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Autoanticorps dans l'hypertension artérielle pulmonaire (HTAP) associée aux connectivites et à l'infection par le virus de l'immunodéficience humaine (VIH)

Benjamin Thoreau

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École doctorale Hématologie, Oncogénèse et Biothérapies (ED 561)

Institut Cochin (INSERM U 1016 / CNRS UMR 8104)

Équipe Neutrophile et Vascularites

***Autoanticorps dans l'hypertension artérielle
pulmonaire (HTAP) associée aux connectivites et
à l'infection par le virus de l'immunodéficience
humaine (VIH)***

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Thèse de doctorat de **Biothérapies et Biotechnologies**

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Autoanticorps dans l'hypertension artérielle pulmonaire (HTAP) associée aux connectivites et à l'infection par le virus de l'immunodéficience humaine (VIH)

Résumé

L'hypertension artérielle pulmonaire (HTAP) est une pathologie rare, et peut être associée à des connectivites comme la sclérodermie systémique (ScS), le lupus érythémateux systémique (LES) ou la connectivite mixte (CM), ou à d'autres affections comme l'infection par le virus de l'immunodéficience humaine (VIH). La physiopathologie de l'HTAP est complexe et associe une dysfonction endothéliale, l'accumulation de fibroblastes adventitiels et de cellules musculaires lisses vasculaires, et des dérégulations immunitaires, aboutissant au remodelage vasculaire pulmonaire. Des marqueurs d'auto-immunité ont par ailleurs été décrits, au premier rang desquels l'identification d'auto-anticorps au cours des HTAP idiopathique et associée à la ScS, en particulier des anticorps anti-cellules endothéliales vasculaires pulmonaires (AECA) et anti-fibroblaste (AFA) ou anti-cellules musculaires lisses vasculaires. Ce projet avait pour objectifs d'identifier des AECA et des AFA dans les sérums de patients atteints d'HTAP associée à une connectivite autre que la ScS et associée à l'infection par le VIH, d'identifier leurs cibles antigéniques, et d'explorer le potentiel rôle pathogénique de ces auto-anticorps.

Des AECA et des AFA ont été identifiés dans les sérums des patients avec une HTAP associée au LES ou la CM, et à l'HTAP associée au VIH. Les cibles antigéniques de ces auto-anticorps partagées entre ces différentes affections étaient l'annexine A5 (ANXA5), la protéine 14-3-3 ϵ , la vimentine, la triosephosphate isomérase, la galectine 1 et l'elongation facteur 2. La réactivité IgG anti-ANXA5 a été confirmée comme spécifiquement associée à la mise en évidence d'une HTAP chez les patients atteints d'un LES, d'une CM ou d'une infection par le VIH. La réactivité IgG anti-14-3-3 ϵ était elle aussi associée à la mise en évidence d'une HTAP chez les patients atteints d'un LES ou d'une infection par le VIH. Il n'a pas été identifié d'effet pathogène des auto-anticorps anti-ANXA5. Les IgG anti-ANXA5 pourraient constituer un facteur prédictif de survenue d'une HTAP chez les patients atteints de LES, de CM et d'infection par le VIH.

Mots-clés : hypertension artérielle pulmonaire, lupus érythémateux systémique, connectivite mixte, virus de l'immunodéficience humaine, auto-anticorps anti-cellules endothéliales, auto-anticorps anti-fibroblastes, annexine A5, 14-3-3 ϵ .

Autoantibodies in pulmonary arterial hypertension (PAH) associated with connective tissue diseases and human immunodeficiency virus infection

Abstract

Pulmonary arterial hypertension (PAH) is a rare disease, and may be associated with connective tissue diseases such as systemic sclerosis (SSc), systemic lupus erythematosus (SLE) or mixed connective tissue disease (MCTD), or with other conditions such as human immunodeficiency virus (HIV) infection. The pathophysiology of PAH is complex, combining endothelial dysfunction, accumulation of adventitial fibroblasts and vascular smooth muscle cells, and immune dysregulation, leading to pulmonary vascular remodeling. Markers of autoimmunity have also been described, especially the identification of autoantibodies in idiopathic and SSc-associated PAH, including anti-endothelial cells antibodies (AECA), anti-fibroblasts antibodies (AFA) and anti-smooth muscle cell antibodies.

The aims of this project were to identify AECA and AFA in sera from patients with PAH associated with a connective tissue disease other than SSc and associated with HIV infection, to identify their antigenic targets, and to explore the potential pathogenic role of these autoantibodies.

AECA and AFA were identified in the sera of patients with SSc or MCTD-associated PAH, and in the sera of patients with HIV-associated PAH. The antigenic targets of these autoantibodies shared between these different conditions were annexin A5 (ANXA5), 14-3-3 ϵ , vimentin, triosephosphate isomerase, galectin 1 and elongation factor 2 proteins. Anti-ANXA5 IgG reactivity was confirmed as specifically associated with PAH in patients with SLE, MCTD or HIV infection. Anti-14-3-3 ϵ IgG reactivity was also associated with evidence of PAH in patients with SLE or HIV infection. We failed to demonstrate any pathogenic effect of anti-ANXA5 autoantibodies. Anti-ANXA5 IgG could be a predictive factor for the development of PAH in patients with SLE, MCTD and HIV infection.

Keywords: pulmonary arterial hypertension, systemic lupus erythematosus, mixed connective tissue disease, human immunodeficiency virus, anti-endothelial cell autoantibodies, anti-fibroblast autoantibodies, annexin A5, 14-3-3 ϵ .

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Abréviations

AECA : anticorps anti-cellules endothéliales

AFA : anticorps anti-fibroblaste

ALK1 : activin receptor-like kinase 1

Anticorps anti-CMLV : anticorps anti-cellules musculaires lisses vasculaires

ANXA5 : annexine A5

AQP1 : aquaporin-1

ARE : antagonistes des récepteurs de l'endothéline

ATP13A3 : ATPase 13A3

BMPR2 : bone morphogenetic protein receptor de type 2

BMP9 et 10 : gènes bone morphogenetic protein 9 (GDF2) et 10

CAV1 : cavéoline-1

CCL-2 : C-C motif chemokine ligand-2

CM : connectivite mixte

EIF2AK4 : gène de contrôle général non répressible-2 (GCN-2)

ENG : endogline

ESC : European Society of Cardiology

ERS : European Respiratory Society

ET-1 : endothéline-1

GGCX : gamma-glutamyl carboxylase

HAART : traitements antirétroviraux hautement actifs

H-HTAP : hypertension artérielle pulmonaire (HTAP) de forme héritable

HTAP : hypertension artérielle pulmonaire

HTAP-CM : hypertension artérielle pulmonaire associée à la connectivite mixte

HTAPi : hypertension artérielle pulmonaire Idiopathique

HTAP-LES : hypertension artérielle pulmonaire associée au lupus érythémateux systémique

HTAP-ScS : hypertension artérielle pulmonaire associée à la sclérodermie systémique

HTAP-SS : hypertension artérielle pulmonaire associée au syndrome de Sjögren primaire

HTP : hypertension pulmonaire

ICAM1 : intercellular adhesion molecule 1

Ig : immunoglobulines

IL-1 : interleukine 1

KCNK3 : membre 3 de la sous-famille K des canaux potassiques sensible à l'acide (TASK-1)

KLK1 : Kallikrein 1

KTD : cathétérisme cardiaque droit

LES : lupus érythémateux systémique

NO : oxyde nitrique

PAPm : pression artérielle pulmonaire moyenne

PAPs : pression artérielle pulmonaire systolique

PCP : pression capillaire pulmonaire

PDE5i : inhibiteurs de la phosphodiesterase-5

PDGF: platelet derived growth factor

ScS : sclérodermie systémique

SMAD : Mothers against decapentaplegic homolog

SOX17 : facteur de transcription high mobility group (HMG)-box lié à la SRY (sex-determining region Y)

SS : syndrome de Sjögren primaire

SUR-1 : récepteur 1 de sulfonylurée (ABCC8)

T-box4 : TBX4

TGF- β : transforming growth factor beta

TNF- α : tumor necrosis factor

VCAM1 : vascular cell adhesion molecule 1

VEGF : vascular endothelial growth factor

VEGFR2 : vascular endothelial growth factor receptor 2

VIH : virus de l'immunodéficience humaine

Partie I – Contexte scientifique

I.1 – Introduction

L'hypertension pulmonaire (HTP) correspond à une élévation anormale de la pression dans les artères pulmonaires. Elle peut être la conséquence de divers mécanismes, et peut être associée une large diversité de pathologies cardiovasculaires, respiratoires et de maladies systémiques. La classification clinique de l'HTP est divisée en 5 groupes détaillés dans le

Tableau 1. L'hypertension artérielle pulmonaire (HTAP) correspondant au groupe 1 des HTP.

L'HTAP est une maladie vasculaire pulmonaire chronique caractérisée par une vasoconstriction et un remodelage vasculaire progressif des petites artères pulmonaires, engendrant une augmentation des résistances vasculaires pulmonaires et une augmentation anormale de la pression au sein des artères pulmonaires. Cette hypertension peut provoquer un remodelage et une hypertrophie du ventricule droit, une altération de la fonction ventriculaire droite, et à terme une insuffisance cardiaque droite et mettre en jeu le pronostic vital (Humbert et al., 2019).

L'HTAP est considérée comme une maladie rare et potentiellement grave, avec une incidence chez l'adulte évaluée à 6 cas/million et une prévalence entre 48 et 55 cas/million (Leber et al., 2021). Elle peut toucher des personnes de tout âge, y compris des enfants, bien que la prévalence soit plus élevée chez les adultes. Les femmes sont également plus souvent touchées que les hommes, particulièrement dans la forme héritable (Lau et al., 2017).

L'HTAP peut être idiopathique (sans cause sous-jacente retrouvée) représentant la cause la plus fréquente, héritable secondaire à des mutations génétiques identifiées ou non, ou associée à certaines étiologies sous-jacentes comme par exemple des maladies du tissu conjonctif (connectivites), l'infection par le virus de l'immunodéficience humaine (VIH), des

cardiopathies congénitales, ou des exposition à des toxiques ou des médicaments (Humbert et al., 2022). Les HTAP associées aux connectivites représentent la seconde cause la plus fréquente (environ 15% des cas en France), après les formes idiopathiques (50-60% des cas selon les registres) (Humbert et al., 2006; Lau et al., 2017).

Les autres groupes d'HTP sont représentés par les HTP associées à des cardiopathies gauches (groupe 2), à des pathologies pulmonaires et/ou de l'hypoxémie chronique (groupe 3), les HTP associées à des obstructions artérielles pulmonaires (l'hypertension pulmonaire thrombo-embolique chronique par exemple) (groupe 4), et un groupe d'HTP de mécanismes multifactoriels et/ou peu clairs (groupe 5) telles que des hémopathies, la sarcoïdose, des histiocytoses Langerhansiennes pulmonaires, la maladie de Gaucher ou l'insuffisance rénale chronique.

Tableau 1 : Classification clinique des hypertensions pulmonaires (Humbert et al., 2022)

GROUPE 1. Hypertension artérielle pulmonaire (HTAP)

1.1 Idiopathique

1.1.1 Non-répondeurs aux tests de vasoréactivité

1.1.2 Répondeurs aux tests de vasoréactivité en aigu

1.2 Héritable^a

1.3 Associée aux médicaments et aux toxiques^a

1.4 Associée à :

1.4.1 Connectivites

1.4.2 Infection par le VIH

1.4.3 Hypertension portale

1.4.4 Cardiopathie congénitale

1.4.5 Schistosomiase

1.5 HTAP avec des caractéristiques d'atteinte veineuse/capillaire (PVOD/PCH)

1.6 HTP persistante du nouveau-né

GROUPE 2. HTP associé à une cardiopathie gauche

2.1 Insuffisance cardiaque :

2.1.1 avec fraction d'éjection préservée

2.1.2 avec fraction d'éjection réduite ou légèrement réduite^b

2.2 Cardiopathie valvulaire

2.3 Pathologies cardiovasculaires congénitales/acquises entraînant une HTP post-capillaire

GROUPE 3. HTP associé à des maladies pulmonaires et/ou à une hypoxie

3.1 Maladie pulmonaire obstructive ou emphysème

3.2 Maladie pulmonaire restrictive

3.3 Maladie pulmonaire à profil mixte restrictif/obstructif

3.4 Syndromes d'hypoventilation

3.5 Hypoxie sans maladie pulmonaire (par exemple haute altitude)

3.6 Troubles du développement pulmonaires

GROUPE 4. HTP associé à des obstructions de l'artère pulmonaire

4.1 HTP thrombo-embolique chronique

4.2 Autres obstructions de l'artère pulmonaire^c

GROUPE 5. HTP avec mécanismes non précisés et/ou multifactoriels

5.1 Maladies hématologiques^d

5.2 Pathologies systémiques^e

5.3 Maladies métaboliques^f

5.4 Insuffisance rénale chronique avec ou sans hémodialyse

5.5 Microangiopathie thrombotique tumorale pulmonaire

5.6 Médiastinite fibrosante

HTAP, hypertension artérielle pulmonaire ; HTP, hypertension pulmonaire ; IC, insuffisance cardiaque ; PCH, hémangiomasose capillaire pulmonaire ; PVOD, maladie veino-occlusive pulmonaire ; VIH, virus de l'immunodéficience humaine.

