipid-protein complex [7,22]. However, it is interesting to note that SFC-GM$^{+/+}$/GM$^{-/-}$ mice are 30 - 40% larger than there control littermate and had AEC2 hyperplasia (four-fold increases). This observation raised the question of the role of GM-CSF in AEC2 pool regulation in normal mice and their possible contribution to the improvement of SFCT homeostasis in SFC-GM$^{+/+}$/GM$^{-/-}$ mice. By using a mouse model of PAP based on the deletion of the β chain of the GM-CSF-R (GM R$_{βc}$$^{-/-}$ mice), researchers showed that restoration of GM-CSF exclusively in the haematopoietic compartment by transplantation of wild-type bone marrow was sufficient to restore SFCT homeostasis suggesting that the beneficial effect of GM-CSF on SFCT homeostasis was mainly due to its effect on AM [23]. These discoveries, established a mechanistic hypothesis linking GM-CSF signalling impairment, alveolar Macrophages dysfunction and the accumulation of SFCT protein-complex characteristic of PAP.

PAP Classification, and therapeutic approach

Further studies of the GM-CSF and AM functions in PAP patients revealed three main causes leading cause of dysfunction leading to a classification into three distinct forms of PAP.

Autoimmune PAP

Initially called Idiopathic PAP [24], autoimmune PAP (aPAP) is the main form of the disease, representing 90 - 95% of cases, and generally appears during adulthood [2,25-27]. As indicated by its name, this form of PAP is due to the presence in the blood and BALF of these patients of at high concentrations of anti-GM-CSF autoantibodies. Tanaka., *et al.* were the first to report in 1999 the presence in the BALF of patients suffering from PAP of a GM-CSF neutralising factor later identified as antibodies [28]. These antibodies by binding the endogenous GM-CSF, block its interaction with the GM-CSF receptor [29], at the surface notably of AM, preventing their maturation and their ability to catabolise SFCT [2,25]. The induction of the disease in primates by the injection of anti-GM-CSF antibodies purified from the serum of PAP patients clearly established the causal link between circulating neutralising anti-GM-CSF autoantibodies and the disease outbreak [30,31]. Although neutralising anti-GM-CSF autoantibodies are present in both, blood and BALF, the disease severity correlates specifically with its BALF concentration [32]. Active PAP is usually associated with concentrations of anti-GM-CSF antibodies > 19 μg/ml, ranging from 27.4 to 116.5 μg/ml with a median at 59.8 μg/ml [33,34]. Interestingly many healthy subjects presented circulating anti-GM-CSF antibodies concentrations < 4 μg/ml, ranging from 0.63 to 1.72 μg/ml with a median at 1.04 μg/ml. This prompted some researchers to hypothesise that these levels of anti-GM-CSF autoantibodies could play a regulatory role by modulating GM-CSF activity [33]. Other researchers found that anti-GM-CSF autoantibodies represent the dominant anti-cytokine activity from human IgG preparation reinforcing the idea of its expression at a low level in healthy individuals [34]. This raises the question of whether aPAP represents a classical autoimmune disease or the deregulation of a normal regulatory mechanism? Interestingly while autoimmune diseases tend to cluster, the presence of other autoantibodies in PAP is rare, while the presence of anti-GM-CSF autoantibodies was found at low to moderate levels in about 10% of patients (41 out of 425) with other autoimmune diseases [35-37]. Moreover, among the 10% of patients with anti-GM-CSF autoantibodies only around 7% (3 out of 41) had neutralising anti-GM-CSF autoantibodies [35-37].

Autoimmune PAP: Whole lung lavage as the standard therapeutic management

The primary treatment for aPAP is Whole Lung Lavage (WLL), a procedure invented in 1963 by Dr Jose Ramirez-Rivera and consisting of the injection and recovery of a large volume of sterile saline solution (~15 - 25L per lung) in the lung under general anaesthesia [38]. To allow oxygenation of the patients, one lung is treated at a time, starting with the most affected one, while the contralateral lung is ventilated. During the saline instillation, cycles of chest percussion are performed to favour the retrieval of the accumulated lipoproteinaceous material impairing lung function [38]. According to a study by Campo., *et al.* analysing data from WLL performed in 20 centres across 14 countries, the average duration of the procedure is 6 ± 1 hour, followed by mechanical ventilation of both lungs in an intensive care unit for

