AN OFFICIAL JOURNAL OF
THE AMERICAN COLLEGE OF
RHEUMATOLOGY

Adeno-associated virus type 5 infection via PDGFR□ is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Journal:	*Arthritis & Rheumatology*
Manuscript ID	ar-23-0151.R1
Wiley - Manuscript type:	Full Length
Date Submitted by the Author:	18-Aug-2023

Complete List of Authors:	Moroncini, Gianluca; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica; AOU Ospedali Riuniti di Ancona, Medicina Interna Svegliati, Silvia; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica Grieco, Antonella; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica Cuccioloni, Massimiliano; Università di Camerino, Scuola di Bioscienze e Medicina Veterinaria Mozzicafreddo, Matteo; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica Paolini, Chiara; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica Agarbati, Silvia; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica Spadoni, Tatiana; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica Amoresano, Angela; University of Naples Federico II, Scienze Chimiche Pinto, Gabriella; University of Naples Federico II, Scienze Chimiche Chen, Qingxin; German Cancer Research Center, Tumorvirus-specific vaccination Benfaremo, Devis; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica; AOU Ospedali Riuniti di Ancona, Medicina Interna Tonnini, Cecilia; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica Senzacqua, Martina; Polytechnic University of Marche, Medicina Sperimentale e Clinica Alizzi, Silvia; Azienda Ospedaliero Universitaria Citta della Salute e della Scienza di Torino, Immunogenetica e Biologia dei Trapianti Nieto, Karen; German Cancer Research Center, Tumorvirus-specific vaccination Finke, Doreen; Polytechnic University of Marche, Scienze Cliniche e Molecolari - Clinica Medica Viola, Nadia; AOU Ospedali Riuniti di Ancona, Medicina Interna

Amico, Donatella; Azienda Ospedaliera Ospedali Riuniti Marche Nord
Galgani, Mario; University of Naples Federico II, Medicina Molecolare e Biotecnologie Mediche
Gasparini, Stefano; Polytechnic University of Marche, Scienze Biomediche e Sanità Pubblica
Zuccatosta, Lina; AOU Ospedali Riuniti di Ancona, Medicina Interna
Menzo, Stefano; Polytechnic University of Marche, Scienze Biomediche e Sanità Pubblica
Mueller, Martin; German Cancer Research Center, Tumorvirus-specific vaccination
Kleinschmidt, Jurgen; German Cancer Research Center, Research Program Infection and Cancer
Funaro, Ada; Università degli Studi di Torino, Dipartimento di Scienze Mediche
Giordano, Antonio; Polytechnic University of Marche, Dipartimento di Medicina Sperimentale e Clinica
La Cava, Antonio; University of California Los Angeles, Medicine
Dorfmuller, Peter; University Hospitals Giessen and Marburg Campus Giessen, Pathology
Amoroso, Antonio; Azienda Ospedaliero Universitaria Citta della Salute e della Scienza di Torino, Immunogenetica e Biologia dei Trapianti
Pucci, Piero; University of Naples Federico II, Scienze Chimiche
Pezone, Antonio; Università degli Studi di Napoli Federico II Facoltà di Scienze Biotecnologiche
Avvedimento, Enrico; University of Naples Federico II, Medicina Molecolare e Biotecnologie Mediche
Gabrielli, Armando ; Polytechnic University of Marche, Fondazione di Medicina Molecolare e Terapia Cellulare

Keywords: Systemic sclerosis, Scleroderma, Adeno-associated virus, Platelet-derived growth factor receptor α, Autoantibody(ies)

<B>Category: Please select the category from the list below that best describes the content of your manuscript.: Systemic Sclerosis

SCHOLARONE™
Manuscripts

Running head: AAV5 in systemic sclerosis

Adeno-associated virus type 5 infection via PDGFRα is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Gianluca Moroncini MD, PhD[1,2†], Silvia Svegliati PhD[1†], Antonella Grieco PhD [1†], Massimiliano Cuccioloni PhD[4], Matteo Mozzicafreddo PhD[4], Chiara Paolini PhD[1], Silvia Agarbati PhD[1], Tatiana Spadoni PhD[1], Angela Amoresano PhD[5], Gabriella Pinto PhD[5], Qingxin Chen PhD[6], Devis Benfaremo MD[1,2], Cecilia Tonnini BS[1], Martina Senzacqua PhD[7], Silvia Alizzi MD[8], Karen Nieto PhD[6], Doreen Finke PhD[1], Nadia Viola BS[10], Donatella Amico MD, PhD[11], Mario Galgani MD[12], Stefano Gasparini MD[2,13],Lina Zuccatosta MD[2], Stefano Menzo MD[2,13], Martin Müller PhD6 Juergen Kleinschmidt PhD[6], Ada Funaro PhD[8], Antonio Giordano MD[7], Antonio La Cava MD, PhD[14], Peter Dorfmuller MD, PhD[15], Antonio Amoroso MD [8,9], Piero Pucci PhD [5], Antonio Pezone [16], Enrico Vittorio Avvedimento MD, PhD[12*], Armando Gabrielli MD[1,2,3*].

1, Dipartimento di Scienze Cliniche e Molecolari, Clinica Medica, Università Politecnica delle Marche, Ancona, Italy;

2. Azienda Ospedaliero-Universitaria delle Marche, Ancona, Italy

3. Hiller Research Center, University Hospital Düsseldorf, Heinrich Heine University, Düsseldorf, Germany

4. Scuola di BioScienze e Medicina Veterinaria, Università di Camerino, Camerino, Italy

5. Dipartimento di Scienze Chimiche, Federico II University Napoli, Italy

6.Tumorvirus-specific Vaccination (F035), German Cancer Research Center (DKFZ)
Im Neuenheimer Feld 242(ATV), Heidelberg Germany

7. Dipartimento di Medicina Sperimentale e Clinica, Università Politecnica delle Marche. Ancona Italy

8. Dipartimento di Scienze Mediche, Università di Torino, Torino, Italy

9. Servizio di Immunogenetica e Biologia dei Trapianti, Azienda Ospedaliera Universitaria Città della Salute e della Scienza, di Torino, Torino - Italy

10. Servizio di Immunologia Clinica, Ospedali Riuniti, Ancona, Italy.

11. Unità di Pneumologia, "Ospedali Riuniti Marche Nord", Pesaro, Italy

12. Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Federico II University, Napoli, Italy;

For Peer Review

13. Dipartimento di Scienze Biomediche e Sanità Pubblica, Università Politecnica delle Marche. Ancona Italy;

14 Department of Medicine, University of California Los Angeles, Los Angeles, USA.

15 University Hospital Giessen and Marburg, Pathology Department, University of Giessen and Marburg Lung Center (UGMLC), German Center of Lung Research (DZL), Giessen, Germany

16 Dipartimento di Biologia, Università Federico II, Napoli, Italy.

† Contributed equally to the work

*Corresponding Authors:

Armando Gabrielli
a.gabrielli@staff.univpm.it

and :

Enrico V. Avvedimento
avvedim@unina.it

For Peer Review

FUNDING

This work was supported by grants from Fondazione di Medicina Molecolare e Terapia Cellulare, Associazione Italiana lotta alla Sclerodermia (AILS) Gruppo Italiano per la Lotta alla Sclerodermia (GILS) and by Dr. Andrea and Diego Della Valle.

COMPETING INTERESTS

The authors declare no competing interests.

ABSTRACT

Objectives

The etiopathogenesis of systemic sclerosis (SSc) is unknown. Platelet-derived growth factor receptors (PDGFRs) are overexpressed in SSc patients. Since PDGFRα is targeted of the adeno-associated virus type 5 (AAV5), we investigated whether AAV5 forms a complex with PDGFRα exposing epitopes that may induce the immune responses to the virus-PDGFRα complex.

Methods

The binding of monomeric human PDGFRα to the AAV5 capsid was analyzed by *in silico* molecular docking, surface plasmon resonance (SPR), and genome editing of the PDGFRα locus. AAV5 was detected in SSc lungs by *in situ* hybridization, immunohistochemistry, confocal microscopy, and molecular analysis of bronchoalveolar lavage (BAL). Immune responses to AAV5 and PDGFRα were evaluated by SPR using SSc monoclonal anti-PDGFRα antibodies and Immunoaffinity-purified anti-PDGFRα antibodies from sera of SSc patients.

Results

AAV5 was detected in the BAL of 41 out of 66 (62.1%) SSc patients with interstitial lung disease and in 17 of 66 controls (25.75 %; p<0.001). In SSc lungs, AAV5 localized in type II pneumocytes and in interstitial cells. A molecular complex formed of spatially contiguous epitopes of the AAV5 capsid and PDGFRα was identified and characterized. *In silico*

For Peer Review

molecular docking analysis and binding to the agonistic anti-PDGFRα antibodies identified spatially contiguous epitopes derived from PDGFRα and AAV5 that interacted with SSc agonistic antibodies to PDGFRα. These peptides were also able to bind total IgG isolated from SSc patients, not from healthy controls.

Conclusions

These data link AVV5 with the immune reactivity to endogenous antigens in SSc, and provide a novel element in the pathogenesis of SSc.

For Peer Review

Keywords: Systemic sclerosis, scleroderma, adeno-associated virus, PDGFRα, autoimmune disease, autoimmunity

INTRODUCTION

Systemic sclerosis (SSc, scleroderma) is an autoimmune, multisystem disorder of unknown etiology and unclear pathogenesis. It is characterised by microvasculature damage, circulating autoantibodies and fibroblast activation that lead to fibrosis of the skin and visceral organs (1,2). SSc is a major cause of disability and carries high morbidity and mortality associated with the fibrotic and microvascular alterations (3,4).

It has been suggested that SSc can be triggered by viruses (5), and the interferon type I gene expression signature is frequently observed in SSc patients (6-8). However, the search for putative disease-promoting viruses in SSc has so far been inconclusive.

Platelet-derived growth factor receptor (PDGFR) signalling, directly or indirectly, is involved in SSc (9). PDGFRs are the targets of several viruses (10-13) and are typically overexpressed in SSc (14). We also reported the presence of stimulatory anti PDGFRα antibodies in the serum of SSc patients (15), and we cloned agonistic anti PDGFRα autoantibodies from SSc patients (16).

Anti PDGFRα autoantibodies are of particular interest for the following reasons:

i) they induced a SSc -like phenotype in normal human fibroblasts (15); ii) they induced fibrosis ex vivo (17) and iii) Stimulated the proliferation and migration of human pulmonary artery smooth muscle cells (suggesting a role in the formation of the neointima which is the cardinal feature of the vascular abnormalities in SSc) (18); and iv) they triggered a well-characterised signalling pathway leading to fibrosis (19). Furthermore, these antibodies

For Peer Review

selectively recognised specific domains in the receptor. For example, antibodies targeting extracellular domain I of the receptor were biologically inactive, whereas those recognising the extracellular domain II were stimulatory in inducing fibrosis and redox stress (16).

It has to be noted that the detection of these antibodies is not trivial because they cannot be detected by non-functional bioassays (16, 20, 21), underscoring their functional role. Moreover, it has to be considered that these antibodies can have conformational epitopes that are distant in the primary sequence but contiguous in the tertiary structure of the receptor on the cell surface (16). Interestingly, adeno-associated virus type 5 (AAV5) is known to enter several cell types through PDGFRα (22-24), making it a candidate worth studying in the induction of anti-PDGFRα in SSc.

AAV5 is a non-enveloped, single-stranded DNA adeno-associated virus that belongs to the genus Dependoparvovirus within the Parvoviridae family (25); it requires a helper virus, such as adenovirus or herpes simplex virus, or cellular stress, to replicate (26). To date, 13 serotypes of AAV have been identified (27). AAV gain access to specific cell surface receptors after attaching to glycans or glycoconjugates on the cell membrane (28). The current knowledge is that AAVs do not cause any human diseases and have been used for in vivo therapeutic gene delivery (29).

Building on these considerations we hypothesised that AAV5 may recognise and bind specific domains in the receptor and may target the immune response to the receptor and the viral capsid.

For Peer Review

MATERIALS AND METHODS

Patients

Sixty-six consecutive Caucasian patients with ILD-SSc were studied. All SSc patients fulfilled the EULAR/ACR preliminary criteria for the classification of SSc (30). The patients were classified into subgroups according to whether they had diffuse cutaneous scleroderma (dcSSc) or limited cutaneous scleroderma (lcSSc) (31) and then were divided into those with early (<3 years for dcSSc and <5 years for lcSSc) or late disease (>6 years for dcSSc and >10 years for lcSSc) from the first non-Raynaud's phenomenon symptom (32). All patients had not received any immunosuppressive treatment during the previous six weeks. The presence of ILD was confirmed by X-Ray and/or HRCT. The control group (C) included 66 patients who underwent BAL for lung involvement other than SSc ILD, and, in selected *in vitro*, experiments, healthy subjects (HC). The protocol with patients' information and consent forms were approved by the Ethics Committee of the Università Politecnica delle Marche (# 2017-518). The study was conducted in accordance with the Declaration of Helsinki, 5th edition (2000). Written informed consent was obtained from all patients.

For Peer Review

Molecular docking analysis

The three-dimensional structure of AAV5 envelope subunit (33) was obtained from the Protein Data Bank. Human PDGFRα and anti-PDGFRα V_HPAM-V_κ16F4, a stimulatory human anti-PDGFRα monoclonal autoantibody cloned from B cells of a SSc patient, were homology-modelled as previously reported (16).

The binding partners were uploaded on the ClusPro server (34) and the amino acids involved in the interaction between AAV5 and monomeric PDGFRα and between the predicted AAV5/PDGFRα complex and V_HPAM-V_κ16F4 were identified using PyMol software (PyMOL Molecular Graphics System, Version 2.1). Energy-based docking between trans-peptides, minimized using GROMACS (version 2021.5), and V_HPAM-V_κ16F4 was performed using SwissDock server (35).

Other methods are reported in the *Supplementary Methods* section.

Statistical analyses

The two-sample test for equality of proportions was used to analyze the results of the BAL study, presented as percentages of patients tested positive for AAV5 DNA, and for the analysis of double positive subjects for anti-PDGFRα and anti-AAV5 antibodies. Significance of the differences in AAV5 transduction in wt and PDGFRα-knockout A549 cell line was determined by the Student's *t*-test and the Mann-Whitney test for the analysis of the quantitative PCR results and cytofluorimetric data. Data are expressed as mean ± SD or median value and a range. Data were analyzed using Prism software (Graph-Pad). All reported *P* values are two-sided. *P* values <0.05 were considered statistically significant.

For Peer Review

RESULTS

AAV5 in the lungs of SSc patients with interstitial lung disease

Since the lung is the main disease target in SSc and is responsible for high morbidity and mortality (37,38), we investigated the possibility that it might be affected by the presence of AAV5-infected cells. Immunohistochemistry was performed on frozen lung tissues from five SSc patients and six control lung specimens (from lobectomy or pneumonectomy for localised lung cancer), and the results were confirmed by chromogenic *in situ* hybridization (CISH) on paraffin-embedded lung sections from two patients and two controls (online supplemental table S1). A representative experiment is shown in Figure 1. All samples were analysed in a blinded fashion by two independent investigators (AG and MS). In SSc lungs of five SSc patients, but not in controls, AAV5 was detected mainly in cells lining the alveolar space - many of them with the cytological appearance of type II pneumocytes (figure 1A,B) - and, to a lesser extent, in interstitial macrophage-like cells. In lung tissue of patients with lung cancer used as controls, AAV5 immunoreactivity was only found in interstitial cells. Positivity was found in two other more controls, one affected by primary arterial hypertension (PAH) with fibrotic remodeling and pneumocytic hyperplasia, and the other by nonspecific interstitial pneumonia with extensive interstitial fibrosis (data not shown). Using prosurfactant protein C (pro-SP-C) and TTF 1 as markers of type II pneumocytes (39,40), we found by double-staining and confocal microscopy showed that a consistent number of proSP-C- or TTF1-positive type II pneumocytes were variably positive for AAV5 (17 ± 6.2% and 11± 4.5% respectively; figure 1C and online supplemental figure S1). Of note, AAV5-positive type II pneumocytes expressed PDGFRα, mainly at the apical segment of the cells, and facing the alveolar space (figure 1D). The specificity of the signals was suggested by the strong positivity for PDGFRα -and not for AAV5- of numerous fibroblast-like cells in the

For Peer Review

interstitial space strongly positive for (figure 1D). Extensive quantification of multiple confocal microscopy images of SSc lung sections (n=14), as described in the online supplementary methods, indicated positivity for AAV5 capsid (16 ± 5.1%,) and PDGFRα (22 ± 5.5%), with an overlay percentage of 10.69 ± 4.4% (online supplemental figure S 2A and S 2B; online supplemental table S 2). Interestingly, 63.77 ± 10% of the AAV5 signal overlapped with the PDGFRα signal, while only 46.49 ± 9.8% of the PDGFRα signal overlapped with the AAV5 signal **(**online supplemental figure S2C). A strong linear correlation between the two signals was observed (R2=0.97, online supplemental figure S 2D).

Of note, colocalization of AAV5 and PDGFRα was also observed by confocal microscopy in peripheral blood mononuclear cells(PBMC) from SSc patients (online supplemental figure S3). Quantification of multiple confocal microscopy images of SSc PBMC (n=11) showed an AAV5 capsid positivity of 11.85 ± 3.4%, and a PDGFRα positivity of 21.5 ± 2.67%, with an overlay percentage of 6.05 ± 2% (online supplemental figure S 4A and S 4B online supplemental table S3). Notably, 52.2 ± 10.9% of the AAV5 signal overlapped with the PDGFRα signal, whereas only 28.4 ± 9.1% of the PDGFRα signal overlapped with the AAV5 signal (online supplemental figure S 4C). Also in this case, the two signals had a strong linear correlation (R2=0.93, online supplemental figure S 4D).

Taken together, the above data indicate that: i) immunostaining for the AAV5 capsid correlates with PDGFRα expression in both lung sections and PBMC of SSc patients; ii) type II pneumocytes coexpress PDGFRα and AAV5 positivity; iii) not all PDGFRα-expressing cells are also positive for the AAV5 capsid.

AAV5 in BAL from SSc patients

For Peer Review

The presence of AAV5 DNA was investigated in the bronchoalveolar lavage (BAL) from 66 SSc patients (53 females and 13 males; mean age 56.9 ± 15.2 years) with interstitial lung disease and 66 control patients with lung disease other than SSc (38 females and 28 males; mean age 62.3 ± 15.1 years). Thirty-seven (56%) SSc patients had the limited cutaneous form of SSc and 29 (44%) had the diffuse cutaneous form. The lung disorders different from SSc in controls are reported in the online supplemental table S4. The results showed positivity for AAV5 DNA in the BAL from 41 SSc patients (62.1%; 95% C.I.: 49.3-73.8%), and 17 controls (25.75%; 95% C.I: 15.8-38.0%) ($p<0.001$) (figure 2A). It is worth mentioning that seven additional SSc patients lacking viral DNA in the BAL had positivity for AAV5 DNA in their PBMCs (online supplemental figure S 3), for a totality of 48 positive patients (72.7%). The only feature distinguishing SSc patients with AAV5-positive BAL from those with AAV5-negative BAL was disease duration (SSc AAV5 positive: 5.3 ± 0.9 years vs. SSc AAV5 negative: 2.4 ± 0.8 years; $p<0.05$) (online supplemental table S5). Quantitatively the AAV5 DNA content was comparable between the BAL from SSc patients and the controls who tested positive for AAV5 ($p=0.191$) (figure 2B). To rule out the possibility that a mutated AAV5 could have accounted for the virus detection in the SSc lungs, we verified the complete nucleotide sequence of the viral *cap* gene in the BAL of three patients and three controls they were identical to the prototypical European AAV5 prototype (acc. n° Y18065.1) in both groups.

The AAV5 capsid binds human PDGFRα

The interaction between the AAV5 capsid and PDGFRα was studied through *in silico* molecular docking using the homology modelled three-dimensional structures of human monomeric PDGFRα (16) and the crystal structure of the AAV5 capsid monomeric subunit.

For Peer Review

It was found that the predominant binding at the external surface of the capsid with the receptor occurred at a region spanning the second and third extracellular Ig-like domains of PDGFRα (figure 3A). These regions of the receptor discriminate agonistic from non-functional anti PDGFRα antibodies that bind to the receptor's domain I of the receptor (16). The AAV5-PDGFRα interacting sequences are shown in online supplemental figure S 5 A,B.

To validate the in silico model we analysed the binding of PDGFRα to purified AAV5 capsid protein by surface plasmon resonance (SPR) using histidine-tagged recombinant human monomeric PDGFRα (rhPDGFRα-His) immobilised and folded into a native-like conformation (16). The capsid protein of AAV2, a close serotype belonging to the same viral family, was tested in parallel as a control. The rhPDGFRα was found to bind AAV5 with a 370-fold higher affinity as compared to AAV2, with a faster recognition phase (higher value of k_{ass}) and a greater stability of the complex (lower value of k_{diss}) (figure 3B).

Next, functional role of human PDGFRα in AAV5 transduction was evaluated with a CRISPR/Cas9 PDGFRα knockout variant in the A549 cell line (a type II pulmonary epithelial cell line) and related control. Transduction efficiency of the AAV5 virus expressing luciferase (AAV5-FF-Luc) in A549-PDGFRα knockout cells was significantly reduced as compared to that of A549 control or cells cotransduced with AAV2-FF-Luc (figure 3 C, D).

The type I transmembrane protein KIAA0319L is a cellular receptor (AAVR) involved in the cellular entry of several AAV serotypes including AAV5 (23). The comparison of binding of the predicted sites of AAV5 to AAVR (41) and PDGFRα showed that the AAV5 capsid binding site for AAVR was distinct from that used to engage hPDGFRα (online supplemental figure S 5A, 5B). Also, when AAVR was immobilised onto the SPR device and tested for binding to the AAV5 capsid, the binding to AAV5 of AAVR (online supplemental figure S 5C) displayed an equilibrium dissociation constant in the sub-micromolar range, similar to that

For Peer Review

reported previously for the the AAV2-AAVR interaction (23). On the other hand, The interaction between AAV5 and rhPDGFRα displayed a 100-fold higher affinity, associated with a faster kinetic association phase (figure 3B).