^aLes patients ayant une HTAP héritable ou une HTAP associée à des médicaments et à des toxiques peuvent être répondeurs aux tests de vasoréactivité en aigu.

^bFraction d'éjection ventriculaire gauche pour l'IC avec fraction d'éjection réduite : ≤ 40 % ; pour l'IC avec une fraction d'éjection légèrement réduite : 41 à 49 %.

^cLes autres causes d'obstruction de l'artère pulmonaire comprennent : les sarcomes (grades intermédiaire ou élevé ou angiosarcome), d'autres tumeurs malignes (par exemple carcinome rénal, carcinome utérin, tumeurs germinales des testicules), tumeurs non malignes (par ex. léiomyome utérin), artérite sans connectivite, sténoses artérielles pulmonaires congénitales et hydatidose.

^dY compris l'anémie hémolytique chronique héréditaire et acquise et l'anémie chronique des syndromes myéloprolifératifs.

^eY compris la sarcoïdose, l'histiocytose pulmonaire à cellules de Langerhans et la neurofibromatose de type 1.

^fY compris les maladies du stockage du glycogène et la maladie de Gaucher.

Les symptômes de l'HTAP peuvent inclure une asthénie, une dyspnée à l'effort, des malaises, des douleurs thoraciques et/ou une tachycardie, ou être complètement asymptomatique à la phase initiale. Ces symptômes sont le résultat des altérations hémodynamiques et de leur retentissement sur la fonction cardiaque et l'hypoxie tissulaire qui en résulte.

Un diagnostic précoce et un traitement adéquat sont essentiels pour ralentir la progression de la maladie, améliorer la qualité de vie et la survie des patients atteints d'HTAP. Le traitement de l'HTAP vise à réduire la pression artérielle pulmonaire et corriger les autres anomalies hémodynamiques, à améliorer les symptômes et la qualité de vie des patients, et à prévenir la progression de la maladie. Il repose schématiquement sur l'utilisation de traitements médicamenteux spécifiques de l'HTAP, représentés par des médicaments vasodilatateurs pulmonaires tels que les inhibiteurs de la phosphodiesterase-5 (PDE5i) et le stimulateur de la guanylate cyclase soluble (GCs) agissant sur la voie de l'oxyde nitrique (NO), les antagonistes des récepteurs de l'endothéline (ARE) et les analogues de la prostacycline (**Figure 1**). Des traitements non spécifiques y sont le plus souvent associés, comme l'oxygénothérapie ou les diurétiques en cas de décompensation cardiaque droite. Dans certains cas, une transplantation pulmonaire peut être envisagée chez les patients atteints d'HTAP sévère et résistante ou réfractaire au traitement médical optimal maximal. Outre les traitements médicaux, le maintien d'une activité physique et d'un statut nutritionnel optimal, l'arrêt du

tabagisme et la mise à jour des vaccinations sont des mesures additionnelles essentielles (Galiè et al., 2016, 2019).

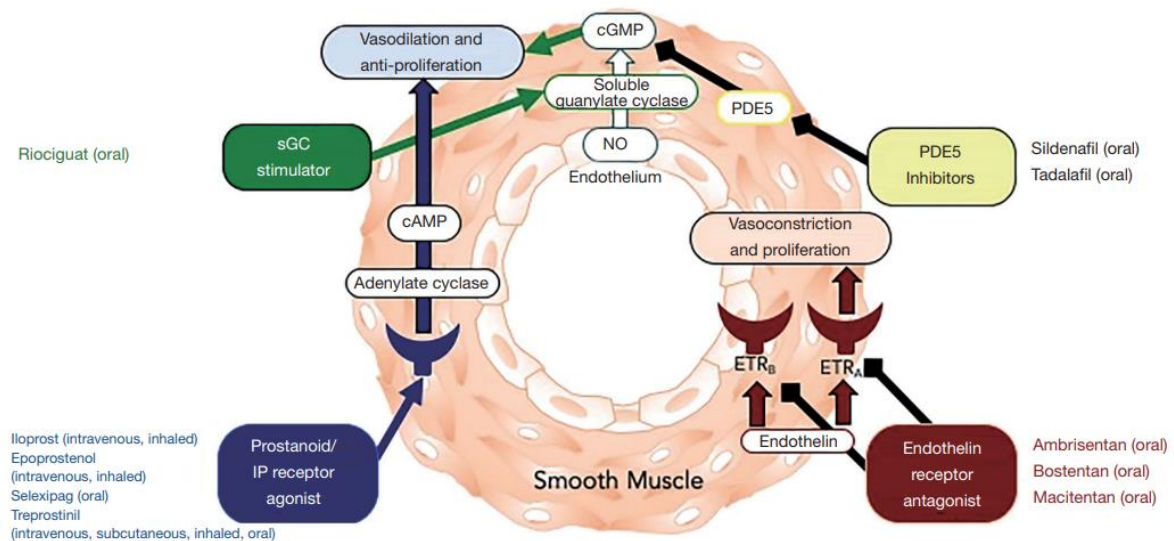


Figure 1 : Cibles moléculaires, voies de signalisation et modes d'action des médicaments du traitement spécifique de l'HTAP (adapté de Humbert et al. (Humbert & Ghofrani, 2016)).

cAMP, adénosine monophosphate cyclique ; ETR_A et ETR_B, récepteur de l'endothéline de type A et de type B ; sGC, guanylate cyclase soluble ; cGMP, guanosine monophosphate cyclique ; NO, oxyde nitrique ; PDE5, phosphodiesterase de type 5.

1.2 – Définition de l'hypertension artérielle pulmonaire (HTAP)

La définition hémodynamique de l'hypertension pulmonaire (HTP) a été récemment modifiée et les critères hémodynamiques mesurés lors du cathétérisme cardiaque droit (KTD) ont été ajustés. Selon les recommandations de l'European Society of Cardiology et de l'European Respiratory Society de 2022 (ESC/ERS 2022), l'HTP est définie par une pression artérielle pulmonaire moyenne (PAPm) supérieure à 20 mmHg au repos (Humbert et al., 2022), plutôt que 25 mmHg précédemment (Galiè et al., 2016). L'HTAP correspond au groupe 1 des HTP, caractérisée par une HTP précapillaire après exclusion principalement d'une pathologie

pulmonaire et d'une d'HTP post embolique. Le caractère précapillaire est défini par une pression capillaire pulmonaire inférieure ou égale à 15 mmHg en fin d'expiration (PCP) associée des résistances vasculaires pulmonaires supérieures à 2 unité Wood, excluant les cardiopathies gauches (Humbert et al., 2022).

I.3 – Maladies associées à l'HTAP

La classification de l'HTP individualise 5 sous-groupes dont l'HTAP représente le premier sous-groupe. L'HTAP est elle-même dichotomisée en plusieurs sous-groupes de patients en fonction des maladies sous-jacentes associées possibles. La forme la plus fréquente est dite idiopathique (HTAPi), situation clinique dans laquelle aucune maladie associée n'est retrouvée. A côté des HTAPi, les principales maladies associées à l'HTAP sont (**Tableau 1**) :

1. HTAP de forme héritable (H-HTAP) : HTAP causée par des mutations génétiques transmises de manière autosomique dominante, impliquant principalement le gène Bone Morphogenetic Protein Receptor Type 2 (BMPR2) et d'autres gènes liés à la voie de signalisation du Transforming Growth Factor beta 2 (TGF- β).
2. Exposition à des toxiques et des médicaments : certaines expositions médicamenteuses comme les anorexigènes (fenfluramine et dexfenfluramine) peuvent être associées au développement d'une HTAP.
3. HTAP associée à des connectivites : notamment la sclérodermie systémique (ScS), le lupus érythémateux systémique (LES), la connectivite mixte (syndrome de Sharp, CM) et le syndrome de Sjögren primaire.
4. HTAP associée à l'infection par le virus de l'immunodéficience humaine (VIH).
5. HTAP associée à l'hypertension portale.

6. HTAP associée à des maladies cardiaques congénitales : telles que les communications interauriculaires, les communications interventriculaires, les malformations de la valve pulmonaire et les cardiopathies cyanogènes.
7. HTAP associée à l'infection parasitaire à Schistosome.
8. L'HTAP dans le cadre de la maladie veino-occlusive pulmonaire avec composante veinulaire et/ou capillaire.
9. Et l'hypertension pulmonaire persistante du nouveau-né.

I.4 – HTAP associée aux connectivites et à l'infection par le VIH

I.4.1 – HTAP associée aux connectivites

Les connectivites sont un groupe complexe et protéiforme de maladies auto-immunes systémiques, caractérisées par des dérégulations du système immunitaire inné et adaptatif, pouvant partager des atteintes cliniques multiples (cutanée, articulaire, musculaire, vasculaire, etc.). Leur développement implique dans une certaine mesure des prédispositions génétiques, l'implication de plusieurs voies moléculaires (interféron de type I, TGF- β , TNF- α , etc.) et parfois des facteurs déclenchants environnementaux (Wahren-Herlenius & Dörner, 2013). On retrouve comme principales connectivites le lupus érythémateux systémique (LES), la sclérodermie systémique (ScS), le syndrome de Sjögren primitif (SS), la connectivite mixte (syndrome de Sharp, CM), qui sont associées avec un risque accru au développement d'une HTAP, tandis que la polyarthrite rhumatoïde (expression articulaire prépondérante) et les myopathies inflammatoires idiopathiques qui ne sont pas associées à un surrisque d'HTAP. Leur traitement implique généralement l'utilisation d'immunosuppresseurs ou de l'hydroxychloroquine en dehors de la ScS.

Les prévalences de l'HTAP associée aux différentes connectivites peuvent varier en fonction de plusieurs facteurs tels que la population étudiée (origine géographique), le délai de suivi, ou les critères diagnostiques utilisés. L'HTAP est fréquente au cours de la ScS et de la CM, non anecdotique au cours du LES principalement en Asie, et plus rare au cours du SS.

La sclérodémie systémique (ScS) est une maladie auto-immune systémique rare caractérisée par une vasculopathie, une fibrose tissulaire, la présence de marqueurs d'auto-immunité, dont la physiopathologie est complexe (Thoreau et al., 2021) (**Annexe 1**). Sa prévalence est évaluée en France entre 132 à 158 cas/million d'habitants, avec une large prédominance féminine (sex-ratio femme/homme de 3:1 à 15:1) (Bergamasco et al., 2019; El Adssi et al., 2013; Le Guern et al., 2004). Elle est classée en forme cutanée diffuse, les lésions cutanées fibrosantes remontant au-dessus des coudes et des genoux, ou forme cutanée limitée, les lésions de sclérose cutanée intéressant la distalité des membres et le visage en épargnant les régions proximales (LeRoy et al., 1988). L'HTAP est une complication fréquente associée la ScS (HTAP-ScS) dont la prévalence évaluée selon les études varie entre 8 et 12 % basée sur le cathétérisme cardiaque droit. Elle représente la première cause de morbidité et de mortalité dépassant parfois selon les registres celle associée à la pneumopathie interstitielle diffuse (Lechartier & Humbert, 2021). Sa prévalence peut être surestimée lorsque seule l'échocardiographie est utilisée pour poser le diagnostic d'HTAP. La ScS représente la première cause d'HTAP associée aux connectivites en Europe et en Amérique du Nord (Avouac et al., 2010; Coghlan et al., 2014; Jais et al., 2008). A contrario, en Asie, la ScS ne représente que 6 à 22% des patients parmi les HTAP associée à une connectivite, supplantée par le LES (Hao et al., 2014; R. Zhang et al., 2011). La prévalence d'une HTP pré-capillaire chez les patients sclérodermiques est évaluée entre 5 à 19%, pouvant correspondre à une HTAP (Avouac et al., 2010; Coghlan et al., 2014; Launay et al., 2017, 2018), être secondaire à une pneumopathie interstitielle diffuse (groupe

3), ou parfois avec des caractéristiques d'atteinte veineuse et capillaire (Günther et al., 2012; Launay et al., 2017). Une atteinte postcapillaire en lien avec une atteinte myocardique spécifique de la ScS est aussi fréquente, résultant d'une dysfonction ventriculaire gauche systolique ou diastolique (groupe 2) (Launay et al., 2017). Du fait de la prévalence et de l'incidence élevées de l'HTAP au cours de la ScS, une stratégie de dépistage dédiée de l'HTAP est recommandée chez les patients asymptomatiques, par une approche multimodale prenant en compte des données cliniques, biologiques (BNP ou NT pro BNP), échocardiographiques (principalement la mesure de la pression artérielle pulmonaire systolique à partir de la vitesse maximale du flux de l'insuffisance tricuspide) et fonctionnelles respiratoires, suivie d'une évaluation annuelle. Plusieurs algorithmes de dépistages existent dont l'algorithme DETECT (Coghlan et al., 2014). La confirmation par KTD doit être systématique. Dans les autres connectivites, le dépistage de l'HTP n'est pas recommandé de manière systématique en absence de point d'appel clinique. L'échocardiographie de dépistage doit être réalisée en présence de symptôme évocateurs. Chez les patients atteints d'une connectivite de chevauchement avec des caractéristiques de ScS (ScS associée à une autre connectivite), une évaluation annuelle du risque de survenue d'une HTAP est recommandée (Humbert et al., 2022).