an average of 5 ± 1.5 hour. In an attempt to mitigate the hypoxic stress caused by WLL, Zhou., *et al.* tested the use of an hyper-oxygenated saline solution and showed that it increases patients oxygen supply during WLL without noticeable side effects [39]. In a retrospective analysis of 231 patients with aPAP, of which 146 undergone WLL and 85 patients did not, Seymour., *et al.* showed that WLL improve significantly the five years survival rate of PAP patients (94 ± 2% vs 85 ± 5%) [35]. According to a review of WLL efficacy in PAP, 85% of patients are showing signs of lung function improvement (e.g. PaO_2, $[A-a]DO_2$, FEV1.0, vital capacity, or diffusion capacity for carbon monoxide) [35]. However, the remaining patients proved to be refractory to the procedure and depending on the severity of the disease, responding patients often required multiple lavages. Indeed, the duration of response (freedom from recurrent symptoms) to WLL in patients with aPAP reported in this study was around 15 months [35], but reduced periods (e.g. 8 and two months) were reported in other studies [40,41]. On average, patients in the WLL group required two procedures in the five years following their diagnosis [35,42]. In another review, Luisetti., *et al.* showed that around 33% of PAP patients required more than one lavage during their follow-up with some refractory patients requiring up to 3 WLL in the three months following the first procedure [41]. In a 2015 study of 120 patients with aPAP, Zhao., *et al.* showed that 80 (66%) patients required WLL [43]. Out of these 80 cases, only 24 (30%) required more than 1 WLL [43]. The authors found that a percentage of predicted standard diffusing lung capacity for carbon monoxide (DLCO% Pred) on admission inferior to 42.1% was a good predictor of the need for a second WLL [43]. In some rare cases, WLL has been associated with adverse effects such as infections, fever, convulsion, pneumothorax, pleural effusion, hypoxemia and in the more dramatic cases death [44].

Due to the complexity of the WLL procedure, the need for frequent repetition and the fact that some patients are refractory to the procedure, alternative therapeutic management have been tested and implemented in recent years, with the intent of treating the cause rather than just the symptoms of aPAP.

Alternative therapeutics in aPAP management

GM-CSF replacement therapy

Since the discovery in 1994 of a PAP-like disease in GM-CSF KO mice, the concept of GM-CSF replacement therapy as a treatment for PAP has gained in popularity [27]. In the context of aPAP, this procedure intends to overcome the neutralising effect of circulating anti-GM-CSF autoantibodies by administrating a surplus of recombinant human GM-CSF (rhGM-CSF). In 1996, the first GM-CSF replacement therapy was tested in a patient with acquired PAP, using subcutaneous injections of rhGM-CSF (5 - 6 µg/kg/day) and resulted in a significant improvement of the alveolar-arterial oxygen gradient [45]. In another study involving 14 patients with aPAP, subcutaneous injections of rhGM-CSF at a dose of 5 mg/kg/day for 6 to 12 weeks improved prospective criteria (i.e. alveolar-arterial oxygen gradient, diffusing capacity of carbon monoxide, and exercise testing) in 5 out of 14 patients. Dose escalation (20 mg/kg/day) resulted in an improvement in a sixth patient leading to an overall response rate of 43% [46]. The benefit of subcutaneous rhGM-CSF in this study lasted a median of 39 weeks and was reproducible with retreatment [46]. No significant side effect was reported, and rhGM-CSF induced eosinophilia was shown to be a good predictor of response to the therapy. In addition to eosinophilia, Seymour., *et al.* showed that a healthy level of serum lactate dehydrogenase was a predictor of good response to subcutaneous GM-CSF replacement therapy [47]. In a 2006 open-label clinical trial involving 25 adults patients with aPAP, Venkateshiah., *et al.* showed that subcutaneous administration of escalating doses of rhGM-CSF improved oxygenation, radiographic findings, and symptoms in 48% of the cohort [48]. Although most studies did not report significant side effects associated with subcutaneous injection of rhGM-CSF, a recent article reported the development of membranous nephropathy in a 43 years old patient successfully treated of her aPAP with a daily injection of rhGM-CSF (750 µg/d). After eight months of treatments her lung function normalised leading to the discontinuation of home oxygen therapy, but 17 months after the start of the treatment she presented signs of membranous nephropathy. Interestingly the recovery of her nephrotic function coincided with the progressive decrease and finally discontinuation of rhGM-CSF injection [49].

As an alternative to subcutaneous injection, Reed., *et al.* established a proof of principle for the use of aerosolised GM-CSF in the treatment of PAP using the GM-CSF deficient mouse model [21]. In a retrospective study of twelve patients having undergone aerosolised

GM-CSF therapy (250 mg b.i.d every other week) in lieu of WLL between 1999 and 2003, William., *et al.* showed that 11 (91.7%) patients responded well to the therapy as assessed by the improvement of their lung function (e.g. arterial oxygen tension, alveolar-arterial oxygen gradient, carbon monoxide diffusing capacity of the lung and forced vital capacity) [50]. Two patients made a complete recovery and were disease-free for 1 and two years after the end of the treatment. Four other patients responded well to the treatment but had a shorter disease-free period requiring further treatment to which they responded well. Lastly, one patient required dose escalation (500 mg b.i.d) to achieve a satisfactory response. Overall the authors showed that aerosolised GM-CSF therapy was a well-tolerated and effective treatment of aPAP, representing an alternative to WLL and subcutaneous injection of GM-CSF [50]. In another prospective clinical trial, involving 35 aPAP patients in Japan, Tazawa., *et al.* tested the efficacy of inhaled rhGM-CSF therapy. Two regimens were tested on 12 weeks, with a high dose (250 µg/day) regimen consisting of 8 days drug administration followed by six days without inhalation and a low dose (125 µg/day) regimen consisting of 4 days administration followed by ten days without inhalation. Both 14 days of high and low dose regimens were repeated six times over 12 weeks followed by a 52 weeks follow-up [51]. The results showed that nebulised rhGM-CSF therapy improved dyspnoea and the 6 min walk distance in 62% of the aPAP cohort [51].