Taken together, these data demonstrate that: i) AAV5 binds with high affinity PDGFRα in human cells; ii) identify the PDGFRα domains bound to the AAV5 capsid and iii) human PDGFRα can be the cell entry receptor for AAV5 as indicated by colocalization studies .

Immune responses to the AAV5-PDGFRα complex

For Peer Review

Next, we investigated whether the PDGFRα–AAV5 complex (figure 3A) associated with anti-PDGFRα immune responses. To this aim, we took advantage of the V_HPAM-V_κ16F4 human monoclonal stimulatory anti-PDGFRα antibody, whose epitopes have been experimentally identified and validated (16). V_HPAM-V_κ16F4 antibody, immobilised onto the SPR device to test binding to AAV5, formed a high affinity complex with AAV5 (figure 4A, left), with an equilibrium dissociation constant in the sub-micromolar range and with about four-fold higher affinity than the control VH_{PAM}-Vκ16F4-AAV2 complex (figure 4A, right). This indicates that the stimulatory antibody to PDGFRα V_HPAM-V_κ16F4 recognises also the AAV5 capsid.

Identification of spatially contiguous epitopes of AAV5 and PDGFRα recognised by SSc autoantibodies

In silico molecular docking was optimised by restricting the AAV5 capsid-PDGFRα interaction to the extracellular domain II recognised by the agonistic antibody V_HPAM-V_κ16F4 - and not by the control antibody V_HPAM-V_k13B8.

To identify the epitopes of the AAV5 capsid and PDGFRα epitopes, jointly recognised by the V_HPAM-V_κ16F4 antibody, we first evaluated *in silico* the interaction between V_HPAM-V_κ16F4, the AAV5 capsid and PDGFRα, focusing on the variable region (Fab) of the monoclonal autoantibody and the AAV5/PDGFRα complex (figure 4B). This analysis showed: that i) the PDGFRα epitope recognised by VH_{PAM}-Vκ16F4 was not included in the PDGFRα domain that interacts with AAV5; ii) the V_HPAM-V_κ16F4 anti-PDGFRα antibody recognized two spatially contiguous peptides formed by the amino acid sequences LYRF (derived from the AAV5 capsid) and PASY (derived from PDGFRα) with high affinity (K_d of 5.182 e^{-8} M). In addition, the V_HPAM-V_κ16F4 anti-PDGFRα antibody recognises other composite peptides formed by the amino acid sequence FTVG (derived from PDGFRα), SARN (K_d = 6.103 e^{-9} M) and SVSA (K_d = 3.124 e^{-9} M) peptides derived from the AAV5 capsid (online supplemental figure S6).

In all, the same PDGFRα epitopes that bound *in silico* the AAV5 capsid were recognised as well by the agonistic anti-PDGFRα antibodies using different techniques (surface plasmon resonance, conformational PDGFRα peptide library and single amino acid mutagenesis) (16).

To confirm the in silico predictions, the rhPDGFRα-coated surface of the biosensor was saturated with AAV5 before addition of the monoclonal antibody V_HPAM-V_κ16F4. The results showed the formation of a receptor-capsid ternary complex (figure 4C, curve A). To rule out that V_HPAM-V_κ16F4 binding was limited to PDGFRα, peptide sequences corresponding to the predicted AAV5 (VDQYLYRF) and PDGFRα (PASYDTFT) binding domains of V_HPAM-V_κ16F4 were synthesised and pre-incubate - alternatively or in combination - with V_HPAM-V_κ16F4 prior to the addition of the rhPDGFRα-AAV5 complex.

For Peer Review

Notably, both peptides, independently, partially inhibited V_HPAM-V_κ16F4 binding (figure 4C, curves B and C) and completely abrogated the binding of the antibody when combined (figure 4C, curve D). Similar results were obtained when V_HPAM-V_κ16F4 was replaced by the immunoaffinity-purified anti-PDGFRα serum antibodies from SSc patients (the polyclonal nature of these antibodies explains the partial inhibition of the binding to the peptides) (figure 4D). Together, these data indicate that anti-PDGFRα autoantibodies purified from the sera of SSc patients as well the cloned V_HPAM-V_κ16F4 PDGFRα autoantibody react to both human PDGFRα and the AAV5 capsomere.

For Peer Review

DISCUSSION

This study shows that: i) PDGFRα allows AAV5 internalization in cells (figure 1 and 3 C,D); ii) a high percentage (62.1%) of SSc patients with ILD harbor AAV5 in the lung; (figure 2) iii) a spatially contiguous epitope composed of a stretch of aminoacids of PDGFRα and the AAV5 capsid is recognized by antibodies *in vivo* that can target PDGFRα (figure 4).

The presence of the AAV5 in BAL of 25.75% of the controls with different lung diseases confirms the notion that AAV5 is endemic in the human population, as also indicated by the presence of anti-AAV5 neutralizing antibodies and circulating INF-γ + T cells that react to AAV5 in 30% and 24% of healthy donors, respectively (42). AAV5 is a nonpathogenic and poorly immunogenic virus. Although it does not directly participate in the pathogenesis of SSc, our data suggest that it can contributes to the generation of neoantigens, some of which can target the immune reactivity to PDGFRα.

After binding to a primary receptor, the capsid protein of AAVs interacts with a co-receptor which leads to virus internalization via endocytosis. Primary cell surface receptors for AAV5 include heparin sulfate proteoglycans, N-terminal galactose and N-or O- linked sialic acid moieties. Secondary receptors for AAV5 are PDGFRα and AAVR, as also shown here (23, 43). Our data confirm previous findings (23) that demonstrate that AAVR (the KIAA0319L transmembrane protein) is a receptor for multiple AAV serotypes including AAV5. Although in our experiments KIAA0319L displayed a lower affinity to AAV5 capsid compared to human PDGFRα (online supplemental figure S 5C), a single nucleotide polymorphism in the *KIAA0319L* locus, rs2275247, was strongly associated with SLE, scleroderma and higher expression of the receptor (44).

The fact that the KIAA0319L sequence the stretch of aminoacids recognizing the AAVR capsid is distinct from the peptide bound to human PDGFRα (online supplemental figure S

5B) suggests that the capsid can bridge a connection between PDGFRα and the KIAA0319L receptor. This trimeric complex sustained by the viral capsid may be recognized as an exogenous protein and thus targeted by immune cells.

The association between viral (exogenous) and endogenous (PDGFRα) antigens has also been implicated in other human infections. For example, CMV, a ubiquitous human herpesvirus, requires PDGFRα to induce a robust inflammatory response (10), and it has also been linked to SSc (45,46). It remains to be solved whether the heterogeneous phenotypes of SSc depend directly or indirectly on the virus involved - CMV or AAV5 or other AAV serotypes - or whether CMV is only one of the helper viruses necessary for a productive AAV5 replication.

The finding that 28.3 % of our cohort of SSc patients did not have AAV5 sequences, could be explained by an on-and-off viral replication and by the consideration that only patients with evidence of ILD underwent BAL for ethical reason. These aspects do not allow at present to formally establish whether the viral infection would occur before or after the clinical evidence of the disease.

Another finding that deserves an explanation is the longer duration of disease in patients with AAV5-positive BAL. We believe that the protracted systemic disease, characterized by increased secretion of disease-related cytokines and growth factors (47), stimulated the expression of PDGFRα in the lungs, increasing chances of local infection or reactivation of AAV5. Incidentally, the virus might also be present in other locations, driving as well the production of antibodies in AAV5-negative BAL subjects as shown by 7 SSc patients without AAV5 in the BAL, but positive for the virus in the peripheral blood.

We propose that high expression of PDGFRα may facilitate AAV5 infection and (re)activation, possibly with helper viruses co-infections, and this, wherever occurring, can

induce, in genetically susceptible individuals, self-reactive immune responses to PDGFRα epitopes physically associated with the virus capsid and AAVR (as indicated by the co-presence of anti PDGFRα and anti AAV5 antibodies; online supplemental figure S7). Of note, the anti-PDGFRα antibodies recognizing the II domain of the receptor (the activation loop physically linked to the capsid; ref.16) are biologically active, present at high levels in patients with early disease (15), and capable to induce the fibrotic phenotype in SSc patients (15-19). However, we cannot rule out that AAV5 can contribute to the development of fibrosis by directly activating mesenchymal-like interstitial cells (figure 1).

The remarkable finding here is also the identification of non-linear epitopes spanning the sequences of two cell receptors, PDGFRα, KIAA0319L, and the AVV5 capsid. The spatial organization of PDGFRα sequences, exposing its II activating domain in this trimeric complex explains the generation of stimulating autoantibodies of PDGFRα. These antibodies isolated from SSc patients have been cloned and functionally tested *in vivo* and *in vitro*. They induce ROS and fibrosis in vivo (17) and in vitro (19) through the PDGFRα activation (16). Our data imply that SSc antibodies recognize a neoantigen composed of two peptides derived from two different proteins, the AAV5 capsid and PDGFRα (figure 4A, C and D). The structure of the complex is better defined by *in silico* modeling (figure 4B) which shows that the AAV5 peptide interacts with the second extracellular domain of PDGFRα in proximity to PDGFRα epitopes targeted by anti- PDGFRα autoantibodies (Fig. 4B).

We acknowledge two limitations of this study: i) the limited number of controls and patients that prevents a conclusive subgroup analysis; ii) the impossibility, for ethical reasons, to perform BAL in patients without HRCT evidence of ILD.

While our results do not indicate that AAV5 is the cause of scleroderma, they suggest that AAV5 favors the formation and presentation of a peculiar and composite peptides complex containing epitopes from different proteins, the PDGFRα, AAVR, and the viral capsid, which stabilizes this complex. Due to the physical proximity of the capsid sequences to cell proteins, PDGFRα or KIAA0319L, it is likely that, immune cells primarily recognize the abundant capsid segment and inefficiently the endogenous cellular proteins (PDGFRα or KIAA0319L) associated with the capsid, probably due to high levels of the capsid. Reduction of the capsid peptides levels with time would increase the visibility of the trimeric complex (PDGFRα-capsid-KIAA0319L) which is recognized as viral antigen by immune cells and consequently enhances the immune response. The appearance of agonistic anti PDGFRα antibodies in genetically susceptible individuals is the result of the trimeric complex which exposes the second domain of the receptor (figure 4B) which is necessary for its activation (16). This scenario also could explain why AAV5-positive BAL is associated with longer disease duration.

For Peer Review

This model excludes molecular mimicry of viral and endogenous proteins as an underlying mechanism inducing autoimmunity. Instead, as shown in Fig.4B, the formation of a single unique complex formed by viral and endogenous protein epitopes would facilitate the emergence of a reactive epitopes.

Spatial association of different and non-contiguous epitopes maintained by viral protein(s) has been suggested by other autoimmune diseases. For example, It has recently been reported that the Epstein Barr virus (EBV) infection dramatically increases the odd of developing multiple sclerosis (48), and while this has been attributed to molecular mimicry between EBV and a glial protein (glialCAM) that is the target of autoantibodies (49,50), it can be also speculated that the GLIACAM peptide(s) might be able to form a complex with viral proteins and the entry receptor (Ephs and Eph-receptor binding proteins, Ephrins) (51).

To conclude, the results from the study presented here point to a new understanding of some aspects of the pathogenesis of SSc and autoimmune diseases that could have diagnostic and therapeutic implications to assess with subsequent work.

REFERENCES

1. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma [review]. N Engl J Med 2009;360(19):1989-2003. DOI: 10.1056/NEJMoa052955

2. Denton CP, Khanna D. Systemic sclerosis [review]. Lancet 2017;390(10103):1685-99. DOI: 10.1016/S0140-6736(17)30933-9

3. Elhai M, Meune C, Boubaya M, et al. Mapping and predicting mortality from systemic sclerosis. Ann Rheum Dis 2017;76(11):1897-905. DOI: 10.1136/annrheumdis-2017-211448

4. Park EH, Strand V, Oh YJ, et al. Health-related quality of life in systemic sclerosis compared with other rheumatic diseases: a cross-sectional study [clinical trial]. Arthritis Res Ther 2019;21(1):61. DOI: 10.1186/s13075-019-1842-x

5. Moroncini G, Mori S, Tonnini C, et al. Role of viral infections in the etiopathogenesis of systemic sclerosis [review]. Clin Exp Rheumatol 2013;31(2 Suppl 76):3-7. Epub 2013 Jul 22. PMID: 23910606

6. Assassi S, Mayes MD, Arnett FC, et al. Systemic sclerosis and lupus: points in an interferon-mediated continuum. Arthritis Rheum 2010;62(2):589-98. DOI: 10.1002/art.27224

7. Brkic Z, van Bon L, Cossu M, et al. The interferon type I signature is present in systemic sclerosis before overt fibrosis and might contribute to its pathogenesis through high BAFF gene expression and high collagen synthesis. Ann Rheum Dis 2016;75(8):1567-73. DOI: 10.1136/annrheumdis-2015-207392

8. Valenzi E, Tabib T, Papazoglou A, et al. Disparate Interferon Signaling and Shared Aberrant Basaloid Cells in Single-Cell Profiling of Idiopathic Pulmonary Fibrosis and

For Review

Systemic Sclerosis-Associated Interstitial Lung Disease. Front Immunol 2021;12:595811. DOI: 10.3389/fimmu.2021.595811

9. Mendoza FA, Piera-Velazquez S, Jimenez SA. Tyrosine kinases in the pathogenesis of tissue fibrosis in systemic sclerosis and potential therapeutic role of their inhibition [review]. Transl Res 2021;231:139-58. DOI: 10.1016/j.trsl.2021.01.001

10. Soroceanu L, Akhavan A, Cobbs CS. Platelet-derived growth factor-alpha receptor activation is required for human cytomegalovirus infection. Nature 2008;455(7211):391-5. DOI: 10.1038/nature07209

11. Cavallin LE, Ma Q, Naipauer J, et al. KSHV-induced ligand mediated activation of PDGF receptor-alpha drives Kaposi's sarcomagenesis. PLoS Pathog 2018;14(7):e1007175. DOI: 10.1371/journal.ppat.1007175

12. Vrijens P, Noppen S, Boogaerts T, et al. Influenza virus entry via the GM3 ganglioside-mediated platelet-derived growth factor receptor beta signalling pathway. J Gen Virol 2019;100(4):583-601. DOI: 10.1099/jgv.0.001235

13. Karabadzhak AG, Petti LM, Barrera FN, et al. Two transmembrane dimers of the bovine papillomavirus E5 oncoprotein clamp the PDGF beta receptor in an active dimeric conformation. Proc Natl Acad Sci 2017;114(35):E7262-E71. DOI: 10.1073/pnas.1705622114

14. Daoussis D, Tsamandas AC, Liossis SN, et al. B-cell depletion therapy in patients with diffuse systemic sclerosis associates with a significant decrease in PDGFR expression and activation in spindle-like cells in the skin [clinical trial]. Arthritis Res Ther 2012;14(3):R145. DOI: 10.1186/ar3879

For Peer Review

15. Svegliati Baroni S, Santillo M, Bevilacqua F, et al. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. N Engl J Med 2006;354(25):2667-76. DOI: 10.1056/NEJMoa052955

16. Moroncini G, Grieco A, Nacci G, et al. Epitope Specificity Determines Pathogenicity and Detectability of Anti-Platelet-Derived Growth Factor Receptor alpha Autoantibodies in Systemic Sclerosis. Arthritis Rheumatol 2015;67(7):1891-903. DOI: 10.1002/art.39125

17. Luchetti MM, Moroncini G, Jose Escamez M, et al. Induction of Scleroderma Fibrosis in Skin-Humanized Mice by Administration of Anti-Platelet-Derived Growth Factor Receptor Agonistic Autoantibodies. Arthritis Rheumatol 2016;68(9):2263-73. DOI: 10.1002/art.39728

18. Svegliati S, Amico D, Spadoni T, et al. Agonistic Anti-PDGF Receptor Autoantibodies from Patients with Systemic Sclerosis Impact Human Pulmonary Artery Smooth Muscle Cells Function In Vitro. Front Immunol 2017;8:75. DOI: 10.3389/fimmu.2017.00075

19. Svegliati S, Marrone G, Pezone A, et al. Oxidative DNA damage induces the ATM-mediated transcriptional suppression of the Wnt inhibitor WIF-1 in systemic sclerosis and fibrosis. Sci Signal 2014;7(341):ra84. DOI: 10.1126/scisignal.2004592

20. Balada E, Simeon-Aznar CP, Ordi-Ros J, et al. Anti-PDGFR-alpha antibodies measured by non-bioactivity assays are not specific for systemic sclerosis. Ann Rheum Dis 2008;67(7):1027-9. DOI: 10.1136/ard.2007.085480

21. Classen JF, Henrohn D, Rorsman F, et al. Lack of evidence of stimulatory autoantibodies to platelet-derived growth factor receptor in patients with systemic sclerosis. Arthritis Rheum 2009;60(4):1137-44. DOI: 10.1002/art.24381

22. Di Pasquale G, Davidson BL, Stein CS, et al. Identification of PDGFR as a receptor for AAV-5 transduction. Nat Med 2003;9(10):1306-12. DOI: 10.1038/nm929

For Peer Review

23. Pillay S, Meyer NL, Puschnik AS, et al. An essential receptor for adeno-associated virus infection. Nature 2016;530(7588):108-12. DOI: 10.1038/nature16465

24. Pilz IH, Di Pasquale G, Rzadzinska A, et al. Mutation in the platelet-derived growth factor receptor alpha inhibits adeno-associated virus type 5 transduction. Virology 2012;428(1):58-63. DOI: 10.1016/j.virol.2012.03.004

25. Berns K, Parrish CR. Parvoviridae. In: *Fields virology*. Knipe DM, Howley PM, Eds.: Lippincott Williams & Wilkins, Philadelphia, PA, ed. 5, 2007, pp. 2437–77.

26. Geoffroy MC, Salvetti A. Helper functions required for wild type and recombinant adeno-associated virus growth [review]. Curr Gene Ther 2005;5(3):265-71. DOI: 10.2174/1566523054064977

27. Lisowski L, Dane AP, Chu K, et al. Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. Nature 2014;506(7488):382-6. DOI: 10.1038/nature12875

28. Huang LY, Halder S, Agbandje-McKenna M. Parvovirus glycan interactions [review]. Curr Opin Virol 2014;7:108-18. DOI: 10.1016/j.coviro.2014.05.007

29. Domenger C, Grimm D. Next-generation AAV vectors-do not judge a virus (only) by its cover [review]. Hum Mol Genet 2019;28(R1):R3-R14. DOI: 10.1093/hmg/ddz148

30. Van den Hoogen F, Khanna D, Fransen J, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European league against rheumatism collaborative initiative. Ann Rheum Dis 2013;**72**,1747–1755. DOI: 10.1136/annrheumdis-2013-204424.

31. LeRoy EC, Black C, Fleischmajer R, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;**15**, 202–5.

For Peer Review

32. Medsger TA Jr. Natural history of systemic sclerosis and the assessment of disease activity, severity, functional status, and psychologic well-being. Rheum Dis Clin North Am 2003;**29**, 255-73. DOI: 10.1016/s0889-857x(03)00023-1.

33. Govindasamy L, DiMattia MA, Gurda BL, et al. Structural insights into adeno-associated virus serotype 5. J Virol 2013;**87**, 11187–99. DOI: 10.1128/JVI.00867-13.

34. Comeau SR, Gatchell DW, Vajda S, et al. ClusPro: an automated docking and discrimination method for the prediction of protein complexes. Bioinformatics 2004;**20**, 45–50. DOI: 10.1093/bioinformatics/btg371.

35. Grosdidier A, Zoete V, Michielin O. SwissDock, a protein-small molecule docking web service based on EADock DSS. Nucleic Acids Res 2011;**39**,W270-7. DOI: 10.1093/nar/gkr366.

36. Moroncini G, Cuccioloni M, Mozzicafreddo M, et al. Characterization of binding and quantification of human toantibodies to PDGFRα using a biosensor-based approach. Anal Biochem 2017;**528**, 26–33. DOI: 10.1016/j.ab.2017.04.011.