La connectivite mixte (ou syndrome de Sharp, CM) associe des manifestations cliniques observées au cours de la ScS, du LES, de la polyarthrite rhumatoïde et des myopathies inflammatoires idiopathiques, la positivité des auto-anticorps anti-U1 RNP (Alarcón-Segovia & Cardiel, 1989), sans remplir les critères de classification de chacune de ces connectivites. Bien que l'épidémiologie de la CM soit mal connue, sa prévalence a été évaluée en 2008 à 38 cas/million d'habitants en Norvège (Gunnarsson et al., 2011), avec un ratio femme/homme de 3 à 12:1 selon les régions géographiques et les critères d'inclusion. La prévalence de l'HTAP

associée à la connectivité mixte (HTAP-CM) a été rapportée comme étant très élevée dans les cohortes historiques, évaluée entre 17 et 24% par des méthodes diagnostiques très hétérogènes principalement basées sur l'échocardiographie (Alpert et al., 1983; Burdt et al., 1999; Sullivan et al., 1984; Wigley et al., 2005). Plus récemment, la prévalence de l'HTP a été rapportée comme étant beaucoup plus faible, de 3,4 % (Gunnarsson et al., 2013). Une méta-analyse récente regroupant les données de 983 patients avec une CM issues de 8 études observationnelles et essais contrôlés randomisés a évalué la prévalence globale de l'HTAP-CM à 12.5% (Hassan et al., 2023). La présence d'une péricardite, d'une pneumopathie interstitielle diffuse, d'une thrombopénie et la positivité des auto-anticorps anti-Sm ou l'absence de polyarthrite a été identifiée comme des facteurs prédictifs indépendant associés à la présence d'une HTAP/HTP au cours des CM (Chaigne et al., 2023). Elle représente la première cause de mortalité au cours des CM.

Le lupus érythémateux systémique (LES) est une maladie auto-immune systémique protéiforme avec des atteintes organiques multiples (cutanéomuqueuse, articulaire, hématologique, sérique, etc.), certaines potentiellement sévères (glomérulonéphrite lupique, neurolupus, etc.), touchant préférentiellement la femme en période d'activité génitale, avec la présence d'anticorps antinucléaires et d'autres auto-anticorps spécifiques (Aringer et al., 2019). Sa prévalence en France a été évaluée à 470 cas/million d'habitants, avec un ratio femme/homme qui augmente avec l'âge des patients, de 3.5:1 à 13:1 (Arnaud et al., 2014). L'atteinte pulmonaire est possible au cours du LES, sous forme d'une atteinte pleurale, exceptionnellement d'une pneumopathie interstitielle diffuse, d'HTAP, d'HTP thromboembolique chronique particulièrement au cours du syndrome des anti-phospholipides (SAPL) associé, ou d'HTP secondaire à une cardiomyopathie (HTP du groupe 2). La prévalence estimée de l'HTAP au cours du LES (HTAP-LES) est faible, évaluée entre 2.8 et 4.2% et mal documentée

car basée exclusivement sur des données d'échocardiographie (PAP systolique, PAPs > 30 mmHg) (T. L. Pan et al., 2000; Prabu et al., 2009; Yang et al., 2013). Il n'y a pas de large cohorte basée sur le KTD qui ait évalué l'incidence de l'HTAP au cours du LES. Bien que la ScS soit reconnue comme la première cause d'HTAP associée aux connectivites dans les pays occidentaux, le LES représente la moitié des connectivites au cours de l'HTAP associée à une connectivite en Asie, et particulièrement en Chine (Hao et al., 2014; R. Zhang et al., 2011). Certains facteurs de risque associés à l'HTAP-LES ont été observés, comme la positivité des auto-anticorps anti-Sm, anti-SSA et anti-SSB. La positivité des auto-anticorps U1-RNP est associée à une meilleure survie (Hachulla et al., 2018). Il a par ailleurs été identifié de manière rétrospective chez des patients chinois deux phénotypes cliniques d'HTAP-LES, avec d'une part un phénotype vascularitique (« *vasculitis cluster* ») illustré par une activité du LES élevée avec des manifestations lupiques systémiques et une HTAP de survenue précoce (généralement < 2 ans), et d'autre part un phénotype clinique vasculopathique (« *vasculopathic cluster* ») avec une activité systémique faible (peu de manifestation systémique) et un phénotype vasculaire pur révélé par l'HTAP de révélation plus tardive et associé à un gain de survie à 3 ans pour ce dernier par rapport au phénotype vascularitique (Sun et al., 2019).

Le syndrome de Sjögren (SS) est aussi une maladie auto-immune systémique, touchant principalement la femme et caractérisée par un dysfonctionnement et une destruction progressive des glandes exocrines avec une infiltration lympho-plasmocytaire et une hyperréactivité immunologique. La symptomatologie est dominée en premier lieu par un syndrome sec (buccal, oculaire, etc.). De nombreux signes extra-glandulaires sont aussi décrits. Au point de vue immunologie, il se caractérise par la présence d'auto-anticorps anti-SSA et/ou anti-SSB (Shiboski et al., 2016). Le syndrome de Sjögren peut être primaire ou secondaire à une autre connectivite. La prévalence de la forme primaire est évaluée à 10 à 100 cas/million

d'habitants, avec un ratio femme/homme de 9:1 (Devauchelle-Pensec et al., 2023; Maldini et al., 2014). L'HTAP est une complication exceptionnelle au cours du syndrome de Sjögren primaire (HTAP-SS) sans estimation étayée de sa prévalence. La fréquence du SS primaire au sein des HTAP associée à une connectivité est beaucoup plus importante en Asie, pouvant aller jusqu'à 15% comparée à sa proportion très minoritaire dans les pays occidentaux (Hao et al., 2014). Dans une étude chinoise récente portant sur 103 patients atteints d'une HTAP-SS prouvée par KTD et comparés à 526 patients avec un SS sans HTAP, les positivités des auto-anticorps anti-SSB (Odds ratio OR 4.1) et anti-U1 RNP (OR 29.5) étaient des facteurs indépendants associés à la présence d'une HTAP (Wang et al., 2020).

I.4.2 – HTAP associée au VIH

Selon l'OMS, à la fin de l'année 2022, environ 39 millions de personnes dans le monde vivaient avec le VIH, représentant un problème de santé publique majeur, avec 630 000 décès en 2022 liés à l'infection par le VIH (World Health Organization (WHO), 2023). L'utilisation moderne des traitements antirétroviraux hautement actifs (HAART) est à l'origine d'une réduction importante de la mortalité induite par le VIH, avec en parallèle une augmentation de la survie à long terme (Antiretroviral Therapy Cohort Collaboration, 2017; Quinn, 2008). Les recommandations thérapeutiques actuelles indiquent l'initiation des antirétroviraux quel que soit la charge virale du VIH et le taux de lymphocytes T CD4⁺ (Gandhi et al., 2023). Ce gain de survie s'accompagne d'une augmentation des maladies chroniques non liées au syndrome d'immunodéficience acquise (SIDA), telles que l'HTAP associée au VIH (Boccaro, 2008). Les causes de décès non liées au SIDA représentent de nos jours plus de 50% de la mortalité chez les patients vivant avec le VIH (Sellier et al., 2023).

L'HTAP associée au VIH (HTAP-VIH) est une complication cardiovasculaire sévère au cours de l'infection par le VIH, associée à un mauvais pronostic quel que soit le stade de l'infection VIH (Nunes et al., 2003). Sa prévalence est évaluée à 0,46% chez les patients séropositifs pour le VIH (Sitbon et al., 2008). Certaines caractéristiques du VIH, notamment l'activation chronique du système immunitaire et l'inflammation systémique semblent être impliquées dans la pathogenèse de l'HTAP, multipliant par 1000 le risque de développer une HTAP chez les sujets vivant avec le VIH par rapport à la population générale (Jarrett & Barnett, 2017). Une méta-analyse récente a identifié comme facteurs de risque associés à la présence d'une HTAP chez les patients vivants avec le VIH un nombre moyen de lymphocytes T CD4⁺ plus bas, une proportion plus importante de patients avec une charge virale du VIH détectable, et l'absence de traitement antirétroviral (Y. Liu et al., 2022). Il n'a pas été identifiée par contre de corrélation entre la sévérité de l'HTAP et le degré d'immunodépression (Mehta et al., 2000; Nunes et al., 2003).

La présentation clinique, échographique et hémodynamique de l'HTAP-VIH partage de nombreuses caractéristiques avec l'HTAPi (Sitbon, 2008). Du fait de la prévalence faible de l'HTAP au cours du VIH, il n'est pas recommandé de dépister l'HTAP chez les patients vivant avec le VIH de manière systématique. Une échocardiographie est recommandée lors de symptôme compatible (dyspnée, malaise, etc.) sans cause explicable afin d'éliminer une autre étiologie (cardiomyopathie, myocardite) ; et un cathétérisme cardiaque droit reste l'examen de référence pour confirmer le diagnostic d'HTAP (Humbert et al., 2022; Sitbon, 2008).

L'HTAP est un facteur de risque de mortalité indépendant au cours de l'infection par le VIH, avec une survie dans les années 1990 évaluée à moins de 50% à 3 ans avant la large disponibilité des antirétroviraux (Nunes et al., 2003). La survie globale à 5 ans des patients

avec une HTAP-VIH est superposable à celle des formes idiopathique/héritable (74% versus 69%) (Salvador et al., 2022). L'impact des HAART sur l'évolution de l'HTAP-VIH reste incertain. Au niveau expérimental, il a été observé une correction de l'HTP induite par l'hypoxie dans un modèle murin traité par antiprotéases (Gary-Bobo et al., 2010), mais ces données sont très préliminaires. Il a tout de même été montré dans une étude observationnelle, une réduction de l'incidence de l'HTAP-VIH dans la période suivant l'introduction des HAART (Zuber et al., 2004), mais d'autres données sont contradictoires (Sitbon et al., 2008). Contrairement à la stratégie moderne de combinaisons thérapeutiques adoptée dans les autres formes d'HTAP, les patients avec une HTAP-VIH traitée bénéficient principalement d'une monothérapie, et plus de 10% n'ont aucun traitement spécifique de l'HTAP, probablement pour des aspects d'interaction thérapeutique (Salvador et al., 2022). Le design rétrospectif de ces études empêche de tirer des conclusions définitives mais il est largement admis et recommandé de ne pas utiliser seuls les HAART chez les patients qui développent une HTAP-VIH (Humbert et al., 2022; Sitbon, 2008).

I.5 – Physiopathologie de l'HTAP associée aux connectivites et à l'infection par le VIH

I.5.1 – Physiopathologie de l'HTAP

La circulation pulmonaire est caractérisée dans les conditions physiologiques par le contraste entre un débit élevé et une faible pression permettant une grande capacité de recrutement vasculaire. L'HTAP est une pathologie complexe dont la physiopathologie n'est pas totalement élucidée. Elle réunit plusieurs mécanismes interconnectés conduisant au remodelage vasculaire pulmonaire qui intéresse préférentiellement les artérioles pulmonaires pré-capillaires entre 500 et 70 μm et les artères pulmonaires. Au point de vue histologique, il est observé une fibrose intimale et de l'adventice, une hyperplasie de la média, des lésions de thrombose in situ et chez les patients avec une forme idiopathique ou héréditaire d'HTAP des

lésions plexiformes correspondant à une prolifération focale de cellules endothéliales entourées de myofibroblastes, de cellules musculaires lisses vasculaires et de matrice extracellulaire. Des données récentes issues d'analyses d'explants pulmonaires d'HTAPi et d'H-HTAP ont fait évoluer l'interprétation de ces lésions complexes rencontrées dans la vasculopathie plexiforme. Actuellement une des hypothèses principales retenue est celle d'un shunt entre la micro vascularisation bronchique et les artérioles pulmonaires (Ghigna et al., 2016). Ces anomalies ont un substratum et une expression à différentes échelles, génétiques, moléculaires, cellulaires et organiques. Elles sont résumées dans la **Figure 2**.

Au niveau cellulaire, il existe une dysfonction endothéliale, une prolifération et une accumulation de plusieurs cellules vasculaires dans la paroi artérielle pulmonaire (cellules endothéliales, cellules musculaires lisses vasculaires, fibroblastes, myofibroblastes et péricytes), une infiltration adventitielles de nombreuses cellules immunitaires (lymphocytes B et T, cellules dendritiques, macrophages, etc.), une raréfaction du lit artériolaire pulmonaire, et des phénomènes de vasoconstriction et de thrombose (Humbert et al., 2019).