Moreover 83 and 66% of the patients did not require further therapy during 1 and 2.5 years respectively [51]. The authors also showed that the high dose regimen resulted in a significant increase in the treatment free period following inhaled rhGM-CSF therapy in the absence of significant clinical side effects [52]. From a mechanistic perspective, Ohashi., *et al.* observed no increase in BALF macrophages or other leucocytes in patients responding positively to inhaled rhGM-CSF therapy. However, they observed a decrease in BALF total protein, and SFCT-A concentrations together with an increase in IL-17 and cancer antigen-125 concentration after aerosolised rhGM-CSF replacement therapy [53]. These results confirm that GM-CSF act by improving macrophage maturation and functionality rather than density [53].

Subcutaneous injection and inhalation, have been the main routes of administration tested in aPAP patients treatment by hGM-CSF replacement therapy. Although both administrations routes showed an ability to improve aPAP symptoms, recent data showed that rhGM-CSF administration by inhalation was the most effective route for aPAP treatment [54]. One of the limiting factors of inhaled rhGM-CSF efficacy is the decreased accessibility of AM due to their embeddedness in the lipoproteinaceous material present in the alveoli, especially in severe cases [55]. In a recent publication, Ohkouchi., *et al.* showed in 5 severe cases of aPAP that hGM-CSF inhalation therapy was most effective when administered after, rather than before WLL [55].

Currently, the primary pharmaceutical sources of rhGM-CSF available are Molgramostin (Molgradex; Savara Pharmaceuticals) a non-glycosylated rhGM-CSF produced in the bacteria *E. coli* and Sargramostim (Leukine, Sanofi-Aventis) a glycosylated rhGM-CSF produced in yeast which has a substitution of leucine at position 23 [56]. Both compounds have been used interchangeably in inhaled rhGM-CSF therapy of aPAP patients although the *in vitro* biological activity of Molgramostin have been shown to be higher than that of Sargramostim [46,53,56]. WLL followed by inhaled rhGM-CSF (i.e. molgramostim) therapy has recently been tested successfully in a paediatric case of aPAP [56]. The effectiveness of inhaled GM-CSF replacement therapy in aPAP is also influenced by the quality of aerosol formation [57]. The progress made in nebulised drug delivery system for patients with other lung diseases such as asthma and chronic obstructive pulmonary disease will also contribute to the increasing effectiveness of aerosolised GM-CSF replacement therapy in aPAP [57].

These data suggest that WLL followed by rhGM-CSF inhalation therapy should be systematically evaluated in severe aPAP to reduce WLL frequency and improve the quality of life of patients with aPAP.

Rituximab

In an attempt to treat the cause of aPAP, namely the production of GM-CSF neutralising autoantibodies, some researchers tested the therapeutic potential of Rituximab, an anti-CD20 monoclonal antibody targeting B cells. Rituximab has been shown to be effective in several au-

toimmune diseases including rheumatoid arthritis and ANCA-associated vasculitis [58-60]. Rituximab act by mediating B cells inhibition through various pathways including direct signalling, complement dependent cellular toxicity and antibody-mediated cell toxicity [61].

In a 2007 case report, Borie., *et al.* reported the beneficial effect of two intravenous administration of Rituximab (1000 mg) at two weeks interval on the disease progression in patients refractory to WLL and GM-CSF therapy [62]. In 2011, using the same protocol in an open-label clinical trial involving 10 patients Kavuru., *et al.* showed that Rituximab therapy of an aPAP patient resulted in a decrease in CD19+ B-Lymphocytes, 15 days post-therapy leading to a decrease in BALF anti-GM-CSF antibody concentration and lung function improvement (e.g. partial pressure of oxygen, Forced Vital capacity), in the absence of significant adverse effects [63]. In another case report, the administration of Rituximab at 375 mg/m^2 weekly for four weeks also resulted in dramatic improvement of lung function in an aPAP patient without adverse effects [64]. Moreover, Rituximab therapy has been shown to increase the expression of the GM-CSF targets PPAR-gamma (2.8 fold) and ABCG1 (5.3 fold) in AM of PAP patients up to 6 months after the end of the therapy. The authors suggest that Rituximab, by blocking the production of neutralising anti-GM-CSF autoantibodies contributes to the restoration of GM-CSF signalling and lipid catabolism [65]. However, in a retrospective study of Rituximab treatment (1000 mg twice at two weeks interval) efficacy in 13 patients, Soyez., *et al.* found no significant improvement in lung function despite a significant decrease in serum anti-GM-CSF antibody concentration [44]. In this last study, the authors did not report the evolution of anti-GM-CSF concentration in BALF even though aPAP severity is more closely related to BALF than systemic anti-GM-CSF autoantibodies concentrations [32]. These conflicting results suggest that, although promising, more studies are required to determine the relevance of Rituximab therapy in aPAP and that BALF anti-GM-CSF concentration should be monitored to set the optimal regimen.