37. Kuwana M, Distler O. Recent progress and missing gaps to achieve goal in the care of systemic sclerosis-associated interstitial lung disease [editorial]. J Scleroderma Relat Disord 2020;5(2 Suppl):3-5. DOI: 10.1177/2397198320902551

38. Hoffmann-Vold AM, Allanore Y, Alves M, et al. Progressive interstitial lung disease in patients with systemic sclerosis-associated interstitial lung disease in the EUSTAR database. Ann Rheum Dis 2021;80(2):219-27. DOI: 10.1136/annrheumdis-2020-217455

39. Whitsett JA, Weaver TE. Hydrophobic surfactant proteins in lung function and disease. N Engl J Med 2002; 347 (26): 2141. DOI: 10.1056/NEJMra022387

40. Balka G, Ladinig A, Ritzmann M, et al. Immunohistochemical characterization of type II pneumocyte proliferation after challenge with type I porcine reproductive and respiratory syndrome virus. J Comp Pathol 2013;149(2-3):322-30. DOI: 10.1016/j.jcpa.2012.12.006

41. Zhang R, Xu G, Cao L, et al. Divergent engagements between adeno-associated viruses with their cellular receptor AAVR. Nat Commun 2019;10(1):3760. DOI: 10.1038/s41467-019-11668-x

42. Kruzik A, Fetahagic D, Hartlieb B, et al. Prevalence of Anti-Adeno-Associated Virus Immune Responses in International Cohorts of Healthy Donors. Mol Ther Methods Clin Dev 2019;14:126-133. DOI: 10.1016/j.omtm.2019.05.014

43. Summerford C, Johnson JS, Samulski RJ. AAVR: A Multi-Serotype Receptor for AAV [comment]. Mol Ther 2016;24(4):663-6. DOI: 10.1038/mt.2016.49

44. Martin JE, Assassi S, Diaz-Gallo LM, et al. A systemic sclerosis and systemic lupus erythematosus pan-meta-GWAS reveals new shared susceptibility loci. Hum Mol Genet 2013;22(19):4021-9. DOI: 10.1093/hmg/ddt248

45. Lunardi C, Bason C, Navone R, et al. Systemic sclerosis immunoglobulin G autoantibodies bind the human cytomegalovirus late protein UL94 and induce apoptosis in human endothelial cells. Nat Med 2000;6(10):1183-6. DOI: 10.1038/80533

46. Lunardi C, Dolcino M, Peterlana D, et al. Antibodies against human cytomegalovirus in the pathogenesis of systemic sclerosis: a gene array approach. PLoS Med 2006;3(1):e2. DOI: 10.1371/journal.pmed.0030002

47. Bonner JC. Regulation of PDGF and its receptors in fibrotic diseases [review]. Cytokine Growth Factor Rev 2004;15(4):255-73. DOI: 10.1016/j.cytogfr.2004.03.006

For Peer

48. Bjornevik K, Cortese M, Healy BC et al Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis Science 2022;375(6578):296-301. DOI: 10.1126/science.abj8222

49. Lanz TV, Brewer RC, Ho PP, et al. Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. Nature 2022;603(7900):321-27. DOI: 10.1038/s41586-022-04432-7

50. Robinson WH, Steinman L. Epstein-Barr virus and multiple sclerosis Science 2022; 375:264-265 DOI: 10.1126/science.abm7930

51. Golan M, Krivitsky A, Mausner-Fainberg K et al. Increased Expression of Ephrins on Immune Cells of Patients with Relapsing Remitting Multiple Sclerosis Affects Oligodendrocyte Differentiation. Int J Mol Sci. 2021; 22: 2182. DOI: 10.3390/ijms22042182

.

iew

ACKNOWLEDGMENTS

We thank Prof. Rosaria Gesuita, Università Politecnica delle Marche, Ancona, Italy, for statistical analysis; Prof. Michael S. Chapman and Dr. O. Davulcu (Oregon Health & Science University, Portland, Oregon, USA) for the bacterially expressed fusion protein of PKD domains 1-5 of adeno-associated virus receptor (AAVR) described by Pillay et al (17); Dr Lorena Zentilin of International Centre for Genetic Engineering and Biotechnology (ICGEB) Trieste, Italy for AAV5 and AAV2 capsids; Dr. Gianni Di Maio for his help in the preparation of the manuscript.

AUTHOR CONTRIBUTION

A.G. and E.V.A. performed the experimental design, the data acquisition and interpretation, and wrote the manuscript. G.M. performed in silico molecular docking, SPR experiments, data acquisition and interpretation and provided critical edits to the text. S.S. performed the immunoaffinity purification of anti PDGFRa antibodies. A. La Cava provided relevant contribution to set up the AAV5 PCR ,and provided critical edits to the text. A.A. and S. A. provided critical analysis of the experimental procedures and provided critical edits to the text. D.A., S. G. and D.B. contributed to BAL and blood samples collection and patient clinical data analysis. S.M. performed the AAV5 capsid gene sequencing. A.G. and M.S. performed the confocal microscopy experiments, interpreted the data and provided critical edits to the text. P.P. and A. Amoresano provided critical edits to the text. P.D.,T.S. and D.F. performed the immunohistochemistry and in situ hybridization experiments and interpreted the data. A.F. cloned the human monoclonal anti PDGFRa antibodies from SSc PBMCs. M.C. and M.M. performed in silico molecular docking, SPR experiments, data acquisition and interpretation. M.G. provided crucial input for PBMCs stimulation experiments and

interpreted the data. A. G. C.P., S.A. C.T. and N.V. performed the PCR experiments. M.G., G.P., Q.C., K.N. M.M. and J.K. performed the AAV5 particles production, the experiments with the A549 cell line and provided valuable information regarding AAV5. **A.P.** Performed the quantification of AAV5 in SSc lung section and in peripheral blood mononuclear cells.

DATA AVAILABILITY STATEMENT

Access to primary datasets (generated during the study) and referenced datasets (datasets analyzed in the study) are available

For

Figure legends

Figure 1. AAV5 and PDGFRα expression in lung tissue of SSc patients

A representative experiment from one SSc patient is shown **A**,**left,** Immunohistochemical staining of the lung of one SSc patient shows AAV5 in cells lining the alveolar space resembling the cytological appearance of type II pneumocytes (arrows). **A, right**, No staining was detected with an isotype control antibody Scale bar, 60 μm. **B**, **left**, Representative microscopic images of chromogenic *in situ* hybridization for AAV5 was applied to paraffin-embedded lung sections from two SSc patients and two control subjects. A labeled DNA probe was used to hybridize to AAV5 DNA sequence. Strong signals for AAV5 set specifically in pneumocytes (arrows). **B, right**, Antisense probe was used as control**.** Scale bar, 60 μm. **C,** A lung section from one SSc patient is shown at low magnification. AAV5 capsid-specific staining (left panels, green) is present in several epithelial cells lining the alveolar cavities (Alv), that are also positive for the type II pneumocytes (P) marker proSP-C (middle panels, red). The larger area framed in the right upper panel is enlarged in the lower panels, while the smaller area is shown as insets of the upper panels. They both show at higher magnification proSP-C-positive pneumocytes infected by AAV5 (arrows). Asterisks (*) indicate bulk of collagen and other extracellular matrix components that exhibit autofluorescence. Semiquantitative analysis showed that 17 ± 6.2% of pro SP-C + type II pneumocytes expressed AAV5**.** **D,** AAV5-positive pneumocytes (P; left panel, green) are variably positive for PDGFRα (middle panel, red) that is mainly detectable on their cellular luminal side (arrowheads), indicating colocalization of the two (right panel). In the interstitial space (Int), several fibroblast-like cells (F) with their projections (f) are strongly positive for PDGFRα. Alv, alveolar cavity; Cap, capillary.

Figure 2. AAV5 genomic sequences in bronchoalveolar lavage fluid from SSc patients and controls

For Peer Review

A, Percentage of SSc patients (n=66) and controls with lung disease other than SSc (n=66) positive for AAV5 DNA in cells recovered from bronchoalveolar lavage fluid (BAL. **B,** AAV5 DNA copy number in cells recovered from BAL of SSc patients (n=41) and controls (n=17**)** who tested positive for AAV5. Data are expressed as a median value and a range (SSc: median 20,595; range, 260-1.3x10^6. Controls: median 3800; range 720-16 x10^4).

*** $p < 0.001$.

Figure 3. Binding of AAV5 to human PDGFRα

A. Molecular docking model predicting the binding of AAV5 capsid monomeric subunit (green) to the extracellular region of monomeric PDGFRα (gray). AAV5 capsomer binding site lies between the second and third PDGFRα Ig-like extracellular domains (indicated in roman numerals from V to I)**. B,** Binding curves (measured in arc/seconds [arcsec] over time) of histidin-tagged monomeric recombinant human PDGFRα immobilized on the biosensor chip, after saturation with different concentrations (expressed in nanomoles, nM) of AAV5 capsid monomeric subunits. AAV2 capsid monomeric subunits were tested as control. Dissociation constants (Kd) are indicated in the boxes. **C,** The immunoblot of A549 cells before (wild-type, wt) and after PDGFRα knockout (KO) by CRISP/Cas 9 technology. **D**, AAV5 and AAV2 transduction in wt and PDGFRα KO A549 cells (A549-KO) is shown as a mean ± SD of three independent experiments**.** ***$p < 0.001$.

Figure 4. Immune responses against AAV5-PDGFRα spatially contiguous epitopes

A, Binding curves (measured in arc/seconds [arcsec] over time) of V_HPAM-V_κ16F4 immobilized on the biosensor chip after saturation with different concentrations (expressed in nanomoles, nM) of AAV5 (left panel) and AAV2 (right panel). Dissociation constants (Kd) are indicated in the boxes. **B,** Molecular docking model predicting binding of V_HPAM-V_κ16F4

For Peer Review

to the complex formed by AAV5 capsid monomeric subunit and the extracellular region of monomeric PDGFRα. The predicted epitopes of V_HPAM-V_κ16F4 are indicated as PDGFRα-pep (gray) and AAV5-pep (green). **C,** Binding curves (measured in arc/seconds [arcsec] over time) of V_HPAM-V_κ16F4 antibody probing rhPDGFRα-His immobilized on the biosensor chip and saturated with AAV5 capsid monomeric subunits are shown. V_HPAM-V_κ16F4 binding to this complex was measured before (curve A) and after preincubation with an AAV5-peptide (curve B) or PDGFRα-peptide (curve C) or both mixed (curve D). **D,** Experiment performed as in **D** but V_HPAM-V_κ16F4 was replaced by immunoaffinity purified, SSc anti-PDGFRα antibodies.

For Peer Review

Running head: AAV5 in systemic sclerosis

Adeno-associated virus type 5 infection via PDGFRα is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Gianluca Moroncini MD, PhD[1,2†], Silvia Svegliati PhD[1†], Antonella Grieco PhD [1†], Massimiliano Cuccioloni PhD[4], Matteo Mozzicafreddo PhD[4], Chiara Paolini PhD[1], Silvia Agarbati PhD[1], Tatiana Spadoni PhD[1], Angela Amoresano PhD[5], Gabriella Pinto PhD[5], Qingxin Chen PhD[6], Devis Benfaremo MD[1,2], Cecilia Tonnini BS[1], Martina Senzacqua PhD[7], Silvia Alizzi MD[8], Karen Nieto PhD[6], Doreen Finke PhD[1], Nadia Viola BS[10], Donatella Amico MD, PhD[11], Mario Galgani MD[12], Stefano Gasparini MD[2,13],Lina Zuccatosta MD[2], Stefano Menzo MD[2,13], Martin Müller PhD6 Juergen Kleinschmidt PhD[6], Ada Funaro PhD[8], Antonio Giordano MD[7], Antonio La Cava MD, PhD[14], Peter Dorfmuller MD, PhD[15], Antonio Amoroso MD[8,9], Piero Pucci PhD[5], Antonio Pezone [16], Enrico Vittorio Avvedimento MD, PhD[12*], Armando Gabrielli MD[1,2,3*].

1, Dipartimento di Scienze Cliniche e Molecolari, Clinica Medica, Università Politecnica delle Marche, Ancona, Italy;

2. Azienda Ospedaliero-Universitaria delle Marche, Ancona, Italy

3. Hiller Research Center, University Hospital Düsseldorf, Heinrich Heine University, Düsseldorf, Germany

4. Scuola di BioScienze e Medicina Veterinaria, Università di Camerino, Camerino, Italy

5. Dipartimento di Scienze Chimiche, Federico II University Napoli, Italy

6.Tumorvirus-specific Vaccination (F035), German Cancer Research Center (DKFZ)
Im Neuenheimer Feld 242(ATV), Heidelberg Germany

7. Dipartimento di Medicina Sperimentale e Clinica, Università Politecnica delle Marche. Ancona Italy

8. Dipartimento di Scienze Mediche, Università di Torino, Torino, Italy

9. Servizio di Immunogenetica e Biologia dei Trapianti, Azienda Ospedaliera Universitaria Città della Salute e della Scienza, di Torino, Torino - Italy

10. Servizio di Immunologia Clinica, Ospedali Riuniti, Ancona, Italy.

11. Unità di Pneumologia, "Ospedali Riuniti Marche Nord", Pesaro, Italy

12. Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Federico II University, Napoli, Italy;

For Peer Review

13. Dipartimento di Scienze Biomediche e Sanità Pubblica, Università Politecnica delle Marche. Ancona Italy;

14 Department of Medicine, University of California Los Angeles, Los Angeles, USA.

15 University Hospital Giessen and Marburg, Pathology Department, University of Giessen and Marburg Lung Center (UGMLC), German Center of Lung Research (DZL), Giessen, Germany

16 Dipartimento di Biologia, Università Federico II, Napoli, Italy.

† Contributed equally to the work

*Corresponding Authors:

Armando Gabrielli
a.gabrielli@staff.univpm.it

and :

Enrico V. Avvedimento
avvedim@unina.it

For Peer Review

FUNDING

This work was supported by grants from Fondazione di Medicina Molecolare e Terapia Cellulare, Associazione Italiana lotta alla Sclerodermia (AILS) Gruppo Italiano per la Lotta alla Sclerodermia (GILS) and by Dr. Andrea and Diego Della Valle.

COMPETING INTERESTS

The authors declare no competing interests.

ABSTRACT

Objectives

The etiopathogenesis of systemic sclerosis (SSc) is unknown. Platelet-derived growth factor receptors (PDGFRs) are overexpressed in SSc patients. Since PDGFRα is targeted of the adeno-associated virus type 5 (AAV5), we investigated whether AAV5 forms a complex with PDGFRα exposing epitopes that may induce the immune responses to the virus-receptor complex, resulting in an autoreactive immune response to PDGFRα.

Methods

The binding of monomeric human PDGFRα to the AAV5 capsid was analyzed by *in silico* molecular docking, surface plasmon resonance (SPR), and genome editing of the PDGFRα locus. AAV5 was detected in SSc lungs by *in situ* hybridization, immunohistochemistry, confocal microscopy, and molecular analysis of bronchoalveolar lavage (BAL). Immune responses to AAV5 and PDGFRα were evaluated by SPR using SSc monoclonal anti-PDGFRα antibodies and Immunoaffinity-purified anti-PDGFRα antibodies from sera of SSc patients.

Results

AAV5 was detected in the BAL of 41 out of 66 (62.1%) SSc patients with interstitial lung disease and in 17 of 66 controls (25.75 %; $p<0.001$). In SSc lungs, AAV5 localized in type II pneumocytes and in interstitial cells. A molecular complex formed of spatially contiguous epitopes of the AAV5 capsid and PDGFRα was identified and characterized. *In silico*

For Peer Review

molecular docking analysis and binding to the agonistic anti-PDGFRα antibodies identified spatially contiguous epitopes derived from PDGFRα and AAV5 that interacted with SSc agonistic antibodies to PDGFRα. These peptides were also able to bind total IgG isolated from SSc patients.

Conclusions

These data link AVV5 with the immune reactivity to endogenous antigens in SSc, and provide a novel element in the pathogenesis of SSc.

For Peer Review

Keywords: Systemic sclerosis, scleroderma, adeno-associated virus, PDGFRα, autoimmune disease, autoimmunity

INTRODUCTION

Systemic sclerosis (SSc, scleroderma) is an autoimmune, multisystem disorder of unknown etiology and unclear pathogenesis. It is characterised by microvasculature damage, circulating autoantibodies and fibroblast activation that lead to fibrosis of the skin and visceral organs (1,2). SSc is a major cause of disability and carries high morbidity and mortality associated with the fibrotic and microvascular alterations (3,4).

It has been suggested that SSc can be triggered by viruses (5), and the interferon type I gene expression signature is frequently observed in SSc patients (6-8). However, the search for putative disease-promoting viruses in SSc has so far been inconclusive.

Platelet-derived growth factor receptor (PDGFR) signalling, directly or indirectly, is involved in SSc (9). PDGFRs are the targets of several viruses (10-13) and are typically overexpressed in SSc (14). We also reported the presence of stimulatory anti PDGFRα antibodies in the serum of SSc patients (15), and we cloned agonistic anti PDGFRα autoantibodies from SSc patients (16).

Anti PDGFRα autoantibodies are of particular interest for the following reasons:

i) they induced a SSc -like phenotype in normal human fibroblasts (15); ii) they induced fibrosis ex vivo (17) and iii) Stimulated the proliferation and migration of human pulmonary artery smooth muscle cells (suggesting a role in the formation of the neointima which is the cardinal feature of the vascular abnormalities in SSc) (18); and iv) they triggered a well-characterised signalling pathway leading to fibrosis (19). Furthermore, these antibodies

For Peer Review

selectively recognised specific domains in the receptor. For example, antibodies targeting extracellular domain I of the receptor were biologically inactive, whereas those recognising the extracellular domain II were stimulatory in inducing fibrosis and redox stress (16).

It has to be noted that the detection of these antibodies is not trivial because they cannot be detected by non-functional bioassays (16, 20, 21), underscoring their functional role. Moreover, it has to be considered that these antibodies can have conformational epitopes that are distant in the primary sequence but contiguous in the tertiary structure of the receptor on the cell surface (16). Interestingly, adeno-associated virus type 5 (AAV5) is known to enter several cell types through PDGFRα (22-24), making it a candidate worth studying in the induction of anti-PDGFRα in SSc.

AAV5 is a non-enveloped, single-stranded DNA adeno-associated virus that belongs to the genus Dependoparvovirus within the Parvoviridae family (25); it requires a helper virus, such as adenovirus or herpes simplex virus, or cellular stress, to replicate (26). To date, 13 serotypes of AAV have been identified (27). AAV gain access to specific cell surface receptors after attaching to glycans or glycoconjugates on the cell membrane (28). The current knowledge is that AAVs do not cause any human diseases and have been used for in vivo therapeutic gene delivery (29).

Building on these considerations we hypothesised that AAV5 may recognise and bind specific domains in the receptor and may target the immune response to the receptor and the viral capsid.

For Peer Review

MATERIALS AND METHODS

Patients

Sixty-six consecutive Caucasian patients with ILD-SSc were studied. All SSc patients fulfilled the EULAR/ACR preliminary criteria for the classification of SSc (30). The patients were classified into subgroups according to whether they had diffuse cutaneous scleroderma (dcSSc) or limited cutaneous scleroderma (lcSSc) (31) and then were divided into those with early (<3 years for dcSSc and <5 years for lcSSc) or late disease (>6 years for dcSSc and >10 years for lcSSc) from the first non-Raynaud's phenomenon symptom (32). All patients had not received any immunosuppressive treatment during the previous six weeks. The presence of ILD was confirmed by X-Ray and/or HRCT. The control group (C) included 66 patients who underwent BAL for lung involvement other than SSc ILD, and, in selected *in vitro*, experiments, healthy subjects (HC). The protocol with patients' information and consent forms were approved by the Ethics Committee of the Università Politecnica delle Marche (# 2017-518). The study was conducted in accordance with the Declaration of Helsinki, 5th edition (2000). Written informed consent was obtained from all patients.

Molecular docking analysis

The three-dimensional structure of AAV5 envelope subunit (33) was obtained from the Protein Data Bank. Human PDGFRα and anti-PDGFRα V_HPAM-V_κ16F4, a stimulatory human anti-PDGFRα monoclonal autoantibody cloned from B cells of a SSc patient, were homology-modelled as previously reported (16).

For Peer Review

The binding partners were uploaded on the ClusPro server (34) and the amino acids involved in the interaction between AAV5 and monomeric PDGFRα and between the predicted AAV5/PDGFRα complex and V_HPAM-$V_κ$16F4 were identified using PyMol software (PyMOL Molecular Graphics System, Version 2.1). Energy-based docking between trans-peptides, minimized using GROMACS (version 2021.5), and V_HPAM-$V_κ$16F4 was performed using SwissDock server (35).

Other methods are reported in the *Supplementary Methods* section.

Statistical analyses

The two-sample test for equality of proportions was used to analyze the results of the BAL study, presented as percentages of patients tested positive for AAV5 DNA, and for the analysis of double positive subjects for anti-PDGFRα and anti-AAV5 antibodies. Significance of the differences in AAV5 transduction in wt and PDGFRα-knockout A549 cell line was determined by the Student's *t*-test and the Mann-Whitney test for the analysis of the quantitative PCR results and cytofluorimetric data. Data are expressed as mean ± SD or median value and a range. Data were analyzed using Prism software (Graph-Pad). All reported *P* values are two-sided. *P* values <0.05 were considered statistically significant.

For Peer Review

RESULTS

AAV5 in the lungs of SSc patients with interstitial lung disease

Since the lung is the main disease target in SSc and is responsible for high morbidity and mortality (37,38), we investigated the possibility that it might be affected by the presence of AAV5-infected cells. Immunohistochemistry was performed on frozen lung tissues from five SSc patients and six control lung specimens (from lobectomy or pneumonectomy for localised lung cancer), and the results were confirmed by chromogenic *in situ* hybridization (CISH) on paraffin-embedded lung sections from two patients and two controls (online supplemental table S1). A representative experiment is shown in Figure 1. All samples were analysed in a blinded fashion by two independent investigators (AG and MS). In SSc lungs of five SSc patients, but not in controls, AAV5 was detected mainly in cells lining the alveolar space - many of them with the cytological appearance of type II pneumocytes (figure 1A,B) - and, to a lesser extent, in interstitial macrophage-like cells. In lung tissue of patients with lung cancer used as controls, AAV5 immunoreactivity was only found in interstitial cells. Positivity was found in two other more controls, one affected by primary arterial hypertension (PAH) with fibrotic remodeling and pneumocytic hyperplasia, and the other by nonspecific interstitial pneumonia with extensive interstitial fibrosis (data not shown). Using prosurfactant protein C (pro-SP-C) and TTF 1 as markers of type II pneumocytes (39,40), we found by double-staining and confocal microscopy showed that a consistent number of proSP-C- or TTF1-positive type II pneumocytes were variably positive for AAV5 (17 ± 6.2% and 11± 4.5% respectively; figure 1C and online supplemental figure S1). Of note, AAV5-positive type II pneumocytes expressed PDGFRα, mainly at the apical segment of the cells, and facing the alveolar space (figure 1D). The specificity of the signals was suggested by the strong positivity for PDGFRα -and not for AAV5- of numerous fibroblast-like cells in the

For Peer Review

interstitial space strongly positive for (figure 1D). Extensive quantification of multiple confocal microscopy images of SSc lung sections (n=14), as described in the online supplementary methods, indicated positivity for AAV5 capsid (16 ± 5.1%,) and PDGFRα (22 ± 5.5%), with an overlay percentage of 10.69 ± 4.4% (online supplemental figure S 2A and S 2B; online supplemental table S 2). Interestingly, 63.77 ± 10% of the AAV5 signal overlapped with the PDGFRα signal, while only 46.49 ± 9.8% of the PDGFRα signal overlapped with the AAV5 signal (online supplemental figure S2C). A strong linear correlation between the two signals was observed (R2=0.97, online supplemental figure S 2D).