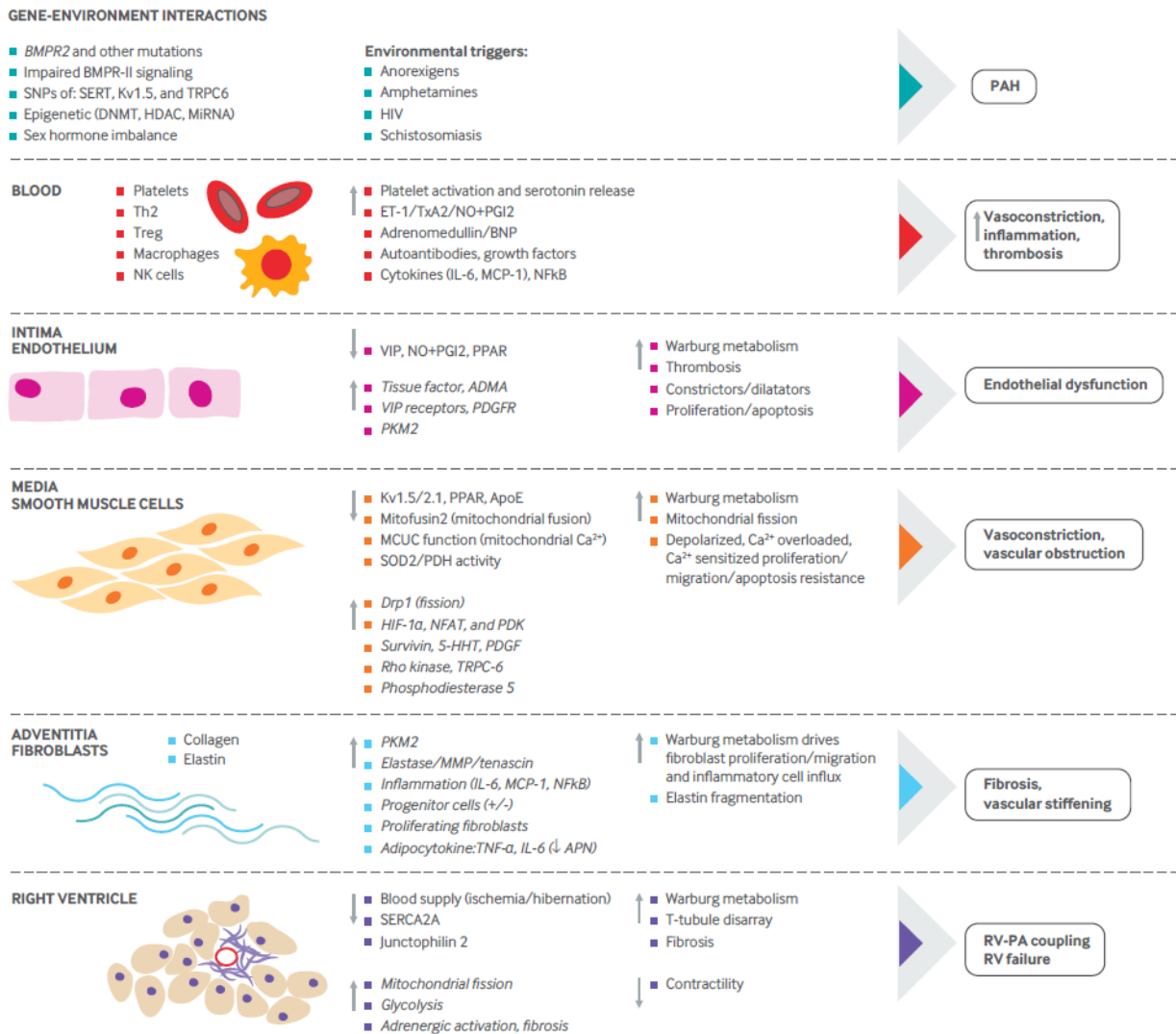


Figure 2 : Mécanismes impliqués dans la physiopathologie de l'HTAP (Thenappan et al., 2018)

Les mécanismes impliqués sont multiples et comprennent :

1. **Des anomalies génétiques** rencontrées principalement dans les formes d'HTAP dites héritables, dont l'hérédité est principalement mendélienne malgré l'existence de cas sporadiques par mutation de novo. Dans un contexte familial, des mutations germinales hétérozygotes perte de fonction du gène du récepteur osseux morphogénétique des protéines de type 2 (*BMPR2*) sont détectées dans au moins 70 à 80 % des cas (Evans et al., 2016; Morrell et al., 2019). Des avancées majeures ont été

faites dans le domaine de la génétique et de la génomique de l'HTAP, avec une estimation à environ 25 à 30% des patients chez qui l'on a diagnostiqué une HTAPi et qui ont finalement une cause génétique mendélienne sous-jacente et qui devraient être classés dans la catégorie des H-HTAP. Pour être actif, le récepteur BMPR2 s'hétérodimérise avec la kinase de type récepteur d'activine (ALK)-1 afin d'être stimulé par ses ligands bone morphogenic protease BMP9 ou BMP10, associé à son corécepteur, l'endogline. Cette stimulation permet notamment de protéger les cellules endothéliales de l'apoptose et d'un excès de prolifération, et diminue la perméabilité vasculaire. La disparition de l'axe BMP9/10-BMPR2 dans ces formes héréditaires est responsable d'une dysfonction endothéliale et d'une transition endothélio-mésenchymateuse à l'origine du remodelage vasculaire. La forte expression de BMPR2 sur les cellules endothéliales pulmonaires explique le tropisme spécifique de cette maladie pulmonaire. Plus récemment, des mutations localisées dans d'autres gènes de différents membres de la superfamille du facteur de croissance transformant (TGF)- β ont été rapportées, et principalement impliquées dans les voies de signalisation dépendantes de BMPR2 (**Tableau 2**). On y retrouve des mutations dans les gènes codant pour le contrôle général non répressible (GCN)-2 (EIF2AK4), kinase de type récepteur d'activine (ALK)-1 (ACVRL1), facteur de transcription T-box 4 (TBX4), BMP9 (GDF2), facteur de transcription HMG-box lié à SRY (SOX17), endogline (ENG), un canal potassique à deux domaines de pores appelé canal potassique sensible à l'acide (TASK)-1 (KCNK3) lié à TWIK, récepteur de sulfonilurée (SUR)-1 (ABCC8), ATPase 13A3 (ATP13A3), membre de la famille SMAD 8 (SMAD9), BMP10 (BMP10), aquaporine-1 (AQP1), SMAD1, SMAD4 et cavéoline-1 (CAV1). Actuellement, on ignore dans la plupart des cas comment ces mutations nouvellement découvertes dans certains cas d'H-HTAP

affectent le maintien de l'intégrité vasculaire pulmonaire et conduisent à l'occlusion des artérioles du poumon. A notre connaissance, il n'a pas été retrouvé de mutation dans la superfamille BMP dans les formes d'HTAP associée à une connectivite.

Tableau 2: Dénominations et fonctions de 16 gènes liés à l'hypertension artérielle pulmonaire (d'après Aldred (Aldred et al., 2022))

Symbole du gène	Localisation chromosomique	Nom complet	Fonction
ABCC8	11p15.1 (38 exons)	sulfonylurea receptor 1 (SUR1)	Ce gène code pour SUR1, un membre de la famille des protéines de transport de l'adénosine triphosphate (ATP) (ATP-binding cassette) qui fonctionnent comme des transporteurs, des canaux ioniques et des régulateurs de canaux.
ACVRL1	12q13.13 (11 exons)	Activin receptor-like kinase-1 (ALK1)	Ce gène code pour un récepteur transmembranaire de type I qui est abondamment exprimé par les cellules endothéliales et qui entraîne l'activation des transducteurs du signal <i>mothers against decapentaplegic homolog</i> SMAD1/5/8.
AQP1	7p14.3 (4 exons)	Aquaporin-1	Ce gène code pour une protéine qui est un canal membranaire permettant un mouvement rapide de l'eau sous l'effet d'un gradient osmotique transmembranaire.
ATP13A3	3q29 (36 exons)	ATPase 13A3	Ce gène code pour une ATPase de type P qui transporte une variété de cations à travers les membranes.
BMP10	2p13.3 (2 exons)	Bone morphogenetic	Ce gène code pour la BMP-10, une protéine sécrétée qui est principalement exprimée dans l'oreille droite. Parmi les BMP, BMP9 et BMP10 sont deux ligands de haute affinité

Symbole du gène	Localisation chromosomique	Nom complet	Fonction
		protein 10 (BMP-10)	pour ALK1 et BMPR2 présents dans un complexe hétéro-tétramérique sur les cellules endothéliales pulmonaires.
BMPR2	2q33.1-q33.2 (13 exons)	Bone morphogenetic protein receptor, type II (BMPRII)	Ce gène code pour un récepteur transmembranaire de type II avec un domaine sérine/thréonine kinase intrinsèque qui est exprimé sur les cellules endothéliales et musculaires lisses vasculaires pulmonaires et sur différents types de cellules inflammatoires. Lors de la liaison du ligand, BMPR2 phosphoryle et active les récepteurs de type I qui activent ensuite SMAD1/5/8.
CAV1	7q31.2 (4 exons)	Caveolin-1	Ce gène code pour une petite protéine d'échafaudage oligomérique nécessaire pour générer la courbure de la membrane dans des structures telles que les cavéoles. Il a été rapporté que la cavéoline-1 se lie à de nombreuses autres protéines, contrôle l'homéostasie du cholestérol et module différentes fonctions cellulaires (endocytose, internalisation des récepteurs, signalisation cellulaire, prolifération et apoptose).
EIF2AK4	15q15.1 (39 exons)	General control nonderepressible 2 (GCN2)	Ce gène code pour la protéine kinase GCN2 qui répond principalement à la privation d'acides aminés en liant les ARN de transfert non chargés. Lorsqu'elle est activée, la GCN2 phosphoryle la sous-unité alpha du facteur 2 d'initiation de la traduction eucaryote (EIF2), induisant une diminution de la synthèse des protéines.
ENG	9q34.11 (15 exons)	Endoglin	Ce gène code pour le récepteur transmembranaire de type III, l'endogline, qui est principalement exprimé sur les cellules

Symbole du gène	Localisation chromosomique	Nom complet	Fonction
			endothéliales en prolifération <i>in vitro</i> et sur les vaisseaux sanguins angiogéniques <i>in vivo</i> .
GDF2	10q11.22 (2 exons)	Bone morphogenetic protein 9 (BMP-9)	Ce gène code pour la BMP-9, principalement exprimée et excrétée par le foie. Parmi les BMP, BMP9 et BMP10 sont deux ligands de haute affinité pour ALK1 et BMPR2 présents dans un complexe hétéro-tétramerique sur les cellules endothéliales pulmonaires.
GGCX	2p11.2 (15 exons)	Gamma-glutamyl carboxylase (GGCX)	Ce gène code pour une protéine membranaire intégrale du réticulum endoplasmique rugueux qui carboxyle les résidus glutamate des protéines dépendantes de la vitamine K en gamma carboxyl glutamate.
KCNK3	2p23.3 (3 exons)	TWIK-related acid-sensitive potassium channel (TASK-1)	Ce gène code pour TASK-1, qui est un canal potassique sensible aux acides, contenant deux domaines P formant un pore contribuant aux potentiels de membrane au repos.
KDR	4q12 (30 exons)	Vascular endothelial growth factor receptor 2 (VEGFR2)	Ce gène code pour le récepteur 2 du facteur de croissance de l'endothélium vasculaire (VEGFR2) qui est considéré comme l'un des principaux récepteurs impliqués dans la prolifération, la migration et la survie des cellules endothéliales.
KLK1	19q13.33 (6 exons)	Kallikrein 1	Ce gène code pour un membre de la sous-famille des kallikréines, des sérine protéases qui sont impliquées dans diverses fonctions physiologiques.

Symbole du gène	Localisation chromosomique	Nom complet	Fonction
SMAD1	4q31.21 (12 exons)	SMAD family member 1 (SMAD1)	Ce gène code pour SMAD1, un des membres de la superfamille de transducteurs de signal de la voie du <i>Transforming growth factor</i> (TGF- β) après fixation à son récepteur de type I ou II. Une fois phosphorylé, SMAD1 se lie à Smad4 et migre vers le noyau.
SMAD4	18q21.2 (12 exons)	SMAD family member 4 (SMAD4)	Ce gène code pour le SMAD4 qui joue un rôle essentiel dans la médiation des effets en aval de la signalisation de la superfamille BMP/TGF- β .
SMAD9	13q13.3 (10 exons)	SMAD family member 8 (SMAD8)	Ce gène code pour SMAD8. Une fois phosphorylé, SMAD8 se lie à Smad4 et migre vers le noyau.
SOX17	8q11.23 (2 exons)	SRY-related HMG-box transcription factor (SOX17)	Ce gène code pour un membre des facteurs de transcription de la famille F des domaines de groupes de haute mobilité liés à Sry (SoxF), qui est un régulateur clé du développement de la lignée endothéliale et hématopoïétique.
TBX4	17q23.2 (11 exons)	T-box transcription factor 4 (TBX4)	Ce gène code pour le facteur de transcription à domaine T TBX4 qui est exprimé dans le mésenchyme pulmonaire où il joue un rôle central dans la régulation transcriptionnelle des gènes nécessaires à la différenciation du mésoderme.