Plasmapheresis

Therapeutic plasmapheresis is a procedure intending to remove pathogenic molecules or substances (e.g. pathogenic antigens, Low-Density Lipoproteins, Autoantibodies) from the plasma fraction of the blood [66]. Depending on the size of the molecules targeted, plasmapheresis can involve plasma exchange, double-filtration plasmapheresis, and plasma adsorption [66]. In a 2009 study Luisetti., *et al.* reported one of the first cases of plasmapheresis used in the treatment of a patient with aPAP refractory to WLL (3 WLL required in 10 months) treatment. The authors observed a decrease in circulating GM-CSF autoantibodies concentration from 250 to 156 μg.mL^{-1} after the completion of ten 1.5L plasma exchanges. After 80 weeks of plasmapheresis, the concentration of circulating GM-CSF autoantibody dropped to 56 μg.mL^{-1}. However, the lung function of the patient did not improve and WLL was required [67]. In 2015, Garber., *et al.* proposed a plasmapheresis protocol for aPAP treatment based on an abridged five days treatment regimen followed by Rituximab administration as opposed to weeks of treatment in previous studies [68]. After five consecutive days of plasmapheresis followed by Rituximab administration they observed a drastic decrease in circulating anti-GM-CSF autoantibodies (from 24.8 to 2.7 mcg/mL) [68]. However, the use in this protocol of Rituximab, immediately after plasmapheresis does not allow an evaluation of the effectiveness of plasmapheresis alone.

Moreover, despite this combination, the authors only reported a decrease in WLL frequency and marginal lung function improvement [68]. Lastly, a 2017 case report of plasmapheresis treatment in an aPAP patients refractory to WLL and GM-CSF therapy reported a failure of this therapeutics to improve aPAP associated lung dysfunction [69]. Recent data from animal models of asthma and influenza infection suggest the possible presence in the lung of a pool of resident B cells distinct from the circulatory pool [70,71]. As said earlier, aPAP severity is more closely related to BALF than systemic anti-GM-CSF concentration [32]. Plasmapheresis mainly affect the blood anti-GM-CSF pool; therefore if BALF anti-GM-CSF autoantibodies do not come from the blood supply but are produced locally by resident lung B cells, this might explain the failure of this therapeutics in aPAP. Further studies are required to determine the source of BALF neutralising anti-GM-CSF autoantibodies in aPAP.

Lung transplantation

PAP is a disease caused by the disruption of GM-CSF signalling, a cytokine whose regulation and downstream effectors involved transcription factors such as NF-kB shared with many other inflammatory mediators [72-74]. To avoid graft rejection after lung transplantations, patients are given high doses of immunosuppressant and anti-inflammatory drugs [75] such as cyclosporine which has been shown to impair GM-CSF secretion by T lymphocytes [76]. Considering the ability of these treatments to disrupt GM-CSF signalling and myeloid cells production it is not surprising that PAP has been reported in several occasion after lung [77-80], kidney [81] or unrelated cord blood haematopoietic stem cell [82] transplantation for conditions other than PAP. Unsurprisingly several cases of PAP recurrence after lung transplantation have been documented [11,83]. However, Turkalj., *et al.* reported in 2016 a successful bilateral lung transplantation in a 12 years old boy with aPAP refractory to WLL and GM-CSF therapy [84]. Following lung transplantation, the patient developed a post-transplant lymphoproliferative disease which was successfully treated with a chemotherapeutic protocol [84]. Therefore, lung transplantation should only be performed in patients refractory to WLL and GM-CSF replacement therapy.

Type	Auto-Immune PAP	Secondary PAP	Congenital/hereditary PAP
% of Total PAP	90%	4%	1%
Causes	High level of circulating and Broncho Alveolar Lavage Fluid neutralising anti GM-CSF autoantibodies [25].	Secondary to: 1. Haematological malignancies [85] 2. Primary Immunodeficiency [2,86] 3. Exposure to inhaled particles [87]	Genetic mutations in the α or β chain of the GM-CSF Receptor [27].
Treatments	Whole Lung Lavage [35] [40, 41] associated or not with GM-CSF replacement therapy [38, 55]. Rituximab [62-65].	Haematopoietic Stem Cell transplant [2, 27]. Rituximab and Bendamustine [85]. Dust exposure discontinuation [42].	Whole Lung Lavage [88], Haematopoietic Stem Cell transplant [89].
Treatments Outcome	A survival rate of 94%, five years post-diagnosis [35]	Median survival after diagnosis is < 15 months [2].	Mostly fatal [17, 90]

Table 1: *Summary of Pulmonary Alveolar Proteinosis (PAP) subtypes characteristics.*

Secondary PAP

The second most frequent form of PAP, representing around 4% of cases, is secondary PAP (sPAP), a heterogeneous form of the disease, usually devoid of high concentration of neutralizing anti-GM-CSF antibodies, and observed in patients with haematological malignancies (e.g. Myelodysplastic Syndrome, Chronic Myelogenous Leukaemia), primary immunodeficiency diseases (e.g. Common Variable Immunodeficiency, DiGeorge syndrome) [27,42,85] or exposure to organic and inorganic dust (e.g. fertilizers, silica, talc, cement, kaolin, aluminium, titanium, indium) [27,42,87]. The disease can be characterised by a low number of AM due to insufficient replenishment by hematopoietic derived progenitors, in patients with haematological malignancies, and primary immunodeficiencies [27,42,85] or AM dysfunction due to dust overload [91,92]. However, in some sPAP cases induced by the exposure to indium, the presence of circulating neutralising anti-GM-CSF autoantibodies have been described [93]. The median survival after diagnosis reported in sPAP is inferior to 15 months [2]. As in aPAP, the first line therapy for sPAP is WLL, but data suggest poorer efficacy as only two patients out of fourteen showed improvement in a clinical study [2]. Therefore, the definitive treatment of sPAP is the treatment of the underlying disease [2,27].