Of note, colocalization of AAV5 and PDGFRα was also observed by confocal microscopy in peripheral blood mononuclear cells(PBMC) from SSc patients (online supplemental figure S3). Quantification of multiple confocal microscopy images of SSc PBMC (n=11) showed an AAV5 capsid positivity of 11.85 ± 3.4%, and a PDGFRα positivity of 21.5 ± 2.67%, with an overlay percentage of 6.05 ± 2% (online supplemental figure S 4A and S 4B online supplemental table S3). Notably, 52.2 ± 10.9% of the AAV5 signal overlapped with the PDGFRα signal, whereas only 28.4 ± 9.1% of the PDGFRα signal overlapped with the AAV5 signal (online supplemental figure S 4C). Also in this case, the two signals had a strong linear correlation (R2=0.93, online supplemental figure S 4D).

Taken together, the above data indicate that: i) immunostaining for the AAV5 capsid correlates with PDGFRα expression in both lung sections and PBMC of SSc patients; ii) type II pneumocytes coexpress PDGFRα and AAV5 positivity; iii) not all PDGFRα-expressing cells are also positive for the AAV5 capsid.

AAV5 in BAL from SSc patients

For Peer Review

The presence of AAV5 DNA was investigated in the bronchoalveolar lavage (BAL) from 66 SSc patients (53 females and 13 males; mean age 56.9 ± 15.2 years) with interstitial lung disease and 66 control patients with lung disease other than SSc (38 females and 28 males; mean age 62.3 ± 15.1 years). Thirty-seven (56%) SSc patients had the limited cutaneous form of SSc and 29 (44%) had the diffuse cutaneous form. The lung disorders different from SSc in controls are reported in the online supplemental table S4. The results showed positivity for AAV5 DNA in the BAL from 41 SSc patients (62.1%; 95% C.I.: 49.3-73.8%), and 17 controls (25.75%; 95% C.I: 15.8-38.0%) (p<0.001) (figure 2A). It is worth mentioning that seven additional SSc patients lacking viral DNA in the BAL had positivity for AAV5 DNA in their PBMCs (online supplemental figure S 3), for a totality of 48 positive patients (72.7%). The only feature distinguishing SSc patients with AAV5-positive BAL from those with AAV5-negative BAL was disease duration (SSc AAV5 positive: 5.3 ± 0.9 years vs. SSc AAV5 negative: 2.4 ± 0.8 years; p<0.05) (online supplemental table S5). Quantitatively the AAV5 DNA content was comparable between the BAL from SSc patients and the controls who tested positive for AAV5 (p=0.191) (figure 2B). To rule out the possibility that a mutated AAV5 could have accounted for the virus detection in the SSc lungs, we verified the complete nucleotide sequence of the viral *cap* gene in the BAL of three patients and three controls they were identical to the prototypical European AAV5 prototype (acc. n° Y18065.1) in both groups.

The AAV5 capsid binds human PDGFRα

The interaction between the AAV5 capsid and PDGFRα was studied through *in silico* molecular docking using the homology modelled three-dimensional structures of human monomeric PDGFRα (16) and the crystal structure of the AAV5 capsid monomeric subunit.

For Peer Review

It was found that the predominant binding at the external surface of the capsid with the receptor occurred at a region spanning the second and third extracellular Ig-like domains of PDGFRα (figure 3A). These regions of the receptor discriminate agonistic from non-functional anti PDGFRα antibodies that bind to the receptor's domain I of the receptor (16). The AAV5-PDGFRα interacting sequences are shown in online supplemental figure S 5 A,B.

To validate the in silico model we analysed the binding of PDGFRα to purified AAV5 capsid protein by surface plasmon resonance (SPR) using histidine-tagged recombinant human monomeric PDGFRα (rhPDGFRα-His) immobilised and folded into a native-like conformation (16). The capsid protein of AAV2, a close serotype belonging to the same viral family, was tested in parallel as a control. The rhPDGFRα was found to bind AAV5 with a 370-fold higher affinity as compared to AAV2, with a faster recognition phase (higher value of k_{ass}) and a greater stability of the complex (lower value of k_{diss}) (figure 3B).

Next, functional role of human PDGFRα in AAV5 transduction was evaluated with a CRISPR/Cas9 PDGFRα knockout variant in the A549 cell line (a type II pulmonary epithelial cell line) and related control. Transduction efficiency of the AAV5 virus expressing luciferase (AAV5-FF-Luc) in A549-PDGFRα knockout cells was significantly reduced as compared to that of A549 control or cells cotransduced with AAV2-FF-Luc (figure 3 C, D).

The type I transmembrane protein KIAA0319L is a cellular receptor (AAVR) involved in the cellular entry of several AAV serotypes including AAV5 (23). The comparison of binding of the predicted sites of AAV5 to AAVR (41) and PDGFRα showed that the AAV5 capsid binding site for AAVR was distinct from that used to engage hPDGFRα (online supplemental figure S 5A, 5B). Also, when AAVR was immobilised onto the SPR device and tested for binding to the AAV5 capsid, the binding to AAV5 of AAVR (online supplemental figure S 5C) displayed an equilibrium dissociation constant in the sub-micromolar range, similar to that

For Peer Review

reported previously for the the AAV2-AAVR interaction (23). On the other hand, The interaction between AAV5 and rhPDGFRα displayed a 100-fold higher affinity, associated with a faster kinetic association phase (figure 3B).

Taken together, these data demonstrate that: i) AAV5 binds with high affinity PDGFRα in human cells; ii) identify the PDGFRα domains bound to the AAV5 capsid and iii) human PDGFRα can be the cell entry receptor for AAV5 as indicated by colocalization studies .

Immune responses to the AAV5-PDGFRα complex

For Peer Review

Next, we investigated whether the PDGFRα–AAV5 complex (figure 3A) associated with anti-PDGFRα immune responses. To this aim, we took advantage of the V_HPAM-$V_κ$16F4 human monoclonal stimulatory anti-PDGFRα antibody, whose epitopes have been experimentally identified and validated (16). V_HPAM-$V_κ$16F4 antibody, immobilised onto the SPR device to test binding to AAV5, formed a high affinity complex with AAV5 (figure 4A, left), with an equilibrium dissociation constant in the sub-micromolar range and with about four-fold higher affinity than the control VH_{PAM}-Vκ16F4-AAV2 complex (figure 4A, right). This indicates that the stimulatory antibody to PDGFRα V_HPAM-$V_κ$16F4 recognises also the AAV5 capsid.

Identification of spatially contiguous epitopes of AAV5 and PDGFRα recognised by SSc autoantibodies

In silico molecular docking was optimised by restricting the AAV5 capsid-PDGFRα interaction to the extracellular domain II recognised by the agonistic antibody V_HPAM-$V_κ$16F4 - and not by the control antibody V_HPAM-V_k13B8.

To identify the epitopes of the AAV5 capsid and PDGFRα epitopes, jointly recognised by the V_HPAM-$V_κ$16F4 antibody, we first evaluated *in silico* the interaction between V_HPAM-$V_κ$16F4, the AAV5 capsid and PDGFRα, focusing on the variable region (Fab) of the monoclonal autoantibody and the AAV5/PDGFRα complex (figure 4B). This analysis showed: that i) the PDGFRα epitope recognised by VH_{PAM}-Vκ16F4 was not included in the PDGFRα domain that interacts with AAV5; ii) the V_HPAM-$V_κ$16F4 anti-PDGFRα antibody recognized two spatially contiguous peptides formed by the amino acid sequences LYRF (derived from the AAV5 capsid) and PASY (derived from PDGFRα) with high affinity (K_d of 5.182 e^{-8} M). In addition, the V_HPAM-$V_κ$16F4 anti-PDGFRα antibody recognises other composite peptides formed by the amino acid sequence FTVG (derived from PDGFRα), SARN (K_d = 6.103 e^{-9} M) and SVSA (K_d = 3.124 e^{-9} M) peptides derived from the AAV5 capsid (online supplemental figure S6).

In all, the same PDGFRα epitopes that bound *in silico* the AAV5 capsid were recognised as well by the agonistic anti-PDGFRα antibodies using different techniques (surface plasmon resonance, conformational PDGFRα peptide library and single amino acid mutagenesis) (16).

To confirm the in silico predictions, the rhPDGFRα-coated surface of the biosensor was saturated with AAV5 before addition of the monoclonal antibody V_HPAM-$V_κ$16F4. The results showed the formation of a receptor-capsid ternary complex (figure 4C, curve A). To rule out that V_HPAM-$V_κ$16F4 binding was limited to PDGFRα, peptide sequences corresponding to the predicted AAV5 (VDQYLYRF) and PDGFRα (PASYDTFT) binding domains of V_HPAM-$V_κ$16F4 were synthesised and pre-incubate - alternatively or in combination - with V_HPAM-$V_κ$16F4 prior to the addition of the rhPDGFRα-AAV5 complex.

For Peer Review

Notably, both peptides, independently, partially inhibited V_HPAM-V_κ16F4 binding (figure 4C, curves B and C) and completely abrogated the binding of the antibody when combined (figure 4C, curve D). Similar results were obtained when V_HPAM-V_κ16F4 was replaced by the immunoaffinity-purified anti-PDGFRα serum antibodies from SSc patients (the polyclonal nature of these antibodies explains the partial inhibition of the binding to the peptides) (figure 4D). Together, these data indicate that anti-PDGFRα autoantibodies purified from the sera of SSc patients as well the cloned V_HPAM-V_κ16F4 PDGFRα autoantibody react to both human PDGFRα and the AAV5 capsomere.

For Peer Review

DISCUSSION

This study shows that: i) PDGFRα allows AAV5 internalization in cells (figure 1 and 3 C,D); ii) a high percentage (62.1%) of SSc patients with ILD harbor AAV5 in the lung; (figure 2) iii) a spatially contiguous epitope composed of a stretch of aminoacids of PDGFRα and the AAV5 capsid is recognized by antibodies *in vivo* that can target PDGFRα (figure 4).

The presence of the AAV5 in BAL of 25.75% of the controls with different lung diseases confirms the notion that AAV5 is endemic in the human population, as also indicated by the presence of anti-AAV5 neutralizing antibodies and circulating INF-γ + T cells that react to AAV5 in 30% and 24% of healthy donors, respectively (42). AAV5 is a nonpathogenic and poorly immunogenic virus. Although it does not directly participate in the pathogenesis of SSc, our data suggest that it can contributes to the generation of neoantigens, some of which can target the immune reactivity to PDGFRα.

After binding to a primary receptor, the capsid protein of AAVs interacts with a co-receptor which leads to virus internalization via endocytosis. Primary cell surface receptors for AAV5 include heparin sulfate proteoglycans, N-terminal galactose and N-or O- linked sialic acid moieties. Secondary receptors for AAV5 are PDGFRα and AAVR, as also shown here (23, 43). Our data confirm previous findings (23) that demonstrate that AAVR (the KIAA0319L transmembrane protein) is a receptor for multiple AAV serotypes including AAV5. Although in our experiments KIAA0319L displayed a lower affinity to AAV5 capsid compared to human PDGFRα (online supplemental figure S 5C), a single nucleotide polymorphism in the *KIAA0319L* locus, rs2275247, was strongly associated with SLE, scleroderma and higher expression of the receptor (44).

The fact that the KIAA0319L sequence the stretch of aminoacids recognizing the AAVR capsid is distinct from the peptide bound to human PDGFRα (online supplemental figure S

5B) suggests that the capsid can bridge a connection between PDGFRα and the KIAA0319L receptor. This trimeric complex sustained by the viral capsid may be recognized as an exogenous protein and thus targeted by immune cells.

The association between viral (exogenous) and endogenous (PDGFRα) antigens has also been implicated in other human infections. For example, CMV, a ubiquitous human herpesvirus, requires PDGFRα to induce a robust inflammatory response (10), and it has also been linked to SSc (45,46). It remains to be solved whether the heterogeneous phenotypes of SSc depend directly or indirectly on the virus involved - CMV or AAV5 or other AAV serotypes - or whether CMV is only one of the helper viruses necessary for a productive AAV5 replication.

The finding that 28.3 % of our cohort of SSc patients did not have AAV5 sequences, could be explained by an on-and-off viral replication and by the consideration that only patients with evidence of ILD underwent BAL for ethical reason. These aspects do not allow at present to formally establish whether the viral infection would occur before or after the clinical evidence of the disease.

Another finding that deserves an explanation is the longer duration of disease in patients with AAV5-positive BAL. We believe that the protracted systemic disease, characterized by increased secretion of disease-related cytokines and growth factors (47), stimulated the expression of PDGFRα in the lungs, increasing chances of local infection or reactivation of AAV5. Incidentally, the virus might also be present in other locations, driving as well the production of antibodies in AAV5-negative BAL subjects as shown by 7 SSc patients without AAV5 in the BAL, but positive for the virus in the peripheral blood.

We propose that high expression of PDGFRα may facilitate AAV5 infection and (re)activation, possibly with helper viruses co-infections, and this, wherever occurring, can

induce, in genetically susceptible individuals, self-reactive immune responses to PDGFRα epitopes physically associated with the virus capsid and AAVR (as indicated by the co-presence of anti PDGFRα and anti AAV5 antibodies; online supplemental figure S7). Of note, the anti-PDGFRα antibodies recognizing the II domain of the receptor (the activation loop physically linked to the capsid; ref.16) are biologically active, present at high levels in patients with early disease (15), and capable to induce the fibrotic phenotype in SSc patients (15-19). However, we cannot rule out that AAV5 can contribute to the development of fibrosis by directly activating mesenchymal-like interstitial cells (figure 1).

The remarkable finding here is also the identification of non-linear epitopes spanning the sequences of two cell receptors, PDGFRα, KIAA0319L, and the AVV5 capsid. The spatial organization of PDGFRα sequences, exposing its II activating domain in this trimeric complex explains the generation of stimulating autoantibodies of PDGFRα. These antibodies isolated from SSc patients have been cloned and functionally tested *in vivo* and *in vitro*. They induce ROS and fibrosis in vivo (17) and in vitro (19) through the PDGFRα activation (16). Our data imply that SSc antibodies recognize a neoantigen composed of two peptides derived from two different proteins, the AAV5 capsid and PDGFRα (figure 4A, C and D). The structure of the complex is better defined by *in silico* modeling (figure 4B) which shows that the AAV5 peptide interacts with the second extracellular domain of PDGFRα in proximity to PDGFRα epitopes targeted by anti- PDGFRα autoantibodies (Fig. 4B).

We acknowledge two limitations of this study: i) the limited number of controls and patients that prevents a conclusive subgroup analysis; ii) the impossibility, for ethical reasons, to perform BAL in patients without HRCT evidence of ILD.

While our results do not indicate that AAV5 is the cause of scleroderma, they suggest that AAV5 favors the formation and presentation of a peculiar and composite peptides complex containing epitopes from different proteins, the PDGFRα, AAVR, and the viral capsid, which stabilizes this complex. Due to the physical proximity of the capsid sequences to cell proteins, PDGFRα or KIAA0319L, it is likely that, immune cells primarily recognize the abundant capsid segment and inefficiently the endogenous cellular proteins (PDGFRα or KIAA0319L) associated with the capsid, probably due to high levels of the capsid. Reduction of the capsid peptides levels with time would increase the visibility of the trimeric complex (PDGFRα-capsid-KIAA0319L) which is recognized as viral antigen by immune cells and consequently enhances the immune response. The appearance of agonistic anti PDGFRα antibodies in genetically susceptible individuals is the result of the trimeric complex which exposes the second domain of the receptor (figure 4B) which is necessary for its activation (16). This scenario also could explain why AAV5-positive BAL is associated with longer disease duration.

This model excludes molecular mimicry of viral and endogenous proteins as an underlying mechanism inducing autoimmunity. Instead, as shown in Fig.4B, the formation of a single unique complex formed by viral and endogenous protein epitopes would facilitate the emergence of a reactive epitopes.

Spatial association of different and non-contiguous epitopes maintained by viral protein(s) has been suggested by other autoimmune diseases. For example, It has recently been reported that the Epstein Barr virus (EBV) infection dramatically increases the odd of developing multiple sclerosis (48), and while this has been attributed to molecular mimicry between EBV and a glial protein (glialCAM) that is the target of autoantibodies (49,50), it can be also speculated that the GLIACAM peptide(s) might be able to form a complex with viral proteins and the entry receptor (Ephs and Eph-receptor binding proteins, Ephrins) (51).

For Peer Review

To conclude, the results from the study presented here point to a new understanding of some aspects of the pathogenesis of SSc and autoimmune diseases that could have diagnostic and therapeutic implications to assess with subsequent work.

REFERENCES

1. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma [review]. N Engl J Med 2009;360(19):1989-2003. DOI: 10.1056/NEJMoa052955

2. Denton CP, Khanna D. Systemic sclerosis [review]. Lancet 2017;390(10103):1685-99. DOI: 10.1016/S0140-6736(17)30933-9

3. Elhai M, Meune C, Boubaya M, et al. Mapping and predicting mortality from systemic sclerosis. Ann Rheum Dis 2017;76(11):1897-905. DOI: 10.1136/annrheumdis-2017-211448

4. Park EH, Strand V, Oh YJ, et al. Health-related quality of life in systemic sclerosis compared with other rheumatic diseases: a cross-sectional study [clinical trial]. Arthritis Res Ther 2019;21(1):61. DOI: 10.1186/s13075-019-1842-x

5. Moroncini G, Mori S, Tonnini C, et al. Role of viral infections in the etiopathogenesis of systemic sclerosis [review]. Clin Exp Rheumatol 2013;31(2 Suppl 76):3-7. Epub 2013 Jul 22. PMID: 23910606

6. Assassi S, Mayes MD, Arnett FC, et al. Systemic sclerosis and lupus: points in an interferon-mediated continuum. Arthritis Rheum 2010;62(2):589-98. DOI: 10.1002/art.27224

7. Brkic Z, van Bon L, Cossu M, et al. The interferon type I signature is present in systemic sclerosis before overt fibrosis and might contribute to its pathogenesis through high BAFF gene expression and high collagen synthesis. Ann Rheum Dis 2016;75(8):1567-73. DOI: 10.1136/annrheumdis-2015-207392

8. Valenzi E, Tabib T, Papazoglou A, et al. Disparate Interferon Signaling and Shared Aberrant Basaloid Cells in Single-Cell Profiling of Idiopathic Pulmonary Fibrosis and

For Peer Review

Systemic Sclerosis-Associated Interstitial Lung Disease. Front Immunol 2021;12:595811. DOI: 10.3389/fimmu.2021.595811

9. Mendoza FA, Piera-Velazquez S, Jimenez SA. Tyrosine kinases in the pathogenesis of tissue fibrosis in systemic sclerosis and potential therapeutic role of their inhibition [review]. Transl Res 2021;231:139-58. DOI: 10.1016/j.trsl.2021.01.001

10. Soroceanu L, Akhavan A, Cobbs CS. Platelet-derived growth factor-alpha receptor activation is required for human cytomegalovirus infection. Nature 2008;455(7211):391-5. DOI: 10.1038/nature07209

11. Cavallin LE, Ma Q, Naipauer J, et al. KSHV-induced ligand mediated activation of PDGF receptor-alpha drives Kaposi's sarcomagenesis. PLoS Pathog 2018;14(7):e1007175. DOI: 10.1371/journal.ppat.1007175

12. Vrijens P, Noppen S, Boogaerts T, et al. Influenza virus entry via the GM3 ganglioside-mediated platelet-derived growth factor receptor beta signalling pathway. J Gen Virol 2019;100(4):583-601. DOI: 10.1099/jgv.0.001235

13. Karabadzhak AG, Petti LM, Barrera FN, et al. Two transmembrane dimers of the bovine papillomavirus E5 oncoprotein clamp the PDGF beta receptor in an active dimeric conformation. Proc Natl Acad Sci 2017;114(35):E7262-E71. DOI: 10.1073/pnas.1705622114

14. Daoussis D, Tsamandas AC, Liossis SN, et al. B-cell depletion therapy in patients with diffuse systemic sclerosis associates with a significant decrease in PDGFR expression and activation in spindle-like cells in the skin [clinical trial]. Arthritis Res Ther 2012;14(3):R145. DOI: 10.1186/ar3879

For Peer Review

15. Svegliati Baroni S, Santillo M, Bevilacqua F, et al. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. N Engl J Med 2006;354(25):2667-76. DOI: 10.1056/NEJMoa052955

16. Moroncini G, Grieco A, Nacci G, et al. Epitope Specificity Determines Pathogenicity and Detectability of Anti-Platelet-Derived Growth Factor Receptor alpha Autoantibodies in Systemic Sclerosis. Arthritis Rheumatol 2015;67(7):1891-903. DOI: 10.1002/art.39125

17. Luchetti MM, Moroncini G, Jose Escamez M, et al. Induction of Scleroderma Fibrosis in Skin-Humanized Mice by Administration of Anti-Platelet-Derived Growth Factor Receptor Agonistic Autoantibodies. Arthritis Rheumatol 2016;68(9):2263-73. DOI: 10.1002/art.39728

18. Svegliati S, Amico D, Spadoni T, et al. Agonistic Anti-PDGF Receptor Autoantibodies from Patients with Systemic Sclerosis Impact Human Pulmonary Artery Smooth Muscle Cells Function In Vitro. Front Immunol 2017;8:75. DOI: 10.3389/fimmu.2017.00075

19. Svegliati S, Marrone G, Pezone A, et al. Oxidative DNA damage induces the ATM-mediated transcriptional suppression of the Wnt inhibitor WIF-1 in systemic sclerosis and fibrosis. Sci Signal 2014;7(341):ra84. DOI: 10.1126/scisignal.2004592

20. Balada E, Simeon-Aznar CP, Ordi-Ros J, et al. Anti-PDGFR-alpha antibodies measured by non-bioactivity assays are not specific for systemic sclerosis. Ann Rheum Dis 2008;67(7):1027-9. DOI: 10.1136/ard.2007.085480

21. Classen JF, Henrohn D, Rorsman F, et al. Lack of evidence of stimulatory autoantibodies to platelet-derived growth factor receptor in patients with systemic sclerosis. Arthritis Rheum 2009;60(4):1137-44. DOI: 10.1002/art.24381

22. Di Pasquale G, Davidson BL, Stein CS, et al. Identification of PDGFR as a receptor for AAV-5 transduction. Nat Med 2003;9(10):1306-12. DOI: 10.1038/nm929

For Peer Review

23. Pillay S, Meyer NL, Puschnik AS, et al. An essential receptor for adeno-associated virus infection. Nature 2016;530(7588):108-12. DOI: 10.1038/nature16465

24. Pilz IH, Di Pasquale G, Rzadzinska A, et al. Mutation in the platelet-derived growth factor receptor alpha inhibits adeno-associated virus type 5 transduction. Virology 2012;428(1):58-63. DOI: 10.1016/j.virol.2012.03.004

25. Berns K, Parrish CR. Parvoviridae. In: *Fields virology*. Knipe DM, Howley PM, Eds.: Lippincott Williams & Wilkins, Philadelphia, PA, ed. 5, 2007, pp. 2437–77.