2. **La dysfonction endothéliale** vasculaire pulmonaire qui est une des altérations les plus précoces participant au remodelage vasculaire et associe des altérations de synthèses de molécules régulatrices du tonus vasculaire, telles qu'une diminution de production

d'oxyde nitrique (NO), un puissant vasodilatateur ; une augmentation de la production d'endothéline-1 (ET-1), un puissant vasoconstricteur, l'ensemble entraînant une vasoconstriction artériolaire pulmonaire ; une diminution des propriétés anticoagulantes de l'endothélium, un phénotype pro-inflammatoire avec une augmentation de l'expression de molécules d'adhésion (intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1)), une synthèse inadaptée locale de cytokines pro-inflammatoires et de chémokines avec une dérégulation des mécanismes d'angiogenèse sous la dépendance du vascular endothelial growth factor (VEGF), du platelet derived growth factor (PDGF), du transforming growth factor (TGF- β), de l'angiopoietine-1, la perte de marqueurs membranaires endothéliaux (CD31, CD34 et VE-cadhérine) et l'acquisition de marqueurs de cellules mésenchymateuses et de cellules musculaires lisses tels que la vimentine ou l' α -smooth muscle actine, l'ensemble favorisant l'obstruction vasculaire (Huertas et al., 2018; Humbert et al., 2019).

- 3. L'accumulation de cellules musculaires lisses vasculaires et de fibroblastes adventitiels** qui participent aux phénomènes de vasoconstriction et de fibrose pariétale vasculaire. L'ensemble est à l'origine d'une perte de compliance vasculaire et une obstruction vasculaire pulmonaire. Plusieurs dysrégulations cumulatives ont été mises en évidence dans ces cellules (Thenappan et al., 2018), telles que l'orientation du métabolisme mitochondrial de l'oxydation du glucose vers une glycolyse découplée (métabolisme Warburg), l'augmentation de la fission mitochondriale (Marsboom et al., 2012), l'augmentation des capacités de prolifération et de migration, et la résistance à l'apoptose. Ces anomalies métaboliques et mitochondriales ont été constatées aussi bien dans les cellules musculaires lisses vasculaires pulmonaires (Bonnet et al., 2006),

les cellules endothéliales (Diebold et al., 2015; Fijalkowska et al., 2010) et les fibroblastes adventitiels (Li et al., 2016), permettant une prolifération cellulaire rapide en évitant l'apoptose mitochondriale (H. Zhang et al., 2017). L'augmentation du calcium cytosolique, via la diminution de contrôle de canaux calcique et potassique voltage dépendant contribue aux phénotypes contractile, hyper-prolifératif et anti-apoptotique des cellules musculaires lisses vasculaires pulmonaires rencontrés au cours l'HTAP (Yu et al., 2009; Yuan et al., 1998). Il a été démontré que la restauration de la polarisation membranaire calcium dépendante des cellules musculaires lisses vasculaires pulmonaires par restauration de l'activité du canal potassique voltage dépendant Kv1.5 améliore les paramètres hémodynamiques dans des modèles d'HTP chez le rat (Pozeg et al., 2003).

4. **Dérégulation du système immunitaire inné et adaptatif** (confère partie spécifique I.5.2 en infra).

I.5.2 – Rôle de l'inflammation et du système immunitaire dans l'HTAP

Le rôle de l'inflammation dans la pathogénie de l'HTAP a été démontrée pour la première fois dans l'HTAPi (Humbert et al., 1995). La proportion élevée de patients atteints de connectivite au sein des patients atteints d'HTAP est un argument supplémentaire en faveur de l'implication du système immunitaire dans sa physiopathologie. Les cellules endothéliales, les cellules musculaires lisses vasculaires, les fibroblastes et les myofibroblastes de patients ayant une HTAPi présentent une signature pro-inflammatoire illustrée par l'augmentation de synthèse de nombreuses cytokines pro-inflammatoires (IL-1, IL-6, TNF- α , etc.), de chémokines (C-C Motif Chemokine Ligand-2 (CCL-2), CCL-5) et des molécules d'adhésion à leurs surfaces (Cracowski et al., 2014; Huertas et al., 2020; Itoh et al., 2006; Le Hiress et al., 2015). Ces constatations dépassent le spectre restreint des HTAPi, puisque ces anomalies ont été bien décrites au cours

de la ScS (Thoreau et al., 2021) (**Annexe 1**). Par ailleurs, il a été observé une infiltration des lésions pariétales vasculaires pulmonaires par de nombreuses cellules immunitaires innées et adaptatives, tel que des lymphocytes T et B, des macrophages, des mastocytes, ou des cellules dendritiques (Cohen-Kaminsky et al., 2014; Dorfmueller et al., 2007; Perros et al., 2007, 2012; Savai et al., 2012). Ces cellules ont été identifiées dans les lésions occlusives plexiformes et l'inflammation périvasculaire est corrélée au remodelage vasculaire de l'intima et de la média (Stacher et al., 2012). Par ailleurs il a été observé une dysrégulation du phénotype de certaines de ces cellules comme un défaut dans les fonctions T régulatrices et une polarisation immunitaire par les cellules T-helper 17 (Hautefort et al., 2015; Huertas et al., 2016) (**Figure 3**). Cela a été observée dans l'HTAPi, mais aussi dans les formes héritables et associées à la ScS (Huertas et al., 2016). Par ailleurs, l'observation de structures anatomiques se rapprochant de follicules lymphoïdes tertiaires le long des vaisseaux pulmonaires remodelés de patients atteints d'HTAPi est un argument plaidant en faveur d'une réponse auto-immune locale (Perros et al., 2012). Des structures s'apparentant à des follicules lymphoïdes ont été observées dans les poumons de quelques patients avec HTAP-ScS (Dumoitier et al., 2017). Chez les patients avec une HTAPi, le nombre des plasmablastes dans le sang circulant semble être plus élevé et associé à de possibles expansions clonales par rapport à des sujets sains donneurs de sang (Blum et al., 2018).

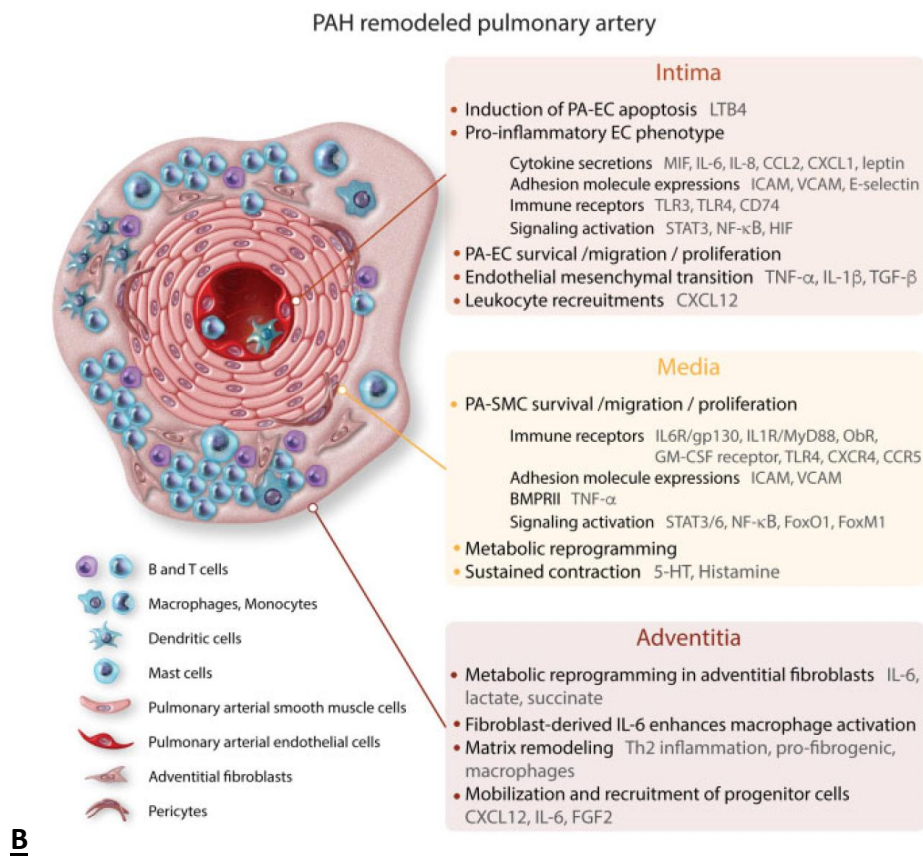
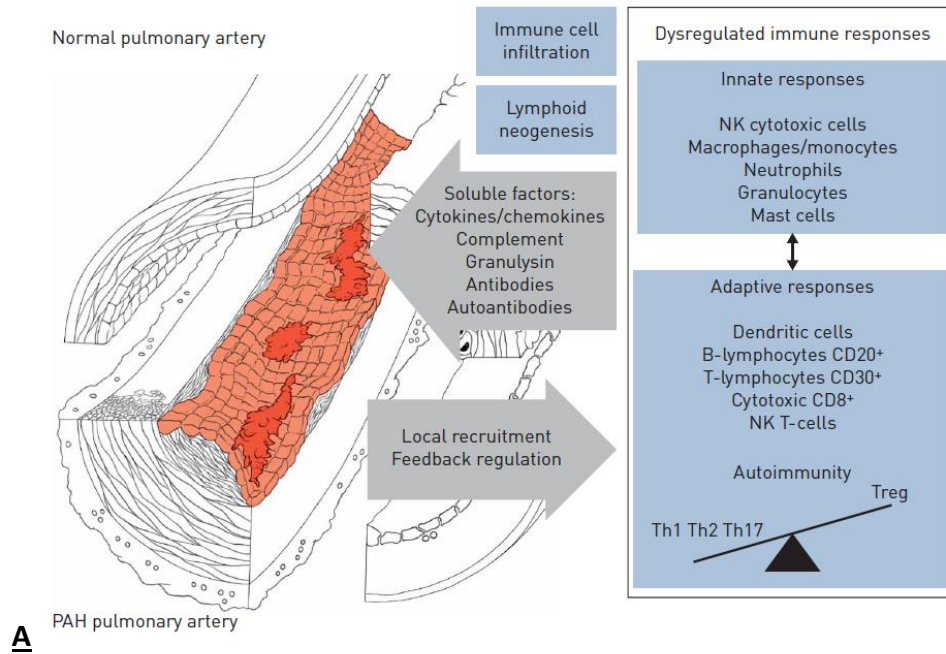


Figure 3 : Perturbations immunitaires associées à HTAP et au remodelage vasculaire pulmonaire (A) (Humbert et al., 2019) et implication de l'inflammation chronique dans les 3 compartiments vasculaires (B) (Huertas et al., 2020)

CCL, Ligand de chimiokine à motif C-C ; CXCL, ligand de chimiokine à motif C-X-C ; EC, cellules endothéliales ; FGF-2, facteur de croissance des fibroblastes 2 ; FoxO1, facteur de transcription Forkhead box O1 ; FoxM1, protéine Forkhead box M1 ; GM-CSF, facteur stimulant les colonies de granulocytes et de macrophages ; HIF, Facteur inductible par l'hypoxie ; 5-HT, récepteur à la sérotonine ; ICAM1, molécule d'adhésion intercellulaire 1 ; IL, interleukine ; IL1R, récepteur de l'interleukine 1 ; LTB4, leucotriène B4 ; MIF, facteur inhibiteur de la migration des macrophages ; MyD88, réponse primaire de différenciation myéloïde 88 ; NF- κ B facteur nucléaire-kap ; B ; ObR, récepteurs de la leptine ; PA-EC, cellules endothéliales artérielles pulmonaires ; STAT3, Transducteur de signal et activateur de transcription 3 ; Th1, lymphocytes CD4+ T helper 1 ; Th2, lymphocytes CD4+ T helper 2 ; TLR, tall like receptor ; TGF- β , facteur de croissance transformant bêta ; TNF- α , Facteur de Nécrose Tumorale alpha ; Treg, lymphocyte T régulateur ; VCAM1, molécule d'adhésion des cellules vasculaires 1.

Par ailleurs, plusieurs auto-anticorps ont été rapportés dans l'HTAP, faisant discuter leur rôle dans la physiopathologie de l'HTAP.

I.5.3 – Auto-anticorps associés à l'HTAP

Des auto-anticorps ont été identifiés au cours de l'HTAP, principalement dans les formes idiopathiques et associée à la ScS (Bussone et al., 2012; Dib et al., 2012; Karmochkine et al., 1996; Negi et al., 1998; Quismorio et al., 1984; Tamby et al., 2005, 2006; Terrier et al., 2008, 2010).