In sPAP caused by haematological malignancies, and primary immunodeficiencies the main treatments have been haematopoietic stem cells transplantation (HSCT) [94,95]. However, Chaulagain., *et al.* also reported in 2014 the successful treatment of a 69-years-old patient with PAP secondary to chronic lymphocytic leukaemia (CLL), using a combination of Rituximab and Bendamustine (a chemotherapeutic drug used in the treatment of CLL) [85]. Regarding secondary PAP due to dust exposure, the efficacy of WLL lavage and GM-CSF replacement therapy is not well documented; however discontinuation of dust exposure is indicated and beneficial to the patients [42].

Congenital/hereditary PAP

The least common but deadliest form of PAP, representing around 1% of cases, is congenital or hereditary PAP (hPAP), a form of the disease that usually appears earlier in life [2,27]. In those patients, mutations in genes involved in SFCT recycling and catabolism are responsible for the disease outbreak [2,27,88]. The most common mutations are found in the genes encoding the alpha (CSF2RA) and beta (CSF2RB) chains of the GM-CSF receptor [17,90]. Depending on the mutation type (e.g. substitution, deletion) the functional impairment of the GM-CSF receptor can vary ranging from no signalling following complete deletion of one or the other receptor (most of the time the CSF2RA) to strong or mild signalling reduction in cases with conservative point mutations [88]. Both CSF2RA and CSF2RB mutations are autosomal recessive but in some cases with incomplete penetrance [6].

In many cases siblings of affected children are asymptomatic while bearing the same mutations [6,88], suggesting that GM-CSF signalling impairment might be compensated in some individuals bearing gene with above average efficiency for other regulators of SFTC homeostasis. Mutations leading to complete deletion without compensation are associated with early onset of the disease whereas complete deletion with compensation or mutation leading to mild signalling impairment might appear later in life. Consistent with this idea, two recent publication reported hPAP associated with CSF2RB and CSF2RA mutations respectively in 36 and 77 years old females [96,97]. In both cases, the patient's peripheral blood mononuclear cells (PBMC) failed to activate STAT5 after a dose-response stimulation with rhGM-CSF up to 1 µg/ml [96,97]. Both patients responded very well to WLL, reinforcing the diagnostic of a mild phenotype. A recent article reported the onset of hPAP in a three-year-old girl with CSF2RA mutation after infection by Mycoplasma pneumoniae. Analysis of her blood cells revealed the absence of the GM-CSF receptor α chain (CD116) and an absence of Stat5 phosphorylation following GM-CSF stimulation. The loss of CD116 was due to the presence in both of her alleles of 2 points mutations [98]. Similar to what was observed with the adult onset of the disease, she responded very well to serial partial lung lavage [98]. Interestingly she had an asymptomatic seven years old sister carrying the same mutations, suggesting that in patients with compensatory mechanisms ageing and infections episodes might precipitate the disease onset [96-98].

Similar to what has been observed in aPAP, patients suffering from hPAP have high GM-CSF concentration in their blood and BALF, as well as accumulation of foamy alveolar macrophages and proteinaceous material in their alveoli. In hPAP as in aPAP, WLL is the primary therapy used to manage the disease activity. Long-term management of hPAP with frequent partial lung lavage of infants and children have been documented [99]. Unsurprisingly, hPAP patients do not respond to subcutaneous or inhaled rhGM-CSF administration [99,100]. Moreover, bilateral lung transplantation resulted in disease recurrence in an hPAP patient with a nonsense mutation in CSF2RB, highlighting the requirement for functional endogenous monocyte-derived AM to maintain SFCT homeostasis [101].

Other Genetic defects associated with PAP

On top of mutations affecting the GM-CSF receptor, several cases of hPAP associated with GATA2 mutation have been reported [102-104]. GATA2 is a zinc finger transcription factors playing a pivotal role in haematopoietic cells development and AM phagocytosis [105]. Therefore impaired AM phagocytosis in GATA2 deficient patients is the likely cause of SFCT accumulation. However, unlike cases of hPAP link to GM-CSF impairment, these patients also present the development of lung fibrosis [103]. Similarly, patients suffering from Lysinuric protein intolerance which is an autosomal recessive disease caused by mutation of the gene Solute Carrier Family 7 Member 7 (SLC7A7) also present macrophage phagocytosis impairment that might explain the development of PAP in these patients [6,106].