26. Geoffroy MC, Salvetti A. Helper functions required for wild type and recombinant adeno-associated virus growth [review]. Curr Gene Ther 2005;5(3):265-71. DOI: 10.2174/1566523054064977

27. Lisowski L, Dane AP, Chu K, et al. Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. Nature 2014;506(7488):382-6. DOI: 10.1038/nature12875

28. Huang LY, Halder S, Agbandje-McKenna M. Parvovirus glycan interactions [review]. Curr Opin Virol 2014;7:108-18. DOI: 10.1016/j.coviro.2014.05.007

29. Domenger C, Grimm D. Next-generation AAV vectors-do not judge a virus (only) by its cover [review]. Hum Mol Genet 2019;28(R1):R3-R14. DOI: 10.1093/hmg/ddz148

30. Van den Hoogen F, Khanna D, Fransen J, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European league against rheumatism collaborative initiative. Ann Rheum Dis 2013;**72**,1747–1755. DOI: 10.1136/annrheumdis-2013-204424.

31. LeRoy EC, Black C, Fleischmajer R, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;**15**, 202–5.

For Peer Review

32. Medsger TA Jr. Natural history of systemic sclerosis and the assessment of disease activity, severity, functional status, and psychologic well-being. Rheum Dis Clin North Am 2003;**29**, 255-73. DOI: 10.1016/s0889-857x(03)00023-1.

33. Govindasamy L, DiMattia MA, Gurda BL, et al. Structural insights into adeno-associated virus serotype 5. J Virol 2013;**87**, 11187–99. DOI: 10.1128/JVI.00867-13.

34. Comeau SR, Gatchell DW, Vajda S, et al. ClusPro: an automated docking and discrimination method for the prediction of protein complexes. Bioinformatics 2004;**20**, 45–50. DOI: 10.1093/bioinformatics/btg371.

35. Grosdidier A, Zoete V, Michielin O. SwissDock, a protein-small molecule docking web service based on EADock DSS. Nucleic Acids Res 2011;**39**,W270-7. DOI: 10.1093/nar/gkr366.

36. Moroncini G, Cuccioloni M, Mozzicafreddo M, et al. Characterization of binding and quantification of human toantibodies to PDGFRα using a biosensor-based approach. Anal Biochem 2017;**528**, 26–33. DOI: 10.1016/j.ab.2017.04.011.

37. Kuwana M, Distler O. Recent progress and missing gaps to achieve goal in the care of systemic sclerosis-associated interstitial lung disease [editorial]. J Scleroderma Relat Disord 2020;5(2 Suppl):3-5. DOI: 10.1177/2397198320902551

38. Hoffmann-Vold AM, Allanore Y, Alves M, et al. Progressive interstitial lung disease in patients with systemic sclerosis-associated interstitial lung disease in the EUSTAR database. Ann Rheum Dis 2021;80(2):219-27. DOI: 10.1136/annrheumdis-2020-217455

39. Whitsett JA, Weaver TE. Hydrophobic surfactant proteins in lung function and disease. N Engl J Med 2002; 347 (26): 2141. DOI: 10.1056/NEJMra022387

40. Balka G, Ladinig A, Ritzmann M, et al. Immunohistochemical characterization of type II pneumocyte proliferation after challenge with type I porcine reproductive and respiratory syndrome virus. J Comp Pathol 2013;149(2-3):322-30. DOI: 10.1016/j.jcpa.2012.12.006

41. Zhang R, Xu G, Cao L, et al. Divergent engagements between adeno-associated viruses with their cellular receptor AAVR. Nat Commun 2019;10(1):3760. DOI: 10.1038/s41467-019-11668-x

42. Kruzik A, Fetahagic D, Hartlieb B, et al. Prevalence of Anti-Adeno-Associated Virus Immune Responses in International Cohorts of Healthy Donors. Mol Ther Methods Clin Dev 2019;14:126-133. DOI: 10.1016/j.omtm.2019.05.014

43. Summerford C, Johnson JS, Samulski RJ. AAVR: A Multi-Serotype Receptor for AAV [comment]. Mol Ther 2016;24(4):663-6. DOI: 10.1038/mt.2016.49

44. Martin JE, Assassi S, Diaz-Gallo LM, et al. A systemic sclerosis and systemic lupus erythematosus pan-meta-GWAS reveals new shared susceptibility loci. Hum Mol Genet 2013;22(19):4021-9. DOI: 10.1093/hmg/ddt248

45. Lunardi C, Bason C, Navone R, et al. Systemic sclerosis immunoglobulin G autoantibodies bind the human cytomegalovirus late protein UL94 and induce apoptosis in human endothelial cells. Nat Med 2000;6(10):1183-6. DOI: 10.1038/80533

46. Lunardi C, Dolcino M, Peterlana D, et al. Antibodies against human cytomegalovirus in the pathogenesis of systemic sclerosis: a gene array approach. PLoS Med 2006;3(1):e2. DOI: 10.1371/journal.pmed.0030002

47. Bonner JC. Regulation of PDGF and its receptors in fibrotic diseases [review]. Cytokine Growth Factor Rev 2004;15(4):255-73. DOI: 10.1016/j.cytogfr.2004.03.006

For Peer

48. Bjornevik K, Cortese M, Healy BC et al Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis Science 2022;375(6578):296-301. DOI: 10.1126/science.abj8222

49. Lanz TV, Brewer RC, Ho PP, et al. Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. Nature 2022;603(7900):321-27. DOI: 10.1038/s41586-022-04432-7

50. Robinson WH, Steinman L. Epstein-Barr virus and multiple sclerosis Science 2022; 375:264-265 DOI: 10.1126/science.abm7930

51. Golan M, Krivitsky A, Mausner-Fainberg K et al. Increased Expression of Ephrins on Immune Cells of Patients with Relapsing Remitting Multiple Sclerosis Affects Oligodendrocyte Differentiation. Int J Mol Sci. 2021; 22: 2182. DOI: 10.3390/ijms22042182

.

iew

ACKNOWLEDGMENTS

We thank Prof. Rosaria Gesuita, Università Politecnica delle Marche, Ancona, Italy, for statistical analysis; Prof. Michael S. Chapman and Dr. O. Davulcu (Oregon Health & Science University, Portland, Oregon, USA) for the bacterially expressed fusion protein of PKD domains 1-5 of adeno-associated virus receptor (AAVR) described by Pillay et al (17); Dr Lorena Zentilin of International Centre for Genetic Engineering and Biotechnology (ICGEB) Trieste, Italy for AAV5 and AAV2 capsids; Dr. Gianni Di Maio for his help in the preparation of the manuscript.

AUTHOR CONTRIBUTION

A.G. and E.V.A. performed the experimental design, the data acquisition and interpretation, and wrote the manuscript. G.M. performed in silico molecular docking, SPR experiments, data acquisition and interpretation and provided critical edits to the text. S.S. performed the immunoaffinity purification of anti PDGFRa antibodies. A. La Cava provided relevant contribution to set up the AAV5 PCR ,and provided critical edits to the text. A.A. and S. A. provided critical analysis of the experimental procedures and provided critical edits to the text. D.A., S. G. and D.B. contributed to BAL and blood samples collection and patient clinical data analysis. S.M. performed the AAV5 capsid gene sequencing. A.G. and M.S. performed the confocal microscopy experiments, interpreted the data and provided critical edits to the text. P.P. and A. Amoresano provided critical edits to the text. P.D.,T.S. and D.F. performed the immunohistochemistry and in situ hybridization experiments and interpreted the data. A.F. cloned the human monoclonal anti PDGFRa antibodies from SSc PBMCs. M.C. and M.M. performed in silico molecular docking, SPR experiments, data acquisition and interpretation. M.G. provided crucial input for PBMCs stimulation experiments and

interpreted the data. A. G. C.P., S.A. C.T. and N.V. performed the PCR experiments. M.G., G.P., Q.C., K.N. M.M. and J.K. performed the AAV5 particles production, the experiments with the A549 cell line and provided valuable information regarding AAV5. **A.P. Performed the quantification of AAV5 in SSc lung section and in peripheral blood mononuclear cells.**

DATA AVAILABILITY STATEMENT

Access to primary datasets (generated during the study) and referenced datasets (datasets analyzed in the study) are available

Figure legends

Figure 1. AAV5 and PDGFRα expression in lung tissue of SSc patients

A representative experiment from one SSc patient is shown **A,left,** Immunohistochemical staining of the lung of one SSc patient shows AAV5 in cells lining the alveolar space resembling the cytological appearance of type II pneumocytes (arrows). **A, right**, No staining was detected with an isotype control antibody Scale bar, 60 μm. **B**, **left**, Representative microscopic images of chromogenic *in situ* hybridization for AAV5 was applied to paraffin-embedded lung sections from two SSc patients and two control subjects. A labeled DNA probe was used to hybridize to AAV5 DNA sequence. Strong signals for AAV5 set specifically in pneumocytes (arrows). **B, right**, Antisense probe was used as control**.** Scale bar, 60 μm. **C,** A lung section from one SSc patient is shown at low magnification. AAV5 capsid-specific staining (left panels, green) is present in several epithelial cells lining the alveolar cavities (Alv), that are also positive for the type II pneumocytes (P) marker proSP-C (middle panels, red). The larger area framed in the right upper panel is enlarged in the lower panels, while the smaller area is shown as insets of the upper panels. They both show at higher magnification proSP-C-positive pneumocytes infected by AAV5 (arrows). Asterisks (*) indicate bulk of collagen and other extracellular matrix components that exhibit autofluorescence. Semiquantitative analysis showed that 17 ± 6.2% of pro SP-C + type II pneumocytes expressed AAV5**.** **D,** AAV5-positive pneumocytes (P; left panel, green) are variably positive for PDGFRα (middle panel, red) that is mainly detectable on their cellular luminal side (arrowheads), indicating colocalization of the two (right panel). In the interstitial space (Int), several fibroblast-like cells (F) with their projections (f) are strongly positive for PDGFRα. Alv, alveolar cavity; Cap, capillary.

For Peer Review

Figure 2. AAV5 genomic sequences in bronchoalveolar lavage fluid from SSc patients and controls

A, Percentage of SSc patients (n=66) and controls with lung disease other than SSc (n=66) positive for AAV5 DNA in cells recovered from bronchoalveolar lavage fluid (BAL. **B,** AAV5 DNA copy number in cells recovered from BAL of SSc patients (n=41) and controls (n=17**)** who tested positive for AAV5. Data are expressed as a median value and a range (SSc: median 20,595; range, 260-1.3x10^6. Controls: median 3800; range 720-16 x10^4).

*** p <0.001.

Figure 3. Binding of AAV5 to human PDGFRα

A. Molecular docking model predicting the binding of AAV5 capsid monomeric subunit (green) to the extracellular region of monomeric PDGFRα (gray). AAV5 capsomer binding site lies between the second and third PDGFRα Ig-like extracellular domains (indicated in roman numerals from V to I)**. B,** Binding curves (measured in arc/seconds [arcsec] over time) of histidin-tagged monomeric recombinant human PDGFRα immobilized on the biosensor chip, after saturation with different concentrations (expressed in nanomoles, nM) of AAV5 capsid monomeric subunits. AAV2 capsid monomeric subunits were tested as control. Dissociation constants (Kd) are indicated in the boxes. **C,** The immunoblot of A549 cells before (wild-type, wt) and after PDGFRα knockout (KO) by CRISP/Cas 9 technology. **D**, AAV5 and AAV2 transduction in wt and PDGFRα KO A549 cells (A549-KO) is shown as a mean ± SD of three independent experiments**.** ***p< 0.001.

Figure 4. Immune responses against AAV5-PDGFRα spatially contiguous epitopes

A, Binding curves (measured in arc/seconds [arcsec] over time) of V_HPAM-V_κ16F4 immobilized on the biosensor chip after saturation with different concentrations (expressed

For Peer Review

in nanomoles, nM) of AAV5 (left panel) and AAV2 (right panel). Dissociation constants (Kd) are indicated in the boxes. **B,** Molecular docking model predicting binding of V_HPAM-V_κ16F4 to the complex formed by AAV5 capsid monomeric subunit and the extracellular region of monomeric PDGFRα. The predicted epitopes of V_HPAM-V_κ16F4 are indicated as PDGFRα-pep (gray) and AAV5-pep (green). **C,** Binding curves (measured in arc/seconds [arcsec] over time) of V_HPAM-V_κ16F4 antibody probing rhPDGFRα-His immobilized on the biosensor chip and saturated with AAV5 capsid monomeric subunits are shown. V_HPAM-V_κ16F4 binding to this complex was measured before (curve A) and after preincubation with an AAV5-peptide (curve B) or PDGFRα-peptide (curve C) or both mixed (curve D). **D,** Experiment performed as in **D** but V_HPAM-V_κ16F4 was replaced by immunoaffinity purified, SSc anti-PDGFRα antibodies.

For Peer Review

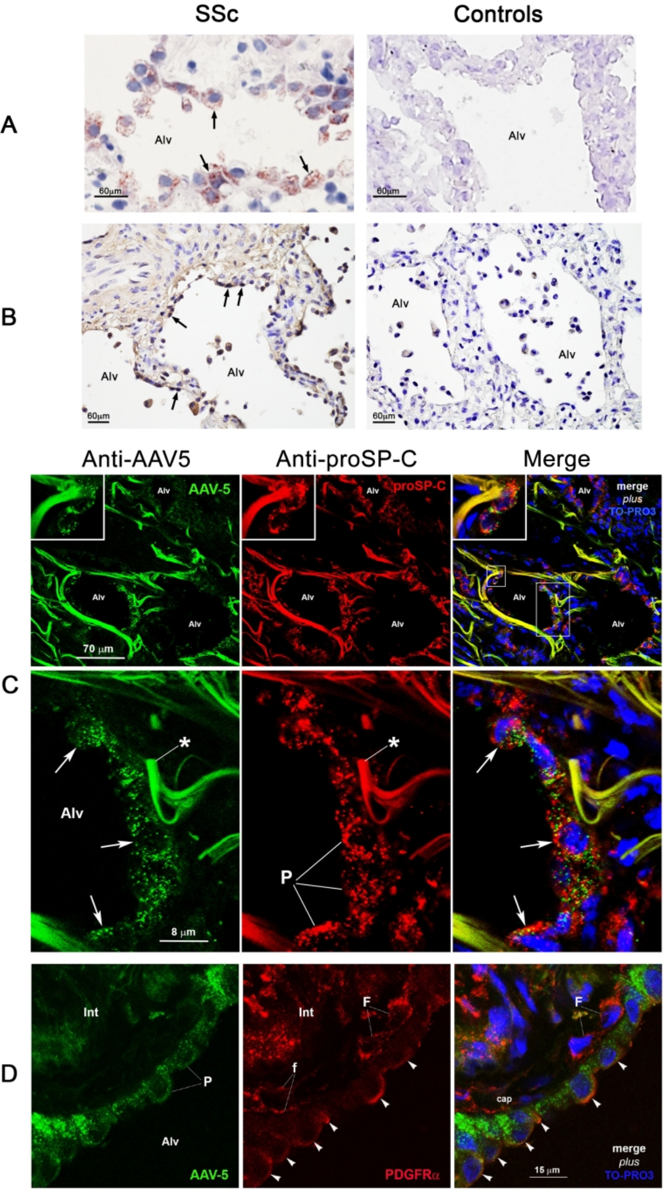


Figure 1

167x296mm (300 x 300 DPI)

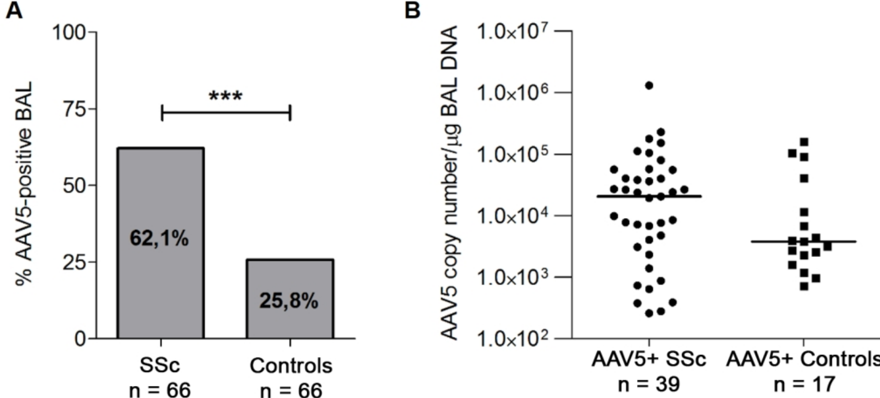


Figure 2

150x80mm (300 x 300 DPI)

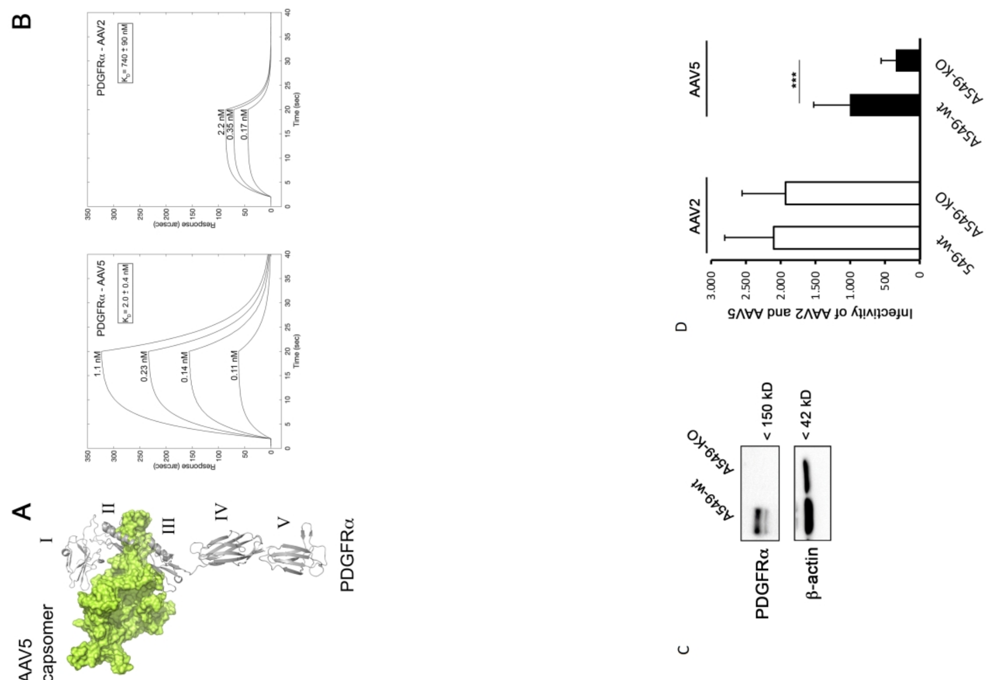


Figure 3

396x281mm (192 x 192 DPI)

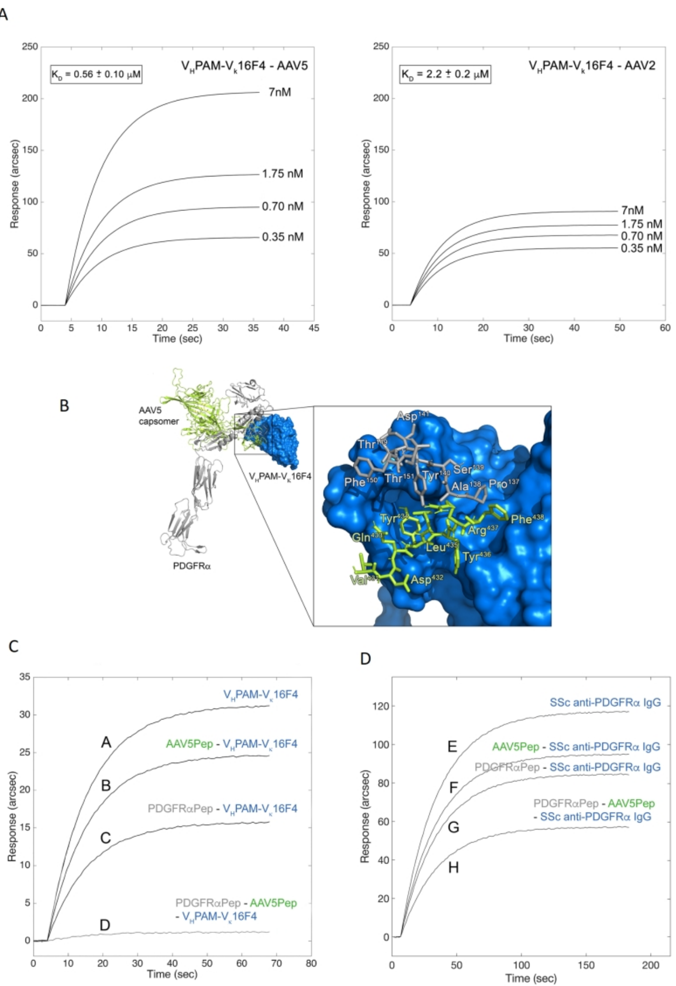


Figure 4

167x279mm (300 x 300 DPI)