Notre équipe a contribué à identifier des auto-anticorps dans les HTAPi et les HTAP-ScS. Les cibles de ces auto-anticorps ont été caractérisées par électrophorèse bi-dimensionnelle (2D) suivie d'un immunoblot sur des extraits protéiques de cellules endothéliales, de fibroblastes et de cellules musculaires lisses vasculaires, puis identifiées par spectrométrie de masse. Des

tests ELISA ont également été réalisés pour confirmer la présence de ces auto-anticorps. Dans la ScS, ces auto-anticorps sont dirigés contre différents types cellulaires impliqués dans la physiopathologie de l'HTAP et présents dans la paroi vasculaire pulmonaire, comme la cellule endothéliale pulmonaire (anticorps anti-cellules endothéliales, AECA), le fibroblaste (anticorps anti-fibroblastes, AFA), et la cellule musculaire lisse vasculaire (anticorps anti-CMLV) (Thoreau et al., 2022) (**Annexe 2**). Certaines cibles antigéniques de ces auto-anticorps ont été identifiées, permettant de soulever des hypothèses quant aux mécanismes physiopathologiques potentiellement impliqués.

I.5.3.1 – Auto-anticorps anti-fibroblastes

Des AFA ont été identifiés dans 40% des sérums de patients atteints d'HTAPi et 30% des sérums de patients avec une HTAP-ScS (Tamby et al., 2006). Les pools sériques d'IgG de patients atteints d'HTAPi, d'H-HTAP, d'HTAP-ScS et de sujets sains incubés avec un extrait protéique de fibroblaste humain reconnaissent respectivement 103 ± 31 , 63 ± 20 , 81 ± 12 et 43 ± 22 spots protéiques. Vingt-et-un de ces spots étaient spécifiquement reconnus par les IgG sériques des patients présentant une HTAP, dont 16 ont pu être identifiés par spectrométrie de masse. Parmi ces 16 spots protéiques la vimentine, la calumenine, la tropomyosine 1, les heat shock proteins (HSP)-27 et 70, la glucose-6-phosphate-dehydrogenase, la phosphatidylinositol 3-kinase et la DAP kinase ont été identifiés. Ces protéines sont impliquées dans la régulation des fonctions du cytosquelette, dans la contraction cellulaire, dans le stress oxydatif ou dans le métabolisme énergétique cellulaire (Terrier et al., 2008).

I.5.3.2 – Auto-anticorps anti-cellules endothéliales

Des AECA ont également été identifiés dans les sérums de patients avec une ScS avec ou sans HTAP et les patients avec HTAPi, reconnaissant respectivement 110, 82, et 37 spots protéiques.

Par exemple, des AECA dirigés contre la lamine A/C, la chaîne β de la tubuline ou la vinculine ont été identifiés dans les sérums des patients avec HTAPi et des patients avec HTAP-ScS. Là aussi, les processus cellulaires identifiés associés à ces auto-anticorps sont multiples, tel que la régulation de la fonction du cytosquelette, le stress oxydatif ou le métabolisme énergétique cellulaire (Dib et al., 2012). Par ailleurs, les AECA de patients avec une HTAP-ScS avaient un profil de réactivité différent de ceux provenant de patients avec une HTAPi (Tamby et al., 2005). D'autres données *in vitro* semblent suggérer un rôle pathogène de certains de ces auto-anticorps, en induisant un phénotype pro-inflammatoire (synthèse d'IL-6), pro-adhérent (synthèse d'E-selectine, ICAM-1, VCAM-1) et la synthèse de chémokine (CCL-2) par les cellules endothéliales pulmonaires (Arends, Damoiseaux, Duijvestijn, Debrus-Palmans, Boomars, et al., 2013), mais ces données n'ont pas été reproduites.

1.5.3.3 – Auto-anticorps anti-cellules musculaires lisses vasculaires

Nos travaux ont par ailleurs mis en évidence la présence d'auto-anticorps anti-CMLV en immunofluorescence chez des patients avec ScS avec ou sans HTAP, des patients avec HTAPi et des sujets sains. Les IgG sériques de ces patients ont induit *in vitro* une contraction plus importante des cellules musculaires lisses vasculaires dans une matrice collagène en comparaison des IgG de sujets sains. Les immunoblot-2D et la spectrométrie de masse ont permis d'identifier comme cibles antigéniques la phosphoprotéine 1 induite par le stress (STIP-1) et l'alpha-enolase. Des méthodes ELISA ont confirmé la positivité des auto-anticorps anti-STIP-1 dans les sérums de 84% des patients ScS sans HTAP, 76% des sérums HTAP-ScS, 24% des sérums HTAPi et dans seulement 3% des sérums de sujets sains (Bussone et al., 2012).

Outre l'effet inhibiteur de la prolifération cellulaire, des auto-anticorps décrits dans l'HTAP semblent pouvoir favoriser l'expression de cytokines pro-inflammatoires, activer des facteurs

pro-apoptotiques, favoriser la prolifération des CMLV, et pourraient donc participer au remodelage vasculaire pulmonaire.

Ces auto-anticorps ont donc été principalement décrits au cours de l'HTAPi et l'HTAP-ScS. Les données relatives à la présence d'auto-anticorps et l'identification de leurs cibles antigéniques au cours de l'HTAP associée aux autres connectivites ou au VIH sont beaucoup plus ténues voir absentes.

I.5.4 – Spécificités de l'HTAP associée aux connectivites

Les connectivites peuvent être associées à plusieurs groupes d'HTP en lien avec leur caractère systémique (atteinte multi-organique). La ScS est la première connectivite associée à l'HTAP en Occident, mais elle peut aussi être associée à une HTP du groupe 3 en lien avec une pneumopathie interstitielle diffuse (ScS-PID), ou du groupe 2 consécutive à une atteinte cardiaque spécifique ou en lien avec une prévalence élevée de dysfonction diastolique du ventricule gauche ; ou une maladie veino-occlusive pulmonaire (MVO) (Haque et al., 2021). Ces HTP du groupe 2 ou 3 peuvent également se rencontrer dans d'autres connectivites comme le LES ou les CM. L'association d'atteintes obstructives veinulaire et artériolaire est par ailleurs plus fréquente dans les HTAP associées à la ScS ou à la CM principalement, avec une prévalence évaluée de 75% (Dorfmueller et al., 2007; O'Callaghan et al., 2011), comme rencontrées au cours de maladie veino-occlusive pulmonaire associée à une connectivite. La distinction entre la maladie veino-occlusive pulmonaire et les autres formes d'HTAP est souvent extrêmement difficile, bien qu'il existe des signes tomodensitométriques évocateurs comme l'association d'opacités en verre dépoli centrolobulaires, des lignes septales et des adénopathies médiastinales (Günther et al., 2012; Remy-Jardin et al., 2021). Par ailleurs, au cours d'une MVO pulmonaire, l'utilisation d'un traitement vasodilatateur (inhibiteurs

calciques ou autre traitement spécifique de l'HTAP) induit une aggravation rapide de la situation clinique et hémodynamique (Montani et al., 2016).

Plusieurs différences histopathologiques entre les connectivites ont été observées dans la vasculopathie associée à l'HTAP. Dans l'HTAP-ScS, le phénotype est principalement fibrotique illustré par la présence d'occlusion vasculaire fibreuse, l'épaississement intimal des artères de taille moyenne et des petits vaisseaux ramifiés, et en contrepartie moins d'éléments inflammatoires observés. La vasculopathie plexiforme est quant à elle rarement observée dans la ScS, tout comme la présence de lésion de vascularite fibrinoïde avec dépôts d'immunoglobulines (Dorfmueller et al., 2007; Sasaki et al., 2011). Par ailleurs, le remodelage vasculaire impliquant les veines et les veinules pulmonaires est plus fréquent dans la ScS, reflétant une prévalence plus élevée de maladie veino-occlusive pulmonaire associée, correspondant à une congestion parcellaire sévère des capillaires avec des hémorragies alvéolaires occultes (Overbeek et al., 2009).

Le phénotype rencontré dans l'HTAP-CM est proche de celui de la ScS ou parfois celui d'une forme frontière entre la ScS et celui du LES, avec un épaississement intimal fibreux prépondérant et plus rarement une vasculopathie plexiforme ou de la vascularite fibrinoïde. Dans l'HTAP-LES, les lésions sont proches de celles observées dans l'HTAPi, avec une artériopathie pulmonaire plexogénique, la présence fréquente d'une vascularite fibrinoïde avec des dépôts de complexes immuns d'IgG, IgM, C3 et C1q, en particulier dans l'intima et la média. Les lésions fibreuses sont plutôt rarement observées (Quismorio et al., 1984; Sasaki et al., 2011). De plus, des occlusions intraluminales par des thrombi dans les artères pulmonaires plus proximales sont possibles, en particulier dans le cadre d'un SAPL associé, pouvant correspondre à une HTP thromboembolique chronique surajoutée (groupe 4) (Lang & Madani,

2014). Les données histopathologiques concernant l'HTAP associée au syndrome de Sjögren primaire sont trop rares pour pouvoir en dégager des spécificités propres.

I.5.5 – Spécificités de l'HTAP associée au VIH

La pathogénie de l'HTAP-VIH est multifactorielle avec encore de très nombreuses inconnues. Elle ferait intervenir notamment des effets pathogènes directs des protéines du VIH, la toxicomanie à la cocaïne, l'inflammation chronique et l'auto-immunité (Jarrett & Barnett, 2017). L'infection directe de l'artère pulmonaire par le VIH n'a pas été documentée, ne permet pas d'expliquer une réaction immunitaire locale déclenchée par la présentation de l'antigène au niveau pulmonaire (Mette et al., 1992). Certaines protéines virales, telles que la protéine virale Gp120, la protéine régulatrice trans-activateur de transcription du VIH (TAT) ou la protéine Nef (Negative Regulatory Factor) ciblent l'endothélium pulmonaire humain, induisant la prolifération, l'activation et l'apoptose endothéliale, ainsi que la synthèse de cytokines pro-inflammatoires comme l'IL-6 et de l'endothéline-1 aux propriétés vasoconstrictrices (Tcherakian et al., 2013). La protéine TAT affecte par ailleurs la voie de signalisation du BMPR2. Un autre mécanisme impliqué serait l'inflammation chronique associée à la déplétion des lymphocytes T CD4⁺ dans l'intestin, induisant la translocation de lipopolysaccharides et la surproduction d'IL-6 (Jarrett & Barnett, 2017). L'ensemble de ses données sont exploratoires et repose sur de petits effectifs d'étude.

Au point de vue histopathologique, 75 % des patients avec HTAP-VIH présentent des lésions typiques rencontrées au cours de l'HTAP (Mehta et al., 2000), et ne se distinguent pas des lésions retrouvées dans l'HTAP associée aux causes non infectieuses (Petitpretz et al., 1994).

I.6 – Places des traitements médicamenteux spécifiques de l'HTAP

Les traitements spécifiques médicamenteux de l'HTAP ciblent la voie de l'endothéline avec les antagonistes des récepteurs de l'endothéline (ARE) (bosentan, macitentan, ambrisentan), la voie de l'oxyde nitrique avec les inhibiteurs de la phosphodiesterase 5 (PDE5i) (sildenafil, tadalafil) et le stimulateur de la guanylate cyclase soluble (riociguat), la voie des prostacyclines avec les analogues de la prostacycline (iloprost, époprostenol, trepostinil) et un agoniste du récepteur de la prostacycline (selexipag).

La stratégie d'utilisation des traitements spécifiques de l'HTAP dans les formes associées aux connectivites est superposable à celle des formes idiopathiques (Humbert et al., 2022). Les patients avec une HTAP associée à une connectivite ont été inclus dans la plupart des essais contrôlés randomisés de validation/autorisation de ces traitements. Leur utilisation est synthétisée dans la revue générale en **Annexe 3**. Cette stratégie est basée sur l'évaluation du risque de mortalité selon un modèle multi-stratifié (risque faible, intermédiaire ou élevé) et en parallèle une stratégie d'escalade thérapeutique dépendante de la réévaluation du risque en termes de réponse thérapeutique (**Figure 4**). Au cours de cette évaluation multimodale du risque, la réalisation pendant le KTD d'un test de vaso-réactivité aux inhibiteurs calciques n'est pas indiqué chez les patients avec une connectivite, contrairement aux formes idiopathique, héritable ou associée à l'exposition toxique potentiellement inductrice d'HTAP. L'utilisation des inhibiteurs calciques n'est donc pas recommandée dans les HTAP associées aux connectivites (Humbert et al., 2022). Chez les patients sans comorbidité cardiopulmonaire et à risque faible à intermédiaire, il est recommandé de débiter une bithérapie par ARE et PDE5i ; alors qu'il sera d'emblée proposé chez les patients à risque élevé de débiter une trithérapie ARE+ PDE5i + analogue des prostacycline (IV ou SC) (**Figure 4**).

En analogie à la stratégie thérapeutique des HTAPi, la combinaison de traitements spécifiques de l'HTAP dans les HTAP associées aux connectivites apporte un bénéfice par rapport à la monothérapie sur le risque de dégradation clinique et l'amélioration de la distance de marche au cours du temps de marche de 6 minutes qui représente le critère de jugement principal consensuel des essais thérapeutiques (J. Pan et al., 2018). À ce jour, 6 essais contrôlés randomisés ont analysé spécifiquement les données concernant les patients atteints d'HTAP associée aux connectivites (SUPER, PHIRST, ARIES, PATENT, GRIPHON, AMBITION) (Badesch et al., 2007; Coghlan et al., 2017; Fischer et al., 2016; Gaine et al., 2017; Galiè et al., 2017; Humbert et al., 2017). Les données relatives sont décrites dans l'**Annexe 3**. Pour finir, deux essais thérapeutiques récents contrôlés randomisés contre de placebo comprenant environ 15% de patients avec une HTAP associée à une connectivite ont démontré l'efficacité du Sotatercept, une protéine de fusion ciblant le domaine extracellulaire du récepteur de l'activine de type IIA (ActRIIA), un facteur de croissance de la voie du TGF- β , sur la réduction des résistances vasculaires pulmonaires et sur l'amélioration de la distance de marche à 6 mois de traitement en association avec d'autres traitements spécifiques de l'HTAP (Hoepfer et al., 2023; Humbert et al., 2021).