Similar to the lung remodelling observed in patients with GATA2 and SLC7A7 deficiency, a mutation affecting the SFCT protein B and C, as well as the transporter ABCA3, or the transcription factor NKX2-1 have been shown to induce a form of PAP with distinct histo-pathological features [6,27,107]. Indeed, whereas in aPAP and hPAP there is no sign of parenchymal remodelling and AEC2 damage, there is in the PAP associated with these mutations various degrees of interstitial remodelling associated with AEC2 hyperplasia [6,27,107]. Therefore, PAP in those cases are not regarded as primary PAP but as surfactant mutation associated disorders and will not be covered in this review as hPAP.

hPAP therapy: Prospective therapeutic approaches

In 1996 Nishinakamura., *et al.* published one of the first experimental therapy for hPAP using CSF2RB (CD131) knock-Out (KO) mice [23]. They showed that bone marrow transplantation and hematopoietic reconstitution of the CSF2RB KO mice with wild-type (WT) bone marrow was able to reverse an established hPAP as assessed by histopathological observations and protein measurement in BALF [23]. Furthermore, they showed that bone marrow transplantation from the RAG2 KO mice which are unable to produce lymphoid cells was also able to improve experimental hPAP reinforcing the concept of a myeloid lineage dysfunction, in particular, macrophage dysfunction in hPAP physiopathology [23]. In 2008, another team successfully treated hPAP in the CSF2RB KO mice model by performing a haemato-poietic stem cell transplantation using genetically corrected autologous bone marrow cells [108].

However, effective hematopoietic reconstitution requires myeloablation which increases the risk of infection and mortality [109]. Moreover, haematopoietic stem cell transplantation from allogeneic donors present a risk of graft versus host disease (GVHD) development [109].

Last year, Frémond., *et al.* reported the first successful treatment of hPAP in a little girl diagnosed at 2.6 years old with hPAP due to the deletion of both CSFR2A alleles [89]. Despite WLL treatments twice a year, her condition deteriorated requiring WLL every three months and continuous oxygen therapy from 5 years old onwards. At 6.3 years old she underwent a haematopoietic stem cell transplantation (HSCT) with cells from a 10/10 HLA matched male donor. Two weeks after the procedure, analysis of her BALF revealed, a reduction in lipoproteinaceous material accumulation, and 94% of AM from donor origin, signing the success of the transplantation [89]. However, 23 days post HSCT she developed skin and gastroenterological manifestations of acute GVHD requiring six successive lines of immuno-suppressant treatment. Fourteen months post-transplantation, she showed signed of lung function deterioration (i.e. tachypnoea, hy-poxia and haemoptysis) due to immunosuppressant treatments (i.e. Rapamycin, corticosteroids and ruxolitinib) which required oxygen therapy. The discontinuation of rapamycin administration allowed a slow recovery of normal lung function on nine months leading to the cessation of oxygen therapy. Around 22 months after HSCT, she was diagnosed with obliterative bronchiolitis. Systemic immunosuppres-sion was discontinued 26 months after HSCT, and despite the onset 40 months after HSCT of ophthalmic GVHD requiring the use of topical immunosuppressant, her lung function remained normal as revealed by a 6 minutes walking test [89].

To bypass the inconvenient of HSCT in human, Suzuki., *et al.* considered lung targeted macrophage transplantation as an alternative [110]. The feasibility of such approach was supported by the discovery that bone-marrow-derived macrophages (BMDM) [111] or mac-rophages from other tissue (e.g. peritoneal macrophage) [112] could differentiate into AM when transplanted in the lung environment. Moreover, AM can self-maintain in the lung for several years as shown by the presence of donors AM in transplanted human lungs two years after transplantation [113]. In this article Suzuki., *et al.* using the CSF2RB KO model showed that a single pulmonary transplantation (intratracheal instillation) of two million WT BMDM was able to cure permanently experimental hPAP as shown by an increased survival of the transplanted mice and the normalization of disease activity markers (e.g. BALF turbidity, SFCT-B and GM-CSF concentrations) two months and one year post-transplantation [110]. Consistent with a self-maintenance of AM, the authors showed that the transplanted BMDM was still present in the lung one year after transplantation. Moreover using a lentiviral-mediated genetic correction, the authors

also showed that transplantation of autologous corrected BMDM from the CSF2RB KO model was an effective therapy in experimental hPAP [110].

Concomitantly, these results were confirmed by a second team in Germany, showing that single lung transplantation of 2 x 10^6 BMDM in CSF2RB KO mice was able to reduce alveolar proteinosis and improve lung function in this model of hPAP [114]. They also used a humanised rodent model of hPAP (huPAP) based on the deletion of RAG2 and IL2RG genes associated with the targeted replacement of the mouse GM-CSF and IL-3 genes by their human counterpart [115]. As a result of GM-CSF signalling disruption, these mice develop hPAP, and their absence of lymphoid cells due to RAG2 and IL-2RG deletion allows the transplantation of human cells [115]. Using the humanized hPAP mice, they showed that lung transplantation therapy using human CD34+-derived macrophage progenitors was associated with the long-term engraftment and AM differentiation of the progenitors cells as well as with an improvement of hPAP markers (i.e. decrease BALF, turbidity, protein and GM-CSF concentration) up to 6.5 months post-transplantation [114].