Supplementary Figures

Figure S1 Confocal microscopy shows AAV5 capsid in TTF 1-positive pneumocytes

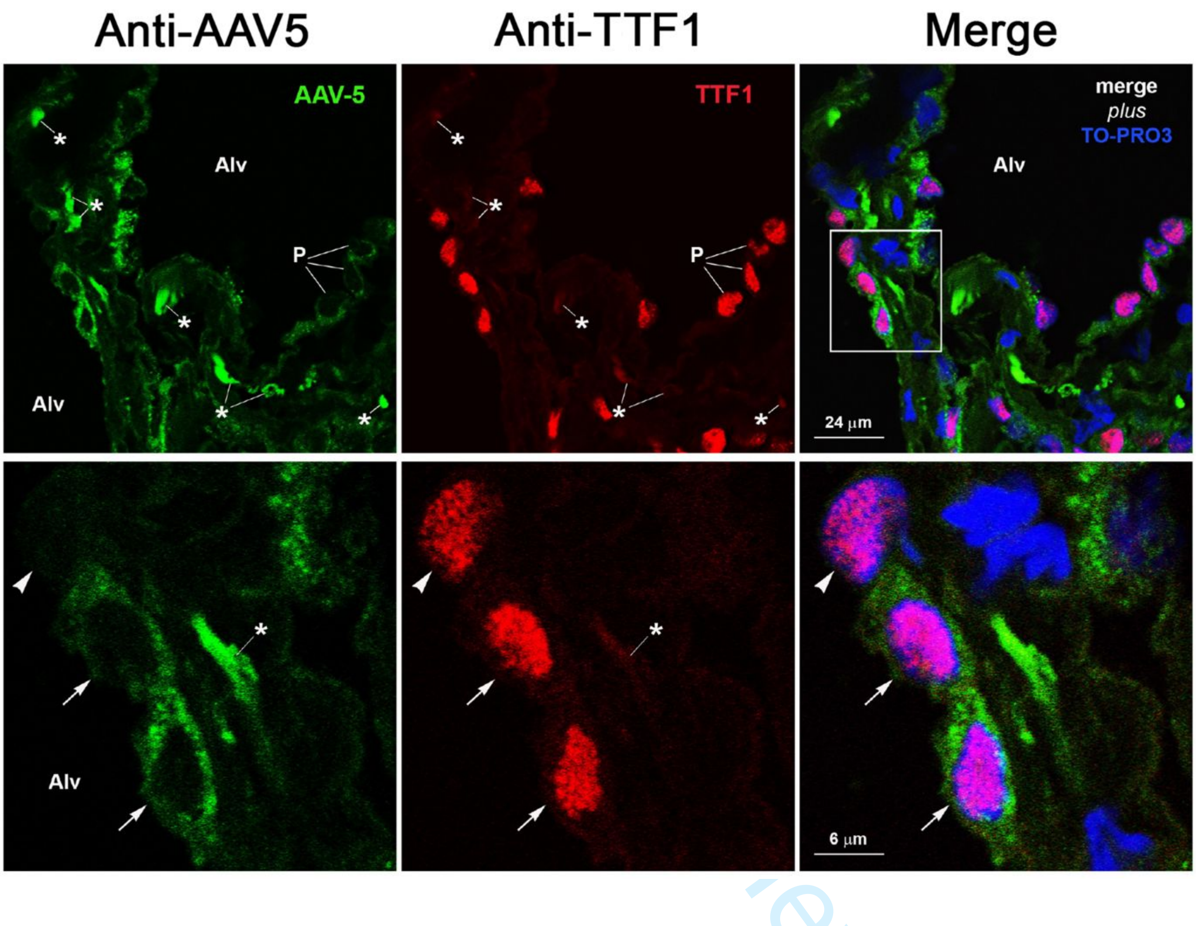


Figure S2 Quantification of AAV5 capsid-PDGFRα co-localization in SSc lung sections.

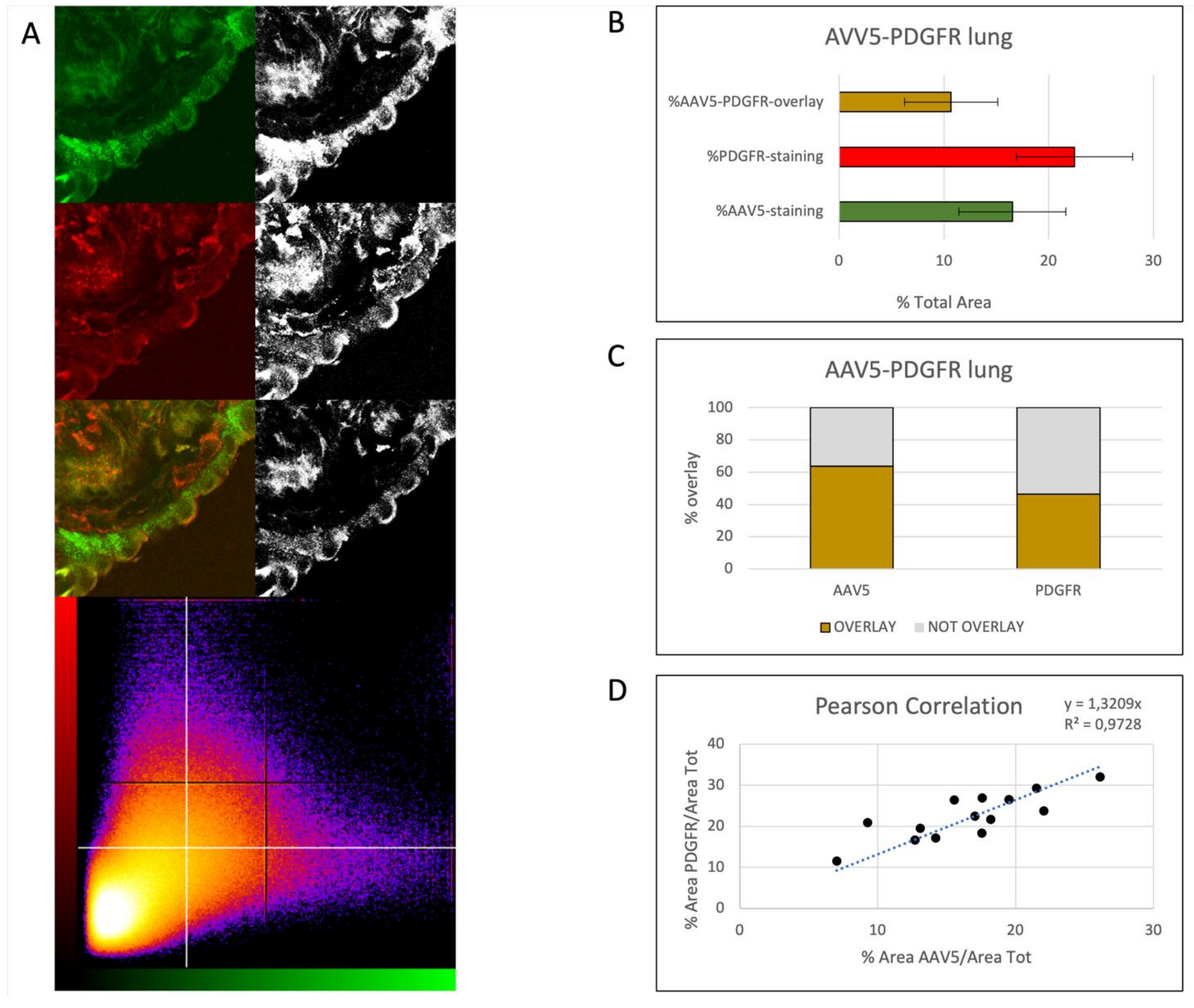
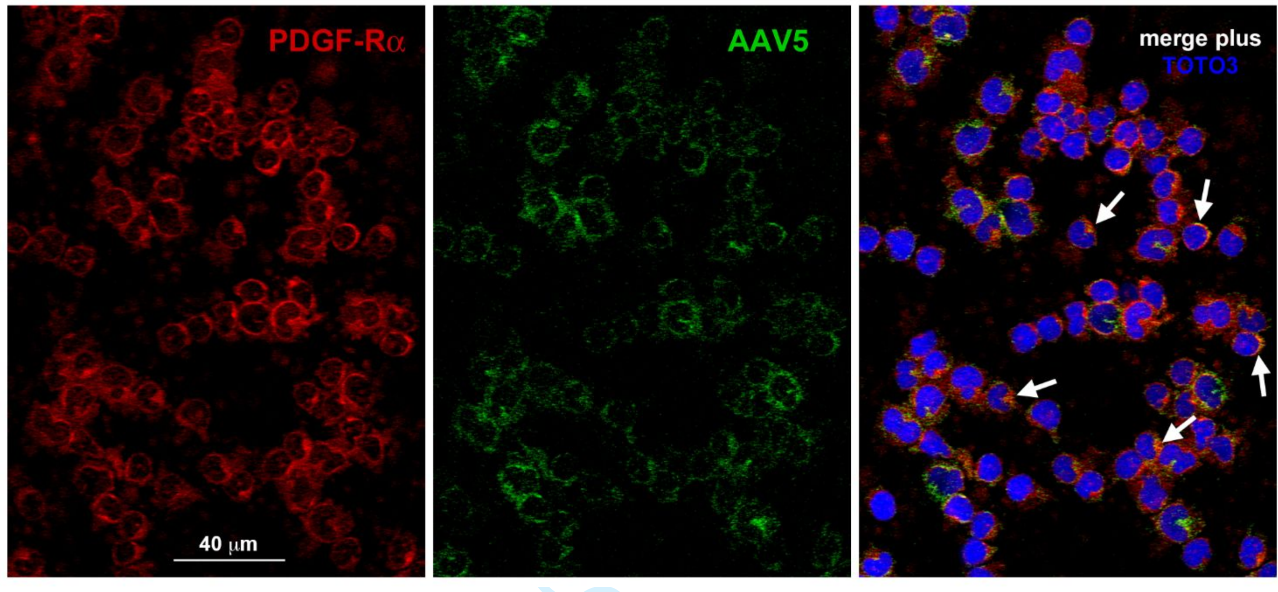
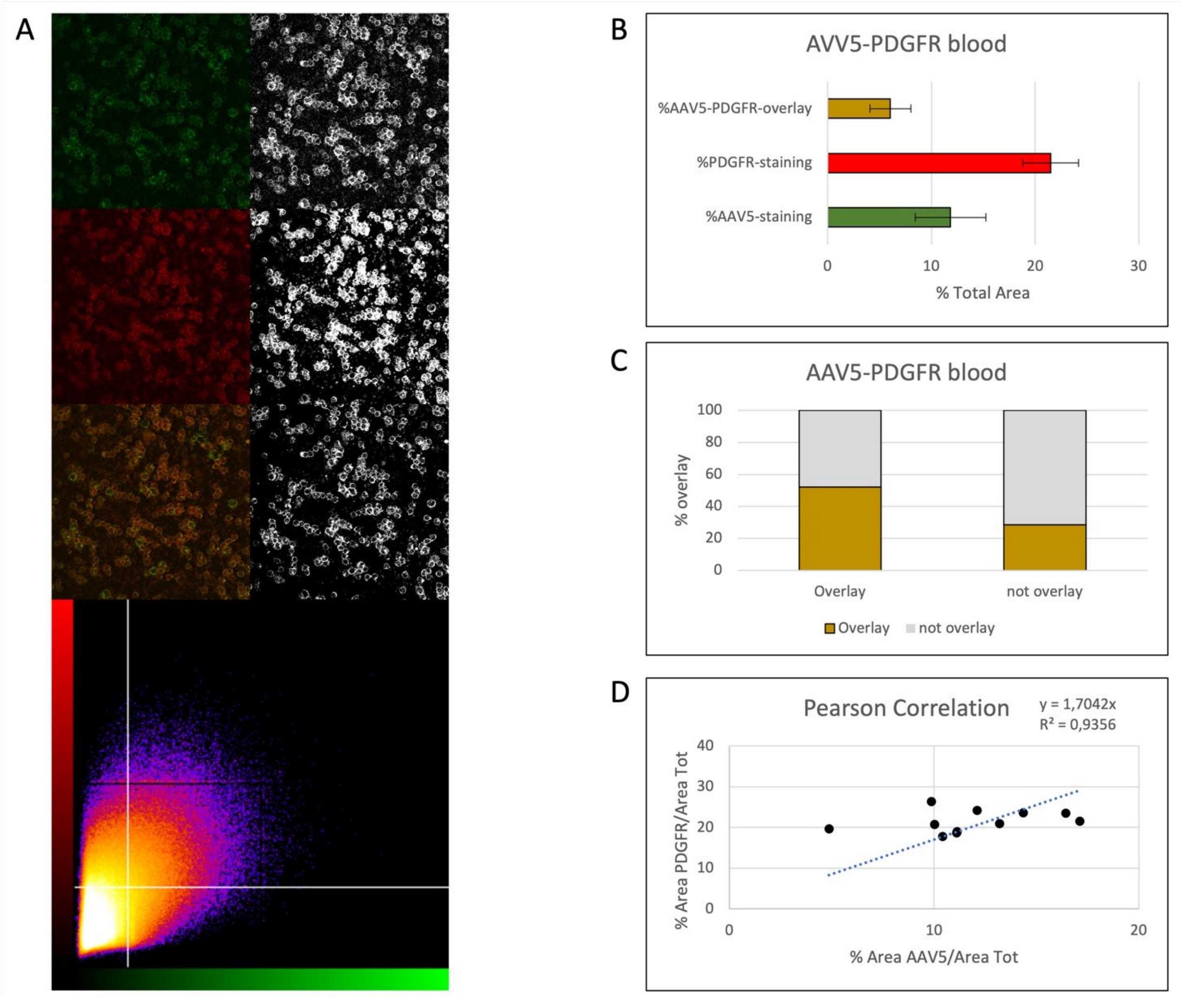


Figure S3 Confocal microscopy shows co-localization of PDGFRα and AAV5 capsid in peripheral blood mononuclear cells of one SSc patient



For Peer Review

Figure S4 Quantification of AAV5 capsid-PDGFRα co-localization in SSc peripheral blood mononuclear cells.



4

Figure S5. Distinct sites and kinetics of AAV5 binding to transmembrane protein KIAA0319L (AAVR) and PDGFRα.

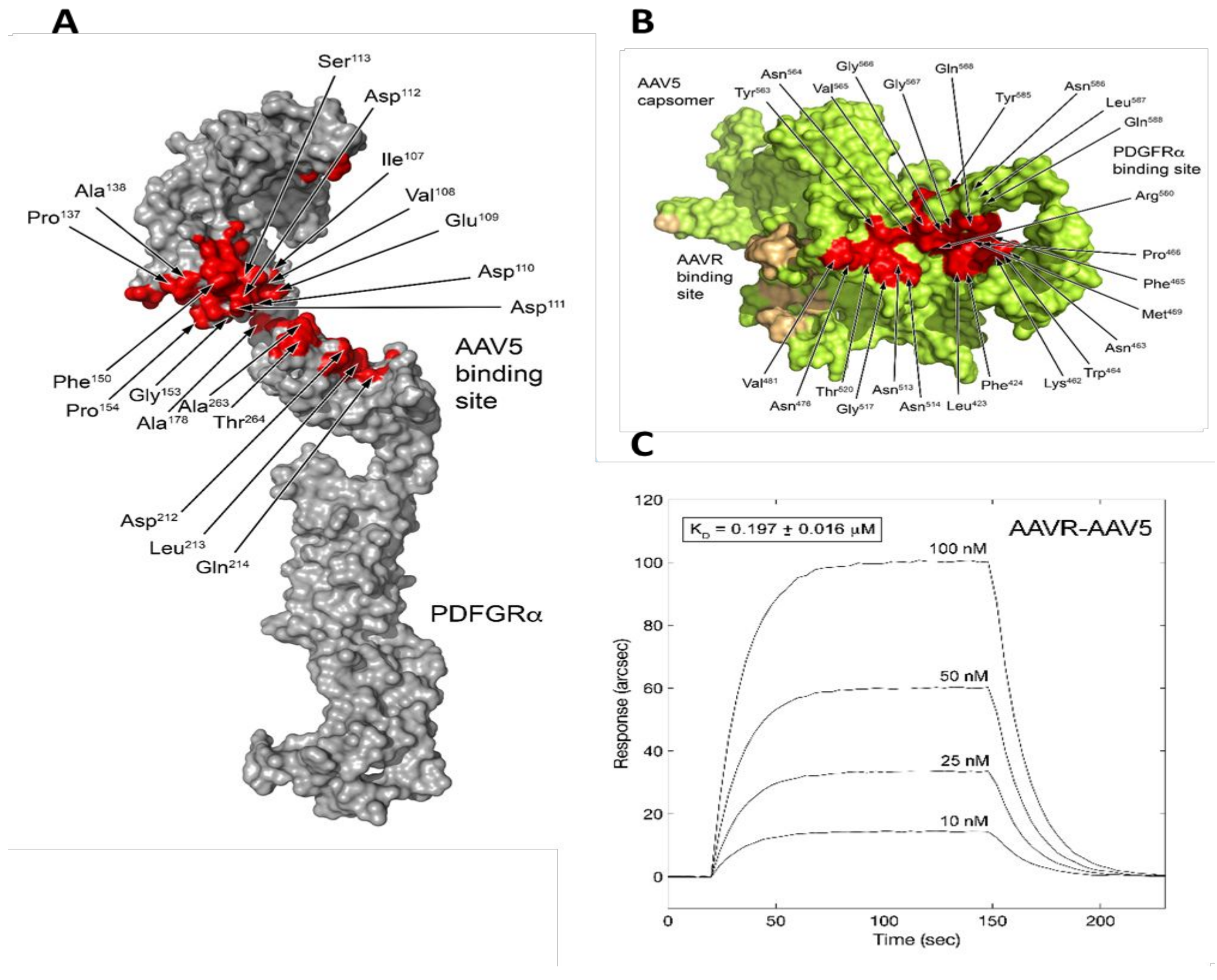


Figure S6. Energy-based docking between trans-peptides and V_HPAM-V_κ16F4

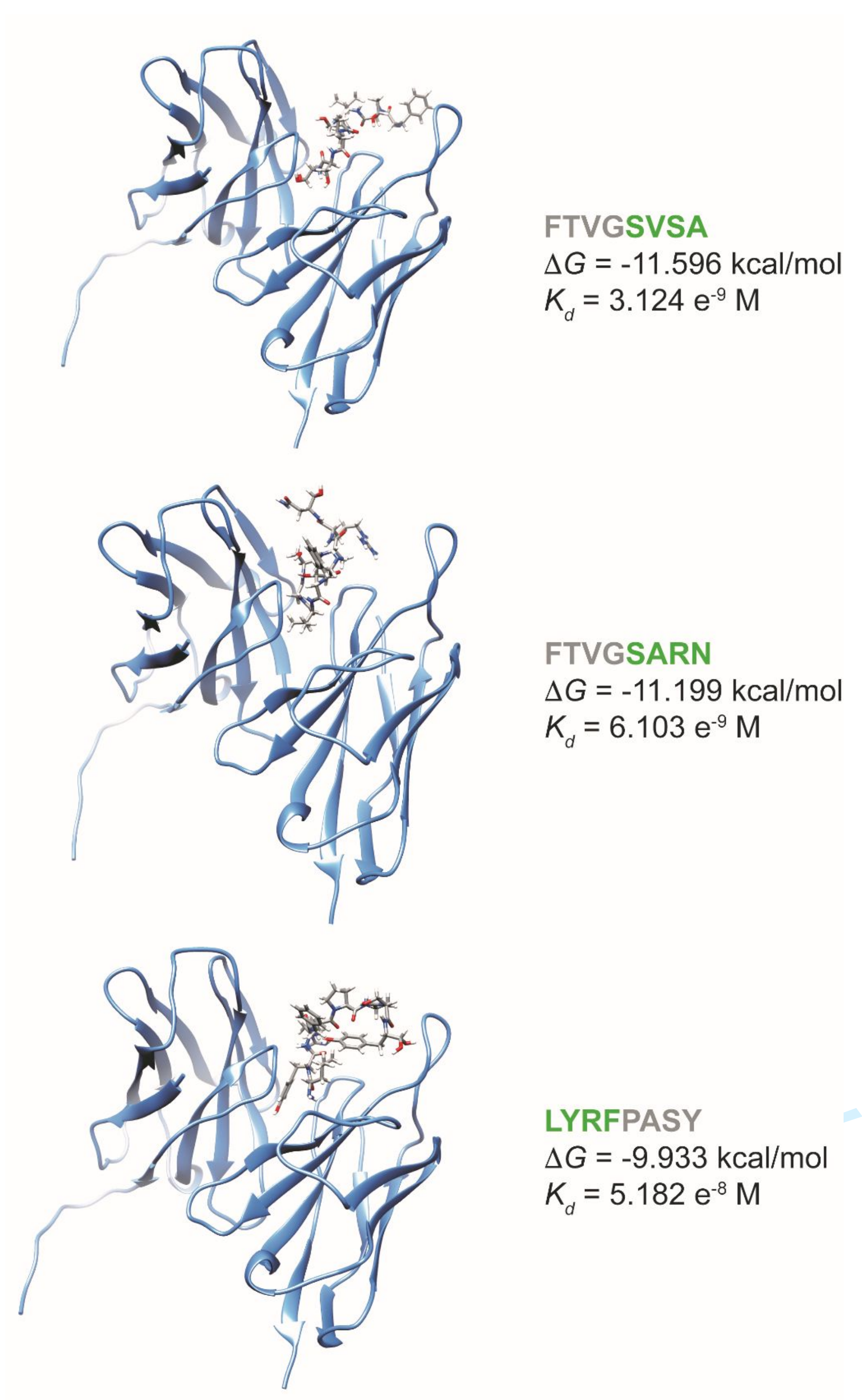
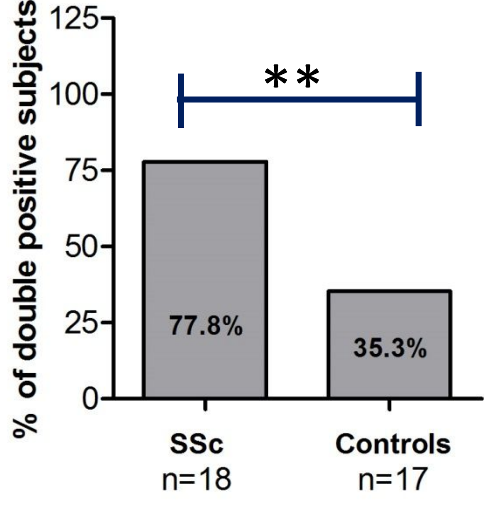


Figure S7 **Antibodies against AAV5 and PDGFRα in SSc patients and controls**



er Review

Legends of supplementary figures

Figure S1. Confocal microscopy shows AAV5 capsid in TTF 1-positive pneumocytes

A lung section from one SSc patient is shown. AAV5 capsid-specific staining (left panels, green) is present in several epithelial cells lining the alveolar cavities (Alv), that are also positive for the type II pneumocytes (P) nuclear marker TTF1 (middle panels, red). The area framed in the right upper panel is enlarged in the lower panels and shows at higher magnification two TTF1-positive pneumocytes strongly infected by AAV5 (arrows). Note the presence of a TTF1-positive pneumocyte devoid of any evident AAV5 staining (arrowheads). Asterisks (*) indicate bulk of collagen and other extracellular matrix components that exhibit autofluorescence. Semiquantitative analysis showed that 11 ± 4.5 % of TTF 1 + type II pneumocytes expressed AAV5.