Malgré une prise en charge médicamenteuse spécifique de l'HTAP optimisée, un certain nombre de patients progresse vers un stade terminal réfractaire de la maladie avec un retentissement fonctionnel sévère et une mortalité très élevée, devant faire discuter la possibilité d'une transplantation pulmonaire ou cœur-poumon (Christie et al., 2012; Humbert et al., 2022; Le Pavec et al., 2018). Dans cette optique thérapeutique de transplantation pulmonaire, la présence d'une connectivite ne constitue pas une contre-indication (Launay et al., 2014; Pradère et al., 2018). Des données récentes relatives à la transplantation pulmonaire pour une pneumopathie interstitielle diffuse associée à la ScS évoluant vers une insuffisance

respiratoire chronique montrent un pronostic et des taux de survie similaires à ceux rencontrés dans la fibrose pulmonaire idiopathique (Crespo et al., 2016). L'indication d'une transplantation pulmonaire nécessite une évaluation multidisciplinaire précoce dans un centre de référence de prise en charge de l'HTAP (Kolaitis, 2023). La prise en compte de spécificités liées à la connectivité est essentielle dans l'évaluation de l'indication et de la faisabilité (par exemple la présence d'une atteinte digestive de la ScS ou d'ulcération digitale active, atteinte musculaire ou cardiaque associée, etc.) (Hachulla et al., 2021). La prise en charge spécifique de la connectivité sous-jacente selon les recommandations en vigueur est bien entendue associée.

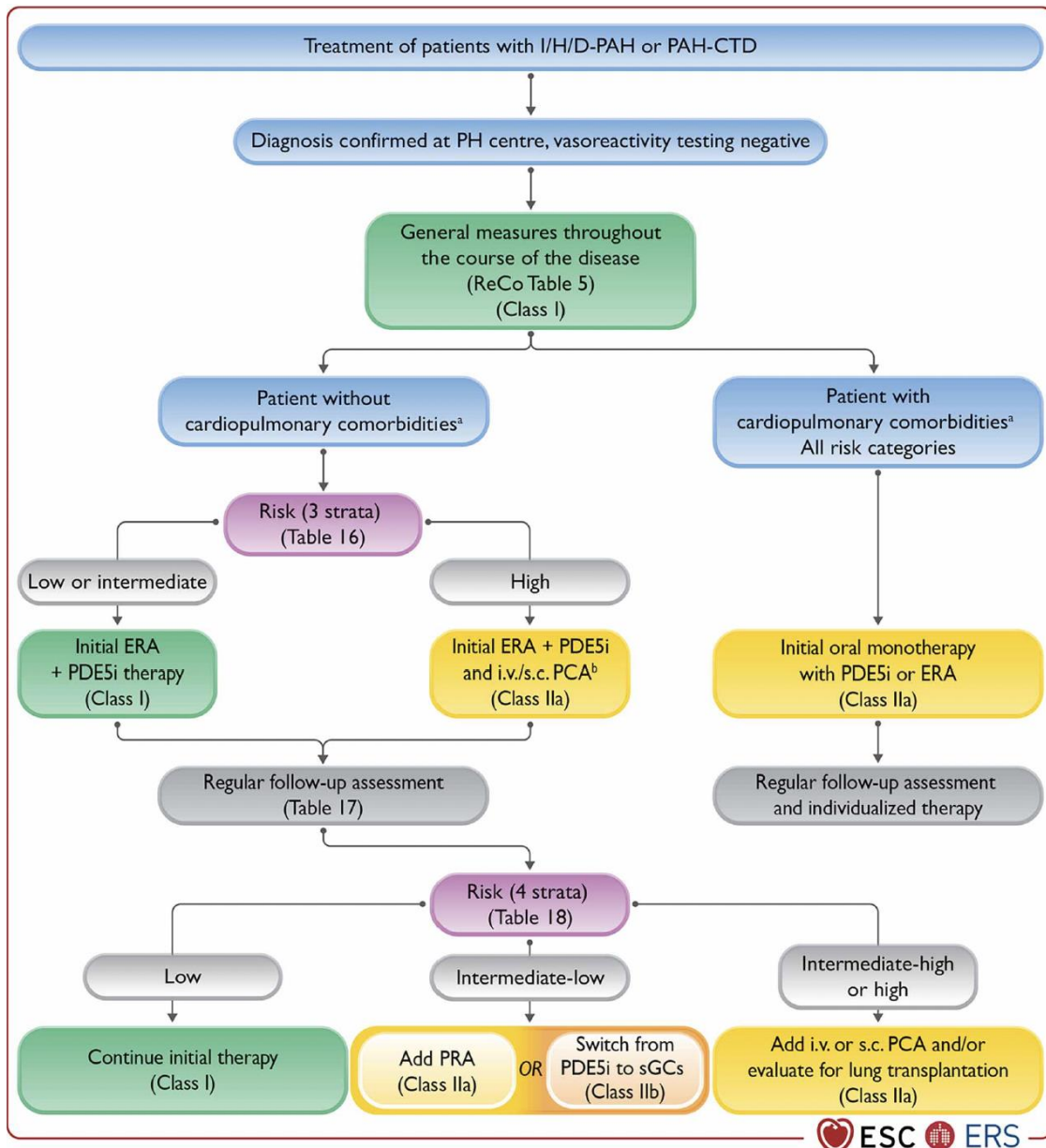


Figure 4 : Algorithme thérapeutique pour les HTAP idiopathique, héritable, induites par les médicaments et associée aux connectivites (Humbert et al., 2022)

DLCO, capacité de diffusion du monoxyde de carbone ; ERA, antagonistes des récepteurs de l'endothéline 1 ; I/H/D-PAH, hypertension artérielle pulmonaire (HTAP) idiopathique (I), héritable (H), associée aux médicaments (D) ; i.v., intraveineux ; PAH-CTD, HTAP associée aux connectivite ; PCA, analogues de la prostacycline ; PDE5i, inhibiteurs de la phosphodiesterase-

5 ; PH, hypertension pulmonaire ; PRA, agoniste du récepteur de la prostacycline ; ReCo, recommandation ; s.c., sous-cutané ; sGCs, stimulateur de la guanylate cyclase soluble.

^aLes comorbidités cardio-pulmonaires sont des entités associées à un risque accru de dysfonction diastolique du ventricule gauche, et comprennent l'obésité, l'hypertension, le diabète sucré et les maladies coronariennes; les comorbidités pulmonaires peuvent inclure des signes de maladie pulmonaire parenchymateuse légère et sont souvent associées à une DLCO faible (<45% de la valeur prédite). ^bÉpoprosténol intraveineux ou tréprostinil i.v./s.c.

I.7 – Place des immunosuppresseurs dans le traitement de l’HTAP

Plusieurs aspects du traitement des HTAP associée aux connectivites diffèrent par rapport aux autres formes d’HTAP et au sein même des connectivites, tel que l’utilisation d’un immunosuppresseur.

L’utilisation combinée de cyclophosphamide et de corticoïdes pourrait apporter un bénéfice hémodynamique et clinique chez les patients présentant une HTAP-LES ou une HTAP-CM (Jais et al., 2008), alors que leur utilisation n’apporte aucun bénéfice et n’est pas recommandée dans l’HTAP associée à la ScS (Humbert et al., 2022; Sanchez et al., 2006). Effectivement, l’utilisation en première ligne du cyclophosphamide pendant 6 à 12 mois, associée habituellement aux corticoïdes, avec ou sans traitement spécifique de l’HTAP, permettrait une stabilisation voir une amélioration des paramètres hémodynamiques chez un tiers à 50% des patients avec une HTAP associée au LES, à une CM ou au SS, définissant une population de patients potentiellement « répondeurs », alors que les patients atteints d’une HTAP-ScS traités par cyclophosphamide sont systématiquement des « non répondeurs » quel que soit les études (Ding et al., 2022). L’utilisation d’immunosuppresseurs dans ces populations de potentiels « répondeurs » est un facteur prédictif d’amélioration de la classe fonctionnelle. La

conception des études principalement rétrospectives avec de petits effectifs, leur hétérogénéité, des méthodes diagnostiques de l'HTAP variables (KTD versus échocardiographie) et le peu d'essai contrôlé randomisé ne permettent pas d'établir des conclusions avec un haut niveau de preuve. Leur utilisation repose donc sur une analyse personnalisée et individuelle. Ces études ont permis tout de même d'identifier une population de patients atteints d'HTAP potentiellement réversible correspondant aux HTAP associées aux connectivites type LES, CM et SS à l'exclusion de la ScS (Jais et al., 2008; Qian et al., 2019; Sanchez et al., 2006).

Les données concernant les autres immunosuppresseurs, conventionnels tel que le tacrolimus, le mycophénolate mofétil, les inhibiteurs de mTor, ou les thérapies ciblées tel que le rituximab ciblant le lymphocyte B ou le tocilizumab ciblant le récepteur de l'IL-6 sont très préliminaires et ne permettent pas d'établir de recommandations spécifiques à l'heure actuelle (Ding et al., 2022; Humbert et al., 2022). Ces données sont développées dans la revue générale sur l'HTAP associées aux connectivites (**Annexe 3**). Le rituximab, un anticorps monoclonal anti-CD20 déplétant les lymphocytes B a été testé au cours d'un essai contrôlé randomisé (1g J1 J15) versus placebo chez 57 patients avec une HTAP-ScS. L'amélioration de la distance de marche au cours du test de marche de 6 min à 6 mois semblait supérieure dans le bras rituximab comparée au bras placebo mais sans atteindre le seuil de significativité (23.6 ± 11.1 mètres vs 0.5 ± 9.7 mètres; $p = 0.12$) (Zamanian et al., 2021). Le tocilizumab (anticorps monoclonal anti-récepteur de l'IL-6) a été testé dans un essai de phase 2 en ouvert (8 mg/kg/4 semaines) chez 19 patients avec une HTAP idiopathique/héritable et 10 associées à une connectivite. Le critère de jugement était la sécurité (incidence d'événement indésirable) associée à l'évolution des résistances vasculaires pulmonaires à 6 mois. Quatre patients ont dû interrompre le tocilizumab pour un événement indésirable grave, et il n'a pas été démontré d'impact sur les

résistances vasculaires pulmonaires (Toshner et al., 2022). L'ensemble de ces résultats sont toutefois un argument plaidant pour l'implication du système immunitaire dans la pathogenèse de l'HTAP.

I.8 – Place des antirétroviraux dans le traitement de l'HTAP

Chez les patients atteints d'une HTAP-VIH, les recommandations de traitement sont superposables à celles de l'HTAPi (Galiè et al., 2016), et l'utilisation des traitements antirétroviraux est encouragée conformément aux recommandations relatives à la prise en charge de l'infection par le VIH. Dans plusieurs études, l'utilisation des antirétroviraux hautement actifs semble être associée à une amélioration du statut fonctionnel et de la survie (Barbaro et al., 2006; Degano et al., 2010; Zuber et al., 2004). Mais aucune étude avec un effectif conséquent et un design prospectif n'a étudié spécifiquement l'effet des antirétroviraux comme traitement de l'HTAP-VIH. La co-administration des antirétroviraux et des traitements spécifiques de l'HTAP a permis quant à elle d'améliorer significativement le pronostic de ces patients (Degano et al., 2010; Opravil & Sereni, 2008). Les recommandations actuelles sont en faveur de l'utilisation des traitements antirétroviraux quel que soit le taux de lymphocyte T CD4⁺ en combinaison des traitements spécifiques médicamenteux de l'HTAP (Humbert et al., 2022). Les patients avec une HTAP-VIH sont habituellement non répondeurs aux inhibiteurs calciques, qui ne sont donc pas recommandés (Montani et al., 2010). Le bosentan comme traitement spécifique de l'HTAP-VIH, évalué de manière prospective dans l'essai BREATHE-4 améliore la classe fonctionnelle, la qualité de vie et les paramètres hémodynamiques (Sitbon et al., 2004). Quelques séries de cas semblent montrer des effets bénéfiques du sildénafil (Carlsen et al., 2002; Schumacher et al., 2001). A noter que chez les patients HTAP-VIH, au vu de l'absence d'essai ayant testé de combinaison de traitements spécifiques de l'HTAP, le traitement initial spécifique de l'HTAP sera une monothérapie, suivi

par une combinaison séquentielle si nécessaire, afin de réduire les risques d'interaction médicamenteuse (Humbert et al., 2022). Effectivement de nombreuses interactions existent entre les ERA ou les PDE5i et les inhibiteurs de protéases, nécessitant un monitoring très étroit.