In the last decade, the invention of inducible pluripotent stem cells (iPSC) by Takahashi., *et al.* paved the way for personalised medicine by offering the ability to generate a large quantity of almost any patients-derived cells types using easily accessible differentiated cells such as skin or blood cells [116]. Since then, many teams published protocols for the generation of mouse [117] and human [117-119] iPSC-derived macrophages, and some research teams decided to use them as a therapeutic tool in experimental hPAP. Earlier this year Mucci., *et al.* showed that single lung transplantation of 2.5 to 4 x 10^6 WT mouse iPSC-derived macrophages in the lung of CSF2RB KO mice was associated with their long-term engraftment, differentiation into AM and the resolution of hPAP [120].

Instead of using iPSC-induced macrophages from allogeneic donors, Lachmann., *et al.* proved that genetic rescuing of GM-CSF receptor deficiency in human, using lentiviral-mediated gene transfer was possible [121]. Using CD34+ bone-marrow cells from a CSF2RA-deficient patient, they generated inducible Pluripotent Stem Cells (hPAP-derived iPSC). These cells were after that transduced with a self-inactivating lentiviral vector construct containing a codon-optimised CSF2RA cDNA or a control one. The transduced hPAP-derived iPSC were after that treated to induce successively hematopoietic, myeloid, monocytic and macrophage differentiation [121]. The authors showed that monocytes and macrophages derived from hPAP-derived iPSC recapitulate the dysfunctions observed in macrophages from patients with hPAP and that genetic correction of CSF2RA mutation in hPAP-derived iPSC resulted in fully functional monocytes and macrophages as assessed by GM-CSF uptake, activation of STAT5, and phagocytic activity [121].

Earlier this year, Happle., *et al.* using the humanised mouse model of hPAP showed the effectiveness of human iPSC-derived macrophage pulmonary transplantation therapy [122]. Similar to what was observed by Suzuki., *et al.* with mouse BMDM, the authors found that human iPSC-derived macrophages pulmonary transplantation resulted in long-term engraftment and differentiation in AM as assessed by gene expression. They also showed that human iPSC-derived macrophages pulmonary transplantation resulted in an improvement of experimental hPAP disease activity parameters such as BALF turbidity, protein and SFCT-D concentrations [122].
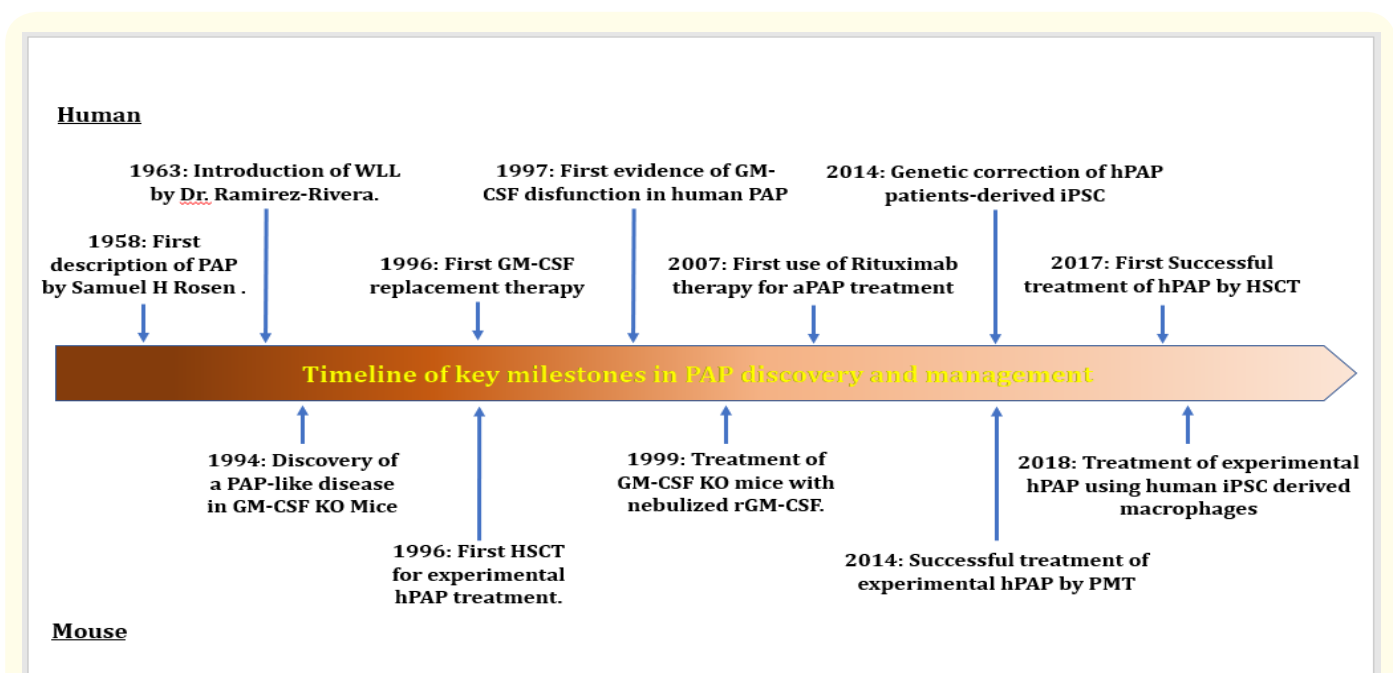
Figure 1: Timeline of the key milestones in PAP discovery and management. PMT: Pulmonary Macrophage Transplantation Therapy