For Peer Review

Figure S2. Quantification of AAV5 capsid-PDGFRα co-localization in SSc lung sections.

A) Confocal microscopy image analysis of AAV5-PDGFRα shown in Figure 1D using the Fiji JACoP plug-in, which performs a collection of recent general indicators of co-location (doi: 10.1111/j.1365-2818.2006.01706.x; doi: 10.1038/s41556-023-01098-9). The dependency of pixels of dual channel section is shown as pixel grey values of two images against each other. Results are then displayed in a pixel distribution diagram called scatter plot or fluorogram. The intensity of a given pixel in the green image is used as the x-coordinate of the scatter plot and the intensity of the corresponding pixel in the red image as the y-coordinate. The obtained colored clouds were used to calculate the different indices shown in the Supplementary table S2. Values of colocalization indicators and comparative analysis of additional images are reported in supplementary table S2. **B)** Percentage of AAV5, PDGFRα, and double positive staining (AAV5- PDGFRα) versus the

total area of multiple SSc lung sections. **C)** Percent overlap between AAV5 and PDGFRα with respect to AAV5 and PDGFRα staining, respectively. **D)** Pearson's correlation between the percentage of AAV5 and PDGFRα in SSC lung sections. For more details see Methods.

Figure S3. AAV5 and PDGFRα expression in peripheral blood mononuclear cells of one SSc patient

PDGFRα is present in several peripheral blood mononuclear cells (left panel, red), while the presence of AAV5 is detectable in the middle panel (green). Arrows indicate co-localization of the two (right panel).

Figure S4. Quantification of AAV5 capsid-PDGFRα colocalization in SSc peripheral blood mononuclear cells.

A) Confocal microscopy image analysis of AAV5- PDGFRα shown in Figure 1D using the Fiji JACoP plug-in, which performs a collection of recent general indicators of co-location (DOI: 10.1111/j.1365-2818.2006.01706.x, DOI: 10.1038/s41556-023-01098-9). Values of colocalization indicators and comparative analysis of additional images are reported in supplementary table S3.The dependency of pixels of dual channel section is shown as pixel grey values of two images against each other. Results are then displayed in a pixel distribution diagram called scatter plot or fluorogram. The intensity of a given pixel in the green image is used as the x-coordinate of the scatter plot and the intensity of the corresponding pixel in the red image as the y-coordinate. The obtained colored clouds were used to calculate the different indices shown in the supplementary table 2. Values of co-location indicators and comparative analysis of additional images are reported in

For Peer Review

supplementary table 2. **B)** Percentage of AAV5, PDGFRα, and double positive staining (AAV5- PDGFRα) versus the total area of multiple SSC lung sections. **C)** Percent overlap between AAV5 and PDGFRα with respect to AAV5 and PDGFRα staining, respectively. **D)** Pearson's correlation between the percentage of AAV5 and PDGFRα in SSC lung sections. For more details see Methods.

For Peer Review

Figure S5. Distinct sites and kinetics of AAV5 binding to transmembrane protein KIAA0319L (AAVR) and PDGFRα.

A, Molecular docking model predicting binding site of AAV5 capsid monomeric subunit to the extracellular region of monomeric PDGFRα (gray). The amino acid residues in the extracellular region of monomeric PDGFRα at interface with AAV5 capsid are colored in red**.** **B,** The amino acid residues of AAV5 capsid at the interface with PDGFRα are colored in red, whereas those previously reported at the interface with AAVR (23) are in gold.

Binding curves (measured in arc/seconds [arcsec] over time) of protein KIAA0319L (AAVR) immobilized on the biosensor chip, after saturation with different concentrations (expressed in nanomoles, nM) of AAV5 capsid monomeric subunits are shown in **C**. Dissociation constant (Kd) is indicated in the box. For comparison with PDGFRα-AAV5 binding curves see figure 3 B left panel.

Figure S6. Energy-based docking between trans-peptides and V_HPAM-V_κ16F4

Molecular docking models predicting the binding of trans-peptides, derived from AAV5 capsid monomeric subunits (green; SVSA, SARN, LYRF) and from the extracellular region of monomeric PDGFRα (gray; FTVG, PASY), and the autoantibody V_HPAM-V_κ16F4. For

each complex are reported the free energies of binding (ΔG) and the equilibrium dissociation constants (K_d).

Figure S7. Antibodies against AAV5 and PDGFRα in SSc patients and controls

A significantly higher percentage of SSc patients tested seropositive for both anti-PDGFRα and anti-AAV5 antibodies as compared to controls by ELISA (77.8%; 95% C.I. 52.4 - 93.6 vs 35.3%; 95% C.I. 14.2 - 61.8; **p = 0.028).

For Peer Review

SUPPLEMENTARY TABLES

Table 1 AAV5 expression in SSc-ILD patients* and controls by immunohistochemistry**

Patients	Interstitial fibrosis§ (degree)	Anti AAV5 capsid mouse mAb^ (degree)
SSc-ILD-PAH 1	-	+
SSc-ILD-PAH 2	++	++
SSc-ILD-PAH 3	+	+
SSc-ILD-PAH 4	+++	++ epith(P)/inter
SSc-ILD-PAH 5	++	++
Control 1	-	(+) inter #
Control 2	-	(+) inter
Control 3	-	(+) inter
Control 4	-	(+) inter
Control 5	-	(+) inter
Control 6	-	(+) inter

* All SSc had ILD and PAH; § Interstitial fibrosis was assessed by an experienced lung pathologist (PD). ** Control lung tissues were from patients undergoing lobectomy or pneumonectomy for lung cancer. The categories - =none, +=mild, ++=moderate, +++=severe fibrosis were assigned after analysis of at least 5 slides stained with HES collagen staining. ^ the mouse monoclonal antibody anti AAV5 capside (ADK 5b) was used; # (+) inter indicates dubious positive interstitial cells. Positivity was evident in pneumocytes.of SSc lungs.

For Peer Review

Table 2 Multiple quantification of AAV5 capsid-PDGFRα colocalization in SSc lung sections.

Overlay Name	Pearson's Coefficient	Spearman's Rank Coefficient	Overlap Coefficient	Area tot	Area A	Area B	Area Overlap	% AreaA/tot	% AreaB/tot	% Area Overlap/tot	% AAV5	% PDGFR
041219_Overlay_01	0.833	0.681	0.920	1048576	148998	181103	102019	14.209	17.271	9.729	68.470	56.332
041219_Overlay_03	0.675	0.713	0.877	1048576	184293	283326	129973	17.575	27.020	12.395	70.525	45.874
041219b_Overlay_02	0.773	0.799	0.897	1048576	274169	336982	233597	26.146	32.137	22.277	85.201	69.320
061119c_Overlay_01	0.625	0.740	0.838	1048576	137409	206421	86511	13.104	19.685	8.250	62.958	41.909
061119c_Overlay_02	0.597	0.748	0.836	1048576	184212	193149	96041	17.567	18.420	9.159	52.136	49.723
091219_Overlay_00b	0.650	0.503	0.876	1048576	133230	176008	80106	12.705	16.785	7.639	60.126	45.512
091219_Overlay_02	0.505	0.601	0.840	1048576	190833	228359	100600	18.199	21.778	9.593	52.716	44.053
091219_Overlay_05	0.473	0.594	0.849	1048576	231223	250088	121679	22.051	23.850	11.604	52.624	48.654
091219_Overlay_09	0.525	0.577	0.849	1048576	97141	220264	56557	9.264	21.006	5.393	58.221	25.676
111219b_Overlay_00	0.633	0.395	0.862	1048576	73785	122038	46768	7.036	11.638	4.460	63.384	38.322
111219b_Overlay_03b	0.490	0.551	0.891	1048576	204649	279019	122698	19.516	26.609	11.701	59.955	43.974
111219b_Overlay_04	0.481	0.464	0.848	1048576	178860	236895	99124	17.057	22.592	9.453	55.419	41.843
191119b_Overlay_02	0.720	0.761	0.865	1048576	163194	277965	124538	15.563	26.508	11.876	76.312	44.803
191119b_Overlay_05	0.726	0.770	0.882	1048576	225671	307853	168763	21.216	29.359	16.094	74.782	54.819

Data are presented as coefficients, positive pixel area, count or number (%) as appropriate. All data was obtained using the Fiji JACoP plug-in.

For more details see Methods

Table 2 Multiple quantification of AAV5 capsid-PDGFRα colocalization in SSC peripheral blood mononuclear cells.

Overlay Name	Pearson's Coefficient	Spearman's Rank Coefi	Overlap Coefficient	Area tot	Area A	Area B	Area Overlap	% AreaA/tot	% AreaB/tot	% Area Overlap/tot	% AAV5	% PDGFR
aav 011019_Overlay_01	0.581	0.526	0.843	1048576	179258	225947	107379	17.095	21.547	10.240	59.901	47.523
aav 300919_Overlay_07	0.485	0.390	0.881	1048576	171938	246379	98198	16.397	23.496	9.364	57.112	39.856
citospin 21-10-21_Overlay_07	0.296	0.132	0.775	1048576	150209	248277	57409	14.325	23.677	5.474	38.219	23.122
citospin 21-10-21_Overlay_08	0.385	0.166	0.773	1048576	138116	219704	58019	13.171	20.952	5.533	42.007	26.407
citospin 21-10-21_Overlay_10bis	0.460	0.222	0.783	1048576	50896	206931	39041	4.853	19.734	3.723	76.707	18.866
citospin 21-10-21_Overlay_11	0.238	0.176	0.794	1048576	126600	254385	54836	12.073	24.260	5.229	43.314	21.556
citospin 21-10-21_Overlay_16	0.433	0.323	0.798	1048576	104824	218012	60384	9.996	20.791	5.758	57.605	27.697
pdgfr-aav5 2_Overlay_00	0.250	0.116	0.812	1048576	103256	276766	44457	9.847	26.394	4.239	43.055	16.063
pdgfr-aav5 2_Overlay_07	0.433	0.282	0.831	1048576	109020	187130	59040	10.396	17.8461	5.630	54.155	31.550
pdgfr-aav5 2_Overlay_08	0.406	0.270	0.836	1048576	116070	196294	58792	11.069	18.720	5.606	50.652	29.950
pdgfr-aav5 2_Overlay_09	0.414	0.261	0.819	1048576	116295	198890	59859	11.090	18.967	5.708	51.471	30.096

Data are presented as coefficients, positive pixel area, count or number (%) as appropriate. All data was obtained using the Fiji JACoP plug-in.

For more details see Methods

Table 4 Demographic characteristics of SSc patients and controls (C)

	SSc (n=66)	C (n=66)	p
Age (years)	56.9 ± 15.2	62.3 ± 15.1	0.04
Sex (F)	53 (80%)	38 (57.6%)	0.004
Disease subgroups	lcSSc 37 (56%) dcSSc 29 (44%)	Sarcoidosis 14 (21.2%) IPF 14 (21.2%) Vasculitis 9 (13.6%) ILD in other CTDs** 12 (18.2%) Other diagnoses# 17 (25.8%)	N/A
Mean duration of disease (years)*	4.2 ± 5.4	3 ± 6	0.21
Autoantibodies	ANA 58 (88%) Anti-centromere 18 (15.1%) Anti-topo I 43 (65.1%)	N/A	N/A
Organ involvements	Lung 66 (100%) Skin 62 (94%) Esophageal 46 (70%) Joint and muscle 5 (7.5%) PAH 2 (3%) Other 3 (4.5%)	Lung 66 (100%) Skin 8 (12.1%) Kidneys 4 (6%) Joint and muscle 8 (12.1%) Vascular 4 (6%) Nervous 2 (3%)	N/A
Mean mRSS	11.8 ± 8.3	N/A	N/A
PFTs ¥	Mean FVC (L) 2.5 ± 0.7 Mean TLC (L) 4.0 ± 1.0 Mean DLCO (mL) 9.8 ± 6.8	Mean FVC (L) 2.7 ± 0.9 Mean TLC (L) 3.9 ± 1.0 Mean DLCO (mL) 14.7 ± 7.4	0.10 0.08 0.004
Therapy	PPI 51 (77%) Corticosteroids 30 (45%)° Intravenous prostanoids 17 (26%) Immunosuppressants 7 (11%)§ ERA 7 (11%)	PPI 25 (37.8%) Corticosteroids 17 (25.7%) Immunosuppressants 2 (3%)	N/A

Data are presented as mean ± SD or number (%) as appropriate. N/A: not applicable. C: controls were patients with lung disease other than SSc ILD; Lc:limited cutaneous systemic sclerosis; dcSSc: diffuse cutaneous systemic sclerosis; IPF: idiopathic pulmonary fibrosis; ILD: interstitial lung disease; CTDs: connective tissue diseases; ANA anti-nuclear antibodies; anti topo I: anti-topoisomerase I

For Peer Review

antibodies; PAH: pulmonary arterial hypertension; mRSS: modified Rodnan Skin Score; PFTs: pulmonary function tests; PPI: protun pump inhibitors; ERS: endothelin receptor antagonists.

*from the first non-Raynaud's symptoms.

° mean dose of corticosteroids was less than 5 mg/day in SSc patients and 10 mg/day in controls

§ Azathioprine 3 patients; cyclosporine 1 patient; methotrexate 1 patient; cyclophosphamide 1 patient; mycophenolate mofetil 1 patient. All Immunosuppressive drugs were discontinued at least 6 weeks before enrollment

** Other CTDs include: systemic lupus erythematosus, polymyositis, mixed connective tissue disease, rheumatoid arthritis.

Other diagnoses include: cryptogenic organizing pneumonia, hypersensitivity pneumonitis, polychondritis, eosinophilic pneumonia.

¥ Available in all patients and controls

Table 5. Comparison between SSc patients with BAL tested positive and negative for AAV5

	BAL positive (N=41)	BAL negative (N=25)	P
Age	57.7 ± 2.2	55.5 ± 3.1	0.57
Female	35 (85.4%)	18 (72%)	0.18
lcSSc	21 (51.2%)	9 (36%)	0.31
Disease duration	5.3 ± 5.9	2.4 ± 4	0.03
Autoantibodies			
ANA	36 (87.8%)	22 (88%)	0.99
Anti-centromere	8 (19.5%)	2 (8%)	0.29
Anti-topo I	23 (56.1%)	20 (80%)	0.06
Organ involvement			
Skin	37 (90.3%)	25 (100%)	0.28
Esophagus	28 (68.3%)	18 (72%)	0.78
Joint and muscle	2 (4.9%)	3 (12%)	0.35
PAH	1 (2.5%)	1 (4%)	0.99
mRSS	12.5 ± 9.6	11.4 ± 7.1	0.64
PFTs			
Mean FVC	2.4 ± 0.8	2.6 ± 0.7	0.19
Mean DLCO	8.7 ± 5.3	11.6 ± 8	0.09
FVC<70%	12 (29.3%)	4 (16%)	0.25
Previous immunosuppressants	21 (51.2%)	8 (32%)	0.21
Therapy			
PPI	29 (70.7%)	22 (88%)	0.13
Steroids	18 (43.9%)	12 (48%)	0.81
Immunosuppressants	6 (14.6%)	1 (4%)	0.23

Data are presented as mean ± SD or number (%) as appropriate.

Differences between groups have been analyzed by Fisher exact test or unpaired Student t test as

For Peer Review

Table 6: Primers used for AAV5 capsid amplification and sequencing analysis.

Primer ID	Sequence (5'- 3')	Sense/Antisense	PCR products (bp)
AAV52158-S	TGGGGATTTTGACGATGCCA	sense	617 bp
AAV52755-AS	CTCCCAAACTTGAGGCTGGT	antisense	
AAV52681-S	GACTCCAAGCCTTCCACCTC	sense	683bp
AAV53345-AS	GAAGAAGCTGCTCCTCTCGG	antisense	
AAV53318-S	TGAACCGCGACAACACAGAA	sense	542bp
AAV53841-AS	CGCTGGTGATGAGCATGTTG	antisense	
AAV53737-S	ATGACCAACAACCTCCAGGG	sense	648 bp
AAV54366-AS	TGGGTTAAAGGGGTCGGGTA	antisense	

Peer Review

ONLINE SUPPLEMENTARY METHODS

Surface plasmon resonance (SPR)

Sensing surfaces containing recombinant human PDGFRα fused to a poly-histidine tag (rhPDGFRα-His) were prepared as previously described (36). Briefly, upon activation of carboxylate groups with an equimolar mixture of N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), rhPDGFRα-His was covalently anchored via the N-terminus of the histidine tail. rhPDGFRα-His was always used at a concentration of 300 µg/mL during surface preparation, to avoid steric hindrance effects and dimerization between adjacent rhPDGFRα macromolecules. Instrumental response upon rhPDGFRα immobilization confirmed the coupling of a partial monolayer (surface density 1.7 ng/mm^2, approximately equivalent to 7 mg/mL). Free carboxylic sites on the surface were inactivated with 1 M ethanolamine pH 8.5, and the surface was equilibrated with PBS. The absence of negative baseline drift signals over time or after multiple PBS washes confirmed that the receptor molecules were irreversibly linked to the sensor surface.

The preservation of the native-like conformation of the receptor upon immobilization and after multiple binding cycles was assessed using conformational ligands, namely PDGF-BB and anti- PDGFRα mab322. The biosensor chamber was kept at 37 °C throughout.

Surfaced-blocked rhPDGFRα was tested for binding to AAV5 and AAV2 capsids at different concentrations of the viral assemblies (range: 0.1-2 nM). Response kinetics were followed up to equilibrium and baseline recovery, i.e., the dissociation of rhPDGFRα-ligand complexes carried out by serial PBS washes was always assessed prior to any new analysis. Kinetic raw data were analyzed according to a mono-exponential model (a bi-

exponential model did not significantly improve quality of the fit as judged by an F-test with 95% confidence).

Sensing surfaces containing bacterially-expressed fusion protein of PKD domains 1-5 of adeno-associated virus receptor (AAVR) described by Pillay et al (23) were assembled using EDC and NHS as coupling agents. AAVR concentration used for surface preparation was 300 µg/mL. Surface density was 1.4 ng/mm^2 with a concentration of 5.2 mg/mL in the sensing volume. Immobilized AAVR was tested for binding to AAV5 capsid at different concentrations (range: 10-100 nM).

PCR on AAV5

For Peer Review

DNA extracted from BAL cells of SSc patients and controls was subjected to qualitative PCR using primers designed to amplify the endonuclease portion of the *rep* gene. The amplification was performed in 50 µl final volume containing: 100 ng DNA, GoTaq Green Master Mix (Promega), 1 µM each of the following: either forward primer F2 5'-GGAAGAAAGCGCGCGTATGAG-3' or F3 5'-CGCGAGACTTCCGGGGTATAAAAG-3'; and common reverse primer R2 5'-CAGGGCGGCCAATTTATACTCG-3. Reactions in T100 Thermal Cycler (Bio-Rad) were as follows: after hot start, 40 cycles at 95°C for 30", annealing at 60°C for 1' 30", extension at 72°C for 1'. 10-fold serial dilutions of viral vector pRC5 containing the entire AAV5 genome was used as positive control. PCR products were subjected to 2% agarose gel electrophoresis in TBE buffer. Gel images were captured under UV light (Chemi Doc, Bio-Rad).