Partie II – Objectifs de la thèse et exposé des travaux

L'inflammation et le système immunitaire sont impliqués dans la physiopathologie de l'HTAP et des auto-anticorps ont été détectés chez les patients atteints d'HTAP idiopathique et associée à la ScS. Au cours de ces maladies, certains auto-anticorps ciblant les cellules de la paroi des artérioles pulmonaires peuvent avoir un effet pathogène, contribuant à la prolifération cellulaire anormale et aux altérations structurelles pariétales.

Cependant, les données relatives aux auto-anticorps dans les HTAP associées à des connectivites autres que la ScS (HTAP-LES, HTAP-CM et HTAP-SS) et à l'infection par le VIH (HTAP-VIH) sont extrêmement limitées, et aucun marqueur biologique ne permet actuellement de prédire le développement d'une HTAP associée à ces maladies. Dans ce contexte, des études supplémentaires sont nécessaires pour mieux comprendre le rôle des auto-anticorps dans les différentes formes d'HTAP, et pour développer des tests de dépistage qui pourraient être utilisables en pratique clinique, et dans ce contexte pourraient permettre d'envisager un traitement précoce voire un traitement préventif des patients à risque de développer une HTAP.

Dans le présent travail, nous nous sommes concentrés sur les formes d'HTAP potentiellement réversibles.

Nous avons formulé plusieurs hypothèses dans le cadre ce projet de recherche, que sont :

1. La détection d'auto-anticorps peut être variable en termes de proportion entre l'HTAP idiopathique, celle associée à la ScS, les HTAP associées aux autres connectivites hors ScS, et l'HTAP de l'infection par le VIH.

2. Les cibles antigéniques de ces auto-anticorps peuvent être distinctes entre l'HTAP idiopathique, l'HTAP-ScS, les HTAP associées aux autres connectivites hors ScS, et l'HTAP associée à l'infection par le VIH.
3. Ces auto-anticorps pourraient être utilisés comme des marqueurs prédictifs et/ou pronostiques pour évaluer le risque de survenue d'une HTAP.
4. Certains de ces auto-anticorps pourraient avoir un effet pathogène direct et contribuer aux mécanismes physiopathologiques de l'HTAP.

Les objectifs de ce projet étaient de :

- I. Détecter la présence d'auto-anticorps spécifiques de l'HTAP ciblant le fibroblaste pulmonaire et la cellule endothéliale microvasculaire pulmonaire dans les HTAP associées aux connectivites hors ScS et l'HTAP associée à l'infection par le VIH.
- II. Identifier les cibles antigéniques de ces auto-anticorps.
- III. Développer un test diagnostique permettant un dépistage sérique.
- IV. Explorer l'association entre la détection des auto-anticorps et les caractéristiques cliniques des patients atteints d'HTAP.
- V. Étudier le rôle pathogénique des auto-anticorps identifiés.

Une première étude a identifié des auto-anticorps anti-fibroblastes et anti-cellules endothéliales spécifiques de la présence d'une HTAP dans les sérums de patients atteints d'une connectivite autre que la ScS (**Article original 1**). Une partie supplémentaire et complémentaire a étudié l'impact clinique des réactivités des auto-anticorps identifiés, et l'exploration du rôle pathogénique des auto-anticorps identifiés.

Une deuxième étude a appliqué la même stratégie expérimentale sur les sérums de patients avec une HTAP associée à l'infection par le VIH comparés aux sérums de patients infectés par le VIH sans HTAP et aux sérums de témoins sains (**Article original 2**).

Une revue générale des données sur les HTAP associées aux connectivites a été réalisée et colligées en **Annexe 3**.

II.1 – Auto-anticorps dans l'HTAP associée au lupus érythémateux systémique, à la connectivite mixte, et au syndrome de Sjögren primaire

II.1.1 – Introduction

L'HTAP est une maladie rare, pouvant être idiopathique ou associée à diverses pathologies comme les connectivites. Les connectivites constituent un groupe hétérogène de maladies auto-immunes systémiques caractérisées par des dérégulations du système immunitaire, pouvant partager de multiples caractéristiques cliniques. Les mécanismes physiopathologiques de l'HTAP sont multiples et complexes, au sein desquels l'inflammation et des dysrégulations du système immunitaire semblent jouer un rôle central. Des auto-anticorps ciblant les composants de la paroi des vaisseaux, notamment les fibroblastes (AFA) et les cellules endothéliales vasculaires pulmonaires (AECA) ont été identifiés dans l'HTAPi et l'HTAP-ScS. Dans les HTAP associées aux autres connectivites comme le lupus érythémateux systémique (LES), la connectivite mixte (CM) ou le syndrome de Sjögren primaire (SS), les données sur la détection d'AFA, des AECA et l'identification de leurs cibles antigéniques sont beaucoup moins étayées.

Nous avons donc supposé que des certains AFA et AECA dirigés contre les composants de la paroi vasculaire pulmonaire pourraient également être identifiés dans les HTAP associée aux

connectivites en dehors de la ScS et pourraient être un facteur prédictif spécifique du développement d'une HTAP associée à une connectivite.

Les objectifs de cette étude étaient donc d'identifier des AFA et AECA spécifiques associés à l'HTAP associée au LES, aux CM et au SS, de caractériser leurs antigènes cibles, de tester la spécificité de ses auto-anticorps pour l'HTAP et en fonction de la cible antigénique tester un potentiel rôle pathogène des immunoglobines G purifiées contenant l'auto-anticorps identifié.

Cette étude a démontré que les IgG sériques des patients avec HTAP associée à une connectivite réagissaient contre des protéines de fibroblastes et de cellules endothéliales vasculaires pulmonaires humaines et que certaines de ces réactivités sont spécifiques de la présence d'une HTAP, en comparaison aux sérums de patients atteints de connectivites sans HTAP ou de sujets sains.

II.1.2 – Résumé

Objectifs

L'HTAP est une maladie rare qui peut être associée à des connectivites. Des mécanismes immunitaires participent à sa physiopathologie parmi lesquels l'identification d'auto-anticorps. Cela a été bien étudié dans l'HTAPi ou l'HTAP associée à la ScS mais peu dans les autres connectivites comme le LES, les CM et le SS. Cette étude a pour objectifs d'identifier des auto-anticorps anti-fibroblaste (AFA) et anti-cellule endothéliale vasculaire pulmonaire (AECA) spécifiques de la présence d'une HTAP associée à une connectivite, d'identifier leurs cibles antigéniques, et de confirmer la réactivité et la spécificité de ces auto-anticorps envers la présence d'une HTAP.

Méthodes

Pour répondre à ces objectifs, nous avons utilisé plusieurs approches expérimentales séquentielles (**Figure 5**).

Les sérums de 86 patients atteints d'une connectivité autre que la ScS (35 LES, 26 CM et 25 SS) et inclus dans le Protocole Hospitalier de Recherche Clinique Auto-HTAP (ClinicalTrials *NCT01208792*) dont 32 associés à une HTAP ont été comparés aux 54 sérums de patients atteints d'une connectivité sans HTAP et à des témoins sains en immunoblot 1 dimension (sérums individuels) et en immunoblot 2 dimensions (sous forme de pool de sérums) afin d'identifier leurs réactivités antigéniques contre des protéines issues d'extraits provenant de cultures cellulaires de fibroblastes pulmonaires humains immortalisés (hTERT) et de cellules endothéliales microvasculaires pulmonaires immortalisées par Sv40 sous forme de spots de réactivités spécifiques de l'HTAP. Des études protéomiques à l'aide d'un système de chromatographie liquide à séparation rapide associée à un spectromètre de masse ont permis d'identifier les cibles antigéniques des auto-anticorps identifiés et reconnues par au moins 2 pools de sérums et partagées entre les connectivités étudiées par la consultation de la base de données SwissProt et la génération de réseau protéique à l'aide du logiciel Ingenuity Pathway Analysis. L'association spécifique des auto-anticorps et de la présence d'une HTAP associée à une connectivité a été ensuite testée en technique ELISA sur des protéines recombinantes humaines et comparée à 170 sérums de patients atteints de ScS (n=46), d'HTAP idiopathique (n=34), 17 patients avec HTAP de forme héritable, atteints de cardiopathie congénitale (n=37), avec hypertension porto-pulmonaire (n=36) et à 150 sérums de contrôles sains. La recherche d'association entre la présence des auto-anticorps et des caractéristiques cliniques des patients avec HTAP a été effectuée par statistiques exploratoires. Des tests fonctionnels ont été réalisés pour explorer le rôle pathogène des auto-anticorps identifiés, en

particulier le rôle pathogène des auto-anticorps anti-annexine A5 par mesure du temps de fibrinof ormation.

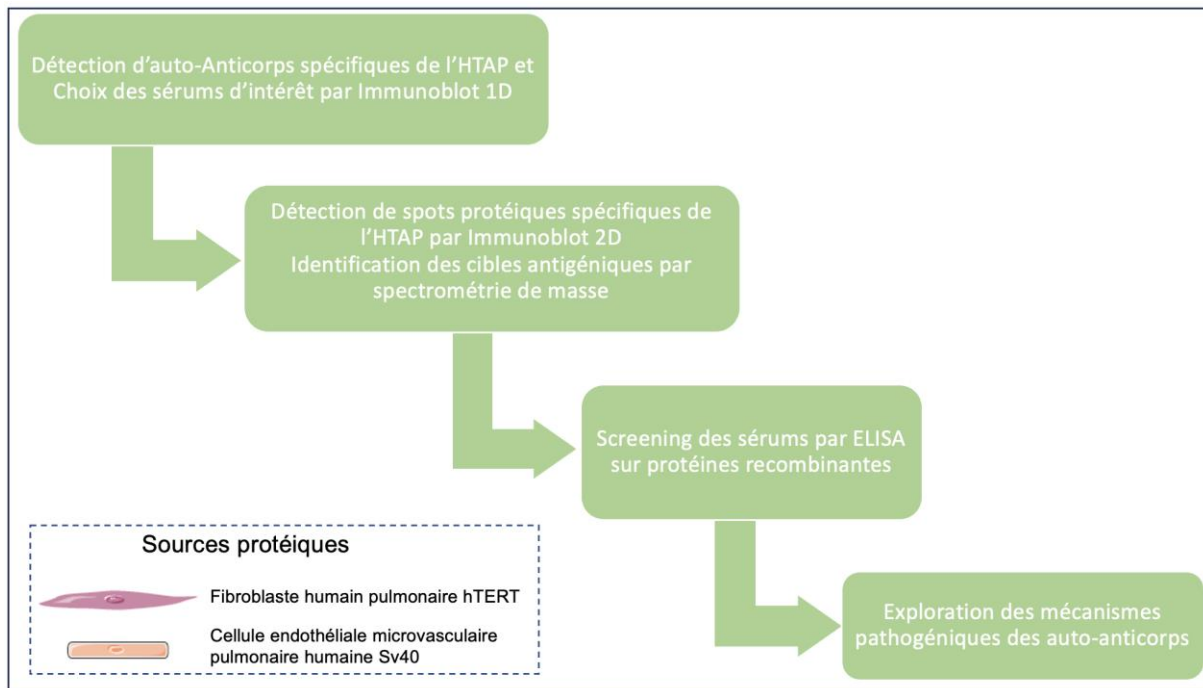


Figure 5 : Approche expérimentale séquentielle du projet de recherche

Résultats

En immunoblot-1D, cinquante-six pourcents (18/32) des sérums de patients avec HTAP associée à une connectivite avaient des bandes de réactivités anti-fibroblaste et 41% (13/32) des bandes de réactivités anti-cellule endothéliale. Trente-neuf protéines cibles issues de l'extrait protéique de fibroblaste et 34 protéines issues de l'extrait de cellules endothéliales étaient spécifiquement reconnues par les pools d'IgG sériques de patients avec une HTAP associée à une connectivite en immunoblot-2D en comparaison des pools d'IgG sériques des patients avec une connectivite sans HTAP associée, et aux contrôles sains. Les protéines identifiées par au moins 2 pools sériques avec HTAP et partagées par les trois différentes connectivites étaient l'Annexine A5 (ANXA5), la protéine 14-3-3 ϵ , la vimentine, la

triosephosphate isomérase, la galectine 1 et l'elongation facteur 2. En ELISA, la réactivité des IgG anti-ANXA5 dans le sérum des 86 patients atteints de connectivite était significativement plus importante chez les patients avec HTAP (44% versus 0%, $p < 0.001$), et particulièrement chez les patients avec une HTAP-LES (33%, $p = 0.009$) et une HTAP-CM (73%, $p < 0.001$) par rapport aux patients sans HTAP. La réactivité des auto-anticorps IgG anti-14-3-3 ϵ étaient significativement plus élevée uniquement chez les patients avec HTAP-LES comparée aux patients LES sans HTAP associée (42% versus 10%, $p = 0.02$). En comparaison avec les 170 autres sérums de patients et des 150 sérums de contrôles sains, la réactivité IgG anti-ANXA5 était spécifiquement associée à l'HTAP secondaire aux connectivites hors ScS. La réactivité des anticorps anti-