hPAP therapy: The way forward

One of the factors preventing the use of human iPSC-derived macrophages pulmonary transplantation therapy in the clinical setting is the risk of reactivation of genes used to induce pluripotency (i.e. Oct3/4, Sox2, c-Myc, and Klf4) that could cause tumorigenesis *in vivo* [123]. Therefore in recent years, researchers developed a method allowing the generation of iPSC with a minimal number of transcription factors. Thus Nakagawa., *et al.* published a method of iPSC generation using mutated form of the proto-oncogene c-Myc or another member of the Myc family named L-Myc, both of which have reduced transformation properties [124]. More recently, Montserrat., *et al.* reported the generation of iPSC in primary renal tubular epithelial cells using the transfection of only two defined factors (i.e. Oct4 and Sox2) [125].

On top of the risk of tumorigenicity, the risk of insertional mutagenesis associated with the use of lentiviral vectors for gene correction is also a factor preventing the use of iPSC in clinics [126]. As an alternative to lentiviral transduction for CSF2RA gene correction Kuhn., *et al.* chosen a gene editing approach based on the use of the restriction enzyme, transcription activator-like effector nucleases (TALENs) [127]. To prevent insertional mutagenesis during gene therapy, scientists are strategically directing the transgene integration into 'genomic safe harbours'. These genomic safe harbours are - chromosomal locations where therapeutic transgenes can integrate and function predictably without perturbing endogenous gene activity or promoting cancer [128]. Using TALENs, Kuhn., *et al.* achieved functional correction of hPAP patients-derived iPSC by inserting a codon-optimised CSF2RA-cDNA in the genomic safe harbours locus AAVS1 [127]. The corrected hPAP patients-derived iPSC were after that differentiated into macrophages resulting in the generation of macrophages with functional GM-CSF signalling, as shown by their expression of CSF2RA (CD113), their capacity to bind GM-CSF and activate STAT5 [127]. Recently Hetzel., *et al.* developed a self-inactivating lentiviral vector expressing a codon-optimised human CSF2RA-cDNA driven from EF1a short promoter [129]. Moreover, they performed a series of nonclinical efficacy and safety studies in macrophages cell lines and primary human cells to validate their self-inactivating vector and showed that it did not adversely affect CD34$^+$ cells proliferation or differentiation program [129].

In recent years many DNA-free reprograming techniques allowing integration-free iPSC generation have been published (e.g. Sendai virus, recombinant proteins, microRNAs, synthetic messenger RNA and small molecules) [130]. In particular, Wen., *et al.* published a method allowing integration-free iPSCs generation from human adult peripheral blood mononuclear cells using an optimal combination of episomal vectors [131].

As an alternative to the use of corrected iPSC, the direct delivery of optimised cDNA encoding CSF2RA or B to hPAP patients AM to restore GM-CSF signalling could be a promising perspective. In recent years liposome-based particles have been used to deliver drugs such as rifampicin [132,133] or nucleic acids [133-135] to monocytes and macrophages. Moreover, liposome designed to deliver drugs specifically to AM through nebulization have been successfully tested opening the door to new therapeutic opportunity in hPAP treatment [132].

Concluding Remarks

Since its description in 1963 by Dr Jose Ramirez-Rivera, WLL has been the leading therapeutic option in the management of PAP. Despite significant technical improvements of the procedure since the 1960s, WLL remain a heavy and invasive procedure, treating the symptoms rather than the cause of PAP. Since the mid-1990s the discovery of GM-CSF signalling impairment by neutralising antibodies and genetic mutations allowed the development of therapy targeting the cause of autoimmune and hereditary PAP. In this review we showed that WLL followed by aerosolised GM-CSF replacement therapy as well as rituximab administration are promising treatments for the long-term management of aPAP.

On the other hand, aerosolised GM-CSF replacement therapy and Rituximab are ineffective in the treatment of hPAP due to the mutations-driven GM-CSF receptor dysfunction. Thereby, the management of hPAP has hardly progressed in the past decades. However,

experimental therapy in mouse models is opening the door to promising therapeutic strategies for the treatment of hPAP. Last year, 21 years after the first successful treatment of experimental hPAP by HSCT, Fremond et al., reported the first successful treatment of hPAP by this therapy in human. Although successful, this procedure was associated with many complications; however, the recent publication by Suzuki et al., and Happle et al., of the long-term treatment of experimental hPAP by lung targeted macrophage transplantation therapy is opening the door to new therapeutic avenues in hPAP. The generation of patients iPSC- or CD34+- derived corrected macrophages have been essential steps in this direction. Although there are still safety concerns associated with the generation of iPSC and genetically engineered cells, the recent development of safer methods should allow the successful long-term treatment of hPAP in the near future.

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