Quantitative PCR

Quantitative (q) real-time PCR (TaqMan) assay was used to determine AAV5 DNA copy numbers in the DNA extracted from BAL of SSc patients and controls. Specific primers were designed using IDT PrimeTime qPCR Assay (Integrated DNA Technologies) as

follows: forward primer, 5′-AGCTGGTGAAAGTGGTCTTC-3′; reverse primer, 5′-CCCAGAATCCACCACCTTATT-3’; probe, 5′(56FAM)TCTTTACCTTGGTGATGGCGACCC(31ABKFQ)-3′. The primers were designed to amplify the helicase portion of the *rep* gene. Amplifications in 25 µl final volume contained 100 ng BAL DNA, 12.5 µl iQ Supermix (Bio-Rad), 1.5 µl 500 nM primers and 250nM probe. A standard curve was generated using five serial dilutions (ranging from 3 x 10^5 to 3 x 10 copy/µg) pRC5 DNA. Reactions were in duplicate for each sample (iCycler, Bio-Rad). Cycling parameters were: 50° C for 2’, 95° C for 10’ followed by 40 cycles at 95° C for 15” and 60° C for 1 min.

AAV particles production

To produce AAV2-Firefly-Luciferase (AAV2-FF-luc), AAV5-Firefly-Luciferase (AAV5-FF-luc) and AAV2-Gam1 particles, 5 x 10^7 HEK293TT cells (ATCC CRL3467), which are devoid of PDGFRα (1) were seeded on five 15-cm dishes and transfected using the three-plasmid system with AAV capsid plasmid, helper plasmid, and firefly reporter plasmid (molar ratio of 1:1:1). After 48-hour transfection, cells were harvested and collected with medium into 50 mL tubes. After spinning at 1,900 rpm for 10’, the pellet was suspended in PBS and centrifuged again. The washed pellet was suspended in 5 mL AAV lysis buffer (50 mM Tris/150 mM NaCl in H_2O, pH 8.5) before undergoing 5 freeze-thaw cycles in alternate liquid nitrogen/37°C. Benzonase (50 U/mL, 1 µL/prep) was added to degrade DNA and RNA without any proteolysis. After incubation at 37° C for 30’ followed by centrifugation at 5,000 rpm for 10’, crude cell lysates containing AAV particles were harvested and added to a Quickseal-Tube to which it followed addition of 1.5 mL iodixanol (Sigma) in PBS-MK/NaCl (1 mM $MgCl_2$, 2.5 mM KCl, 1 M NaCl in

PBS), 1.5 mL 25% iodixanol in PBS-MK with 3 µL phenol-red solution, 1.5 mL 40% iodixanol in PBS-MK, and 3.8 mL 60% iodixanol with 5 µL phenol-red solution. All samples were balanced-sealed with a heating device and centrifuged at 50,000 rpm for 2 hours at 10°C (rotor TFT6513). One mL of AAV particles was collected from the 40% iodixanol phase using a needle and syringe. Purified AAV particles were quantified by real-time amplification of the reporter gene and stored at -20°C or -80°C for long-term preservation until use.

For Peer Review

AAV5 transduction into wild-type (wt) and PDGFRα-knockout A549 cell line

A549-wt, an adenocarcinoma human alveolar basal epithelial cell line (ATCC® CCL-185) and A549-PDGFRα-knockout cells (GenCRISPR™ Custom Cell Line Development Service, GenScript) were seeded prior transduction into 24-well plates at 10^5 cells/well, overnight at 37°C. AAV2-FF-luc and AAV5-FF-luc were prepared at a multiplicity of infection (MOI) of 5 x 10^4 and AAV2-Gam1 at MOI 2.5 x 10^4 for 24 hours coinfection. The AAV protein Gam1 was used to improve AAV5 transduction efficiency in A549 cells (2). To avoid the effects of chemical reagents, AAV2 was used to deliver the Gam1 gene into cells.

For the luciferase assay, medium was discarded after transduction and 100 µL of lysis buffer from Beetle Juice BIG Kit (PIK GmbH) was added to the 24-well plate, and shaken for 15' at room temperature (RT). Subsequently, 20 µL of lysate were transferred into white LIA 96-well plates. All samples were in triplicate. For the firefly luciferase detection, 100 µL of Beetle juice BIG Kit was added to each well. Readout was after 1 min, and luminescence was analyzed using a Wallac workstation.

Lung samples

Lung specimens were obtained at the time of lung transplantation from 5 SSc patients with ILD and WHO Group 1 pulmonary arterial hypertension. Six control lung tissues were taken during lobectomy or pneumonectomy for localized lung cancer, having the pathological tissue always distant from the sample. Specimens were snap frozen or paraffin embedded as described (3).

Interstitial lung fibrosis was assessed by an experienced lung pathologist (PD). The categories 0=none, 1=mild, 2=moderate, 3=severe fibrosis were assigned after analysis of at least 5 slides stained with HES collagen staining.

For Peer Review

Immunohistochemistry

Frozen lung sections were stained with an anti-AAV5 capsid mouse monoclonal antibody (ADK 5b;Progen) for 30' at RT, then washed and incubated with biotinylated goat anti-mouse antibody (Thermo Fisher Scientific) for 10' at RT. HRP-labeled streptavidin and AEC chromogen were used to reveal staining, which was never detected when the primary antibody had been omitted.

Chromogenic *in situ* hybridization.

Digoxigenin (DIG)-labeled probes were obtained from Integrated DNA Technology (IDT). DIG-tagged probes specific for AAV5 DNA sequence were used: sense probe 5'-CTTGTGGAGACCTCCGGCATC-3'; anti-sense probe 5'-ATTGGCTCCGCCCTTCTTTAC-3'. Chromogenic *in situ* hybridization (CISH) was performed on paraffin-embedded sections using Zyto Dot CISH Implementation Kit (ZytoVision), according to the manufacturer's instructions. Sections were deparaffinized, rehydrated and hybridized with DIG-probes overnight at 37°C. The slides were washed and incubated with anti-DIG antibody for 30' at RT, and then with HRP-conjugated secondary antibody for 30' at RT.

The peroxidase ABC method was used with diaminobenzedine hydrochloride as chromogen (Thermo Fisher Scientific).

Confocal microscopy

Immunofluorescence and confocal microscopy analyses were performed on frozen lung sections and cytospin preparations of PBMCs attached onto glass slides and fixed with ethanol for 1 hour at 4° C. In brief, sections and PBMCs preparations were reacted with 0.3% glycine (10 min) and 0.03% sodium dodecyl sulphate (10 min). After rinsing in PBS and blocking for 60 min with 3% normal serum (in 0.2% Triton X-100), incubation was done overnight at 4°C with the primary antibodies mouse monoclonal IgG anti-AAV5 (Progen, ready to use) and rabbit monoclonal IgG anti-PDGFRα (Cell Signaling Technology, dilution 1:50 v/v), rabbit polyclonal IgG anti-prosurfactant protein C (proSP-C, Millipore, dilution 1:8000 v/v) or rabbit monoclonal IgG anti-thyroid transcription factor 1 (TTF1, Cell Signaling Technology, dilution 1:500 v/v) in PBS. The next day, sections were washed twice with PBS and incubated in a cocktail of fluorophore-linked secondary antibodies at a dilution of 1:400 in PBS for 1 h at RT. The secondary antibodies were Alexa Fluor® 488 donkey anti-mouse IgG and Alexa Fluor® 555 donkey anti-rabbit IgG (both from Invitrogen). Sections were washed, counterstained with TO-PRO3-iodide, air-dried, and coverslipped using Vectashield mounting medium (Vector Laboratories). Sections were viewed under a motorized Leica DM6000 microscope at different magnifications. Fluorescence was detected with a Leica TCS-SL spectral confocal microscope equipped with an Argon and He/Ne mixed gas laser. Fluorophores were excited with the 488 nm, 543 nm, and 649 nm lines, and imaged separately. Images (1024 x 1024 pixels) were obtained sequentially from channels using a confocal pinhole of 1.1200 and stored as TIFF files. Brightness and contrast of the final images were adjusted using Photoshop 6 (Adobe Systems). The percentage of type II pneumocytes expressing

For Peer Review

AAV5+ was calculated semiquantitatively as AAV5+/proSP-C+ and AAV5+/TTF1+ in proportion to all type II pneumocytes evaluated as proSP-C+ and TTF1+, respectively. Cell counting was made on five randomly chosen microscopic cellular fields from two sections by two independent observers (AG and MS).

QUANTIFICATION

After the acquisition, the data were processed to isolate relevant regions of interest (ROIs). The fluorescence intensity of a specific marker was quantified within each ROI. The selection of ROI was automated. Segmentation was eroded from a specified number of pixels to identify each marker in the ROIs. This was used to calculate the normalized percentage of overlap between AAV5 and the associated specific marker (4doi: 10.1152/ajpcell.00462.2010)

For Peer Review

Bronchoalveolar lavage

A total volume of about 100 ml of sterile isotonic, saline solution divided into three aliquots was instilled through a bronchoscope in a wedge position within the selected bronchopulmonary segment, with immediate aspiration by gentle suction after each aliquot. A volume >50% was retrieved (5). BAL fluid was strained through a Dacron net (Millipore). Cells were pelleted by centrifugation at 400 rpm for 10 min at 4°C and supernatants poured off. Cells were resuspended in RPMI 1640 medium (Sigma), counted in a Bürker chamber, and total cell viability was determined by trypan blue exclusion. DNA was extracted from cell pellets using QIAamp DNA Mini Kit (Qiagen) following the manufacturer's instructions.

Peripheral blood mononuclear cells

Peripheral blood was obtained by venipuncture and peripheral blood mononuclear cells (PBMCs) were separated from heparinized peripheral blood by Ficoll-Hypaque (Amersham) gradient centrifugation and washed twice in PBS before use.

AAV5 capsid gene sequencing

The complete AAV5 capsid gene sequence was amplified from the DNA extracted from BAL of 6 patients. PCR reactions were performed using Dream Taq DNA Polymerase (Thermo Fisher Scientific) using 2.5 µl of each primer (10 µM), 5 µl 10x Dream Taq Buffer, 1 µl dNTP mix and 1U Dream Taq DNA Polymerase (Thermo Fisher Scientific) with 5 µl DNA extract and water up to a final volume of 50 µl. The thermal cycler program was as following: 94° C for 3', 40 cycles at 94° C for 30'', 60° C for 30", and 72° C for 1' 30'', followed by a last extension at 72° C for 3'. The primers used in the PCR assay are listed in online supplementary table 6. Sanger cycle sequencing was performed with the same primers as for amplification, using the Big Dye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, according to manufacturer's instructions. Sequences were run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems-HITACHI) and further analyzed using Sequencing Analysis, Seqscape and BioEdit programs.

Purification of serum IgG

IgG were purified from serum of SSc patients and healthy controls using gravity flow columns (Pierce), as previously described (6).

Immunoaffinity purification of serum anti-PDGFRα antibodies

The enriched fraction of IgG to PDGFRα was isolated by a pull-down assay procedure. Briefly, recombinant human PDGFRα fused to a poly-histidine tag (rhPDGFRα-His) (a.a. Met 1-Glu 524, Sino Biological Inc.) was immobilized in a HisTrap HP column (Cytiva) prepacked with precharged Ni Sepharose™ High Performance in binding buffer (20 mM

For Peer Review

Na_3PO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4) according to the manufacturer's instructions. A pool of IgG from SSc patients was added to the bead/protein complex and after extensive wash, elution was performed in eluition buffer containing 500 mM imidazole .

Immunoenzymatic assays

Antibodies to AAV5 were detected by enzyme-linked immunosorbent assay (ELISA). Plates were coated overnight at 4° C with 100 µg AAV5 capsid protein or bovine serum albumin (BSA) as negative control. After wash with Tris buffer saline (TBS)/0.1% Tween 20, serum samples (1:100 in TBS/0.1% Tween 20, 50 µl/well) were added to each well and incubated for 1 hour at 37° C. After extensive wash, HRP-conjugated anti-human IgG Fc antibody (dilution 1:10,000, 100 µl/well) was added and incubated for 1 hour at 37° C. After development with 100 µl/well tetramethylbenzidine (BioFX) for 5' at 37° C, the reaction was stopped with 0.5 M H_2SO_4. Optical density (O.D.) was measured at 450 nm. An O.D. value of 0.02 was considered as the cut-off between positive and negative samples. Antibodies to PDGFRα were detected using an immunoenzymatic assay as described using a recombinant receptor fused to a poly-histidine tag produced in-house (7).

For Peer Review

REFERENCES OF ONLINE METHODS

1. C. Summerford, J.S.. Johnson, J. Samulsky. AAVR:a multi-serotype receptor for AAV. *Mol Ther* **24**, 663-6 (2016). DOI: 10.1038/mt.2016.49.

2. D.L. Hacker, E. Derow, F.M. Wurm. The CELO adenoviris Gam 1 protein enhances transient and stable recombinant protein expression in chinese hamster ovary cells. *J Biotechnol* **117**, 21-9 (2005). DOI: 10.1016/j.jbiotec.2005.01.006.

3. Dorfmüller, P. et al. Chemokine RANTES in severe pulmonary arterial hypertension. *Am J Respir Crit Care Med* **165**,534–9 (2002). DOI: 10.1164/ajrccm.165.4.2012112.

4. Dunn KW, Kamocka MM, McDonald JH A practical guide to evaluating colocalization in biological microscopy Am J Physiol Cell Physiol 2011; 300:C723-42. DOI: 10.1152/ajpcell.00462.2010.

5. K.C. Meyer et al. An official American Thoracic Society Clinical Practice Guideline: The clinical utility of bronchoalveolar lavage cellular analysis in interstitial lung disease. *Am J Respir Crit Care Med* **185**,1004–14 (2012). DOI: 10.1164/rccm.201202-0320ST.

6. S. Svegliati Baroni et al. Stimulatory Autoantibodies to the PDGF Receptor in Systemic Sclerosis. *N Engl J Med* **354,** 2667–76 (2006). DOI: 10.1056/NEJMoa052955

7. Moroncini G, Grieco A, Nacci G, et al. Epitope Specificity Determines Pathogenicity and Detectability of Anti-Platelet-Derived Growth Factor Receptor alpha Autoantibodies in Systemic Sclerosis. Arthritis Rheumatol 2015;67(7):1891-903. DOI: 10.1002/art.39125

For Peer Review

ICMJE DISCLOSURE FORM

Date: 17th August 2023
Your Name: Prof. Pietro Pucci
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis
Manuscript number (if known): 23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Review

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

X I certify that I have answered every question and have not altered the wording of any of the questions on this form.

Prof. Pietro Pucci

ICMJE DISCLOSURE FORM

Date: 17/08/2023
Your Name: Stefano Menzo
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis
Manuscript number (if known):___

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Review

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

X **I certify that I have answered every question and have not altered the wording of any of the questions on this form.**

Stefano Menzo

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Donatella Amico
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 15, 2023
Your Name: Antonio Giordano
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 14, 2023
Your Name: Armando Gabrielli
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Antonella Grieco
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Review

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 19, 2023
Your Name: Chiara Paolini
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 15, 2023
Your Name: Cecilia Tonnini
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Devis Benfaremo
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 1, 2023
Your Name: Doreen Finke
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

For Peer Review

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Gabriella Pinto
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Gianluca Moroncini
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Jürgen Kleinschmidt
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 17, 2023
Your Name: Massimiliano Cuccioloni
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Martin Müller
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Review

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 14, 2023
Your Name: Nadia Viola
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Qingxin Chen
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date:_______August 17th 2023
Your Name: Antonio AMOROSO
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis
Manuscript number (if known): ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Review

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

X I certify that I have answered every question and have not altered the wording of any of the questions on this form.

Antonio Amoroso

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Ada Funaro
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date:_August 16, 2023____
Your Name: Antonio La Cava____
Manuscript Title:____
Manuscript number (if known): ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Review

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

__x_ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

Antonio La Cava

ICMJE DISCLOSURE FORM

Date: 18.082023
Your Name: Peter Dorfmüller, MD, PhD
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis
Manuscript number (if known): ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Review

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

X I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 16,2023
Your Name:_VITTORIO ENRICO AVVEDIMENTO
Manuscript Title Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known):ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____????	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

X I certify that I have answered every question and have not altered the wording of any of the questions on this form.

For Peer Review

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name: Karen Nieto
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

For Peer Re

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: **August 17, 2023**____
Your Name:____ **Lina Zuccatosta** ____
Manuscript Title:__ Adeno-associated virus type 5 infection via PDGFRα is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis____
Manuscript number (if known): __ar-23-0151.R1____

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	

For Peer Review

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

X_ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 14, 2023
Your Name: Martina Senzacqua
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

For Peer Review

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name Silvia Agarbati
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 16, 2023
Your Name: Silvia Svegliati
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Re

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: **August 17th, 2023**____

Your Name: **Stefano Gasparini**________________________________

Manuscript Title: Adeno-associated virus type 5 infection via PDGFRα is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known): ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Revi

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	None	
3	Royalties or licenses	None	

4	Consulting fees	None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	None	
6	Payment for expert testimony	None	
7	Support for attending meetings and/or travel	None	
8	Patents planned, issued or pending	None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	None	
11	Stock or stock options	None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	None	
13	Other financial or non-financial interests	None	

Please place an "X" next to the following statement to indicate your agreement:

X I certify that I have answered every question and have not altered the wording of any of the questions on this form.

Ancona, August 17th, 2023

ICMJE DISCLOSURE FORM

Date: August 18, 2023
Your Name:Tatiana Spadoni____
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis

Manuscript number (if known) ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

For Peer Review

4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X__ I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date:__________17/08/2023____
Your Name:______Angela Amoresano____
Manuscript Title:__ Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis__________________________________
Manuscript number (if known):_______ ar-23-0151.R1____

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the <u>current manuscript only</u>.

The author's relationships/activities/interests should be <u>defined broadly</u>. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Review

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

X I certify that I have answered every question and have not altered the wording of any of the questions on this form.

ICMJE DISCLOSURE FORM

Date: August 17, 2023
Your Name: Mario Galgani
Manuscript Title: Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis
Manuscript number (if known): ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

For Peer Review

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
Time frame: Since the initial planning of the work			
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	___X_None	
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	___X_None	
3	Royalties or licenses	___X_None	
4	Consulting fees	___X_None	

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	__X__None	
6	Payment for expert testimony	___X_None	
7	Support for attending meetings and/or travel	__X__None	
8	Patents planned, issued or pending	__X__None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	__X__None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	__X__None	
11	Stock or stock options	___X_None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	__X__None	
13	Other financial or non-financial interests	__X__None	

Please place an "X" next to the following statement to indicate your agreement:

___ **I certify that I have answered every question and have not altered the wording of any of the questions on this form.**

Naples, August 17, 2023

ICMJE DISCLOSURE FORM

Date:_16.08.2023
Your Name:__Matteo Mozzicafreddo
Manuscript Title:__"Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis"__
Manuscript number (if known):__ ar-23-0151.R1

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) No time limit for this item.	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

_X_ **I certify that I have answered every question and have not altered the wording of any of the questions on this form.**

ICMJE DISCLOSURE FORM

Date: ___08/17/2023___
Your Name: __Antonio Pezone___
Manuscript Title: ___ Adeno-associated virus type 5 infection via PDGFRa is associated with Interstitial lung disease in systemic sclerosis and generates composite peptides and epitopes recognized by the agonistic immunoglobulins present in patients with systemic sclerosis. ___
Manuscript number (if known): _____ ar-23-0151.R1 ar-23-0151.R1 ___

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	**Specifications/Comments (e.g., if payments were made to you or to your institution)**
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
Time frame: past 36 months			
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	

Time frame: past 36 months

2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	
5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

	writing, gifts or other services		
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

X I certify that I have answered every question and have not altered the wording of any of the questions on this form.

Antonio Pezone

Dept. of Biology,
University of Naples "Federico II"

For Peer Review

ICMJE DISCLOSURE FORM

Date: 17/08/2023
Your Name: ALIZZI SILVIA
Manuscript Title: AAVS INFECTION VIA PDGFRa IS ASSOCIATED WITH INTERSTITIAL LUNG DISEASE IN S
Manuscript number (if known): /

In the interest of transparency, we ask you to disclose all relationships/activities/interests listed below that are related to the content of your manuscript. "Related" means any relation with for-profit or not-for-profit third parties whose interests may be affected by the content of the manuscript. Disclosure represents a commitment to transparency and does not necessarily indicate a bias. If you are in doubt about whether to list a relationship/activity/interest, it is preferable that you do so.

The following questions apply to the author's relationships/activities/interests as they relate to the current manuscript only.

The author's relationships/activities/interests should be defined broadly. For example, if your manuscript pertains to the epidemiology of hypertension, you should declare all relationships with manufacturers of antihypertensive medication, even if that medication is not mentioned in the manuscript.

In item #1 below, report all support for the work reported in this manuscript without time limit. For all other items, the time frame for disclosure is the past 36 months.

		Name all entities with whom you have this relationship or indicate none (add rows as needed)	Specifications/Comments (e.g., if payments were made to you or to your institution)
		Time frame: Since the initial planning of the work	
1	All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.) **No time limit for this item.**	____None	
		Time frame: past 36 months	
2	Grants or contracts from any entity (if not indicated in item #1 above).	____None	
3	Royalties or licenses	____None	
4	Consulting fees	____None	

5	Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events	____None	
6	Payment for expert testimony	____None	
7	Support for attending meetings and/or travel	____None	
8	Patents planned, issued or pending	____None	
9	Participation on a Data Safety Monitoring Board or Advisory Board	____None	
10	Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid	____None	
11	Stock or stock options	____None	
12	Receipt of equipment, materials, drugs, medical writing, gifts or other services	____None	
13	Other financial or non-financial interests	____None	

Please place an "X" next to the following statement to indicate your agreement:

X **I certify that I have answered every question and have not altered the wording of any of the questions on this form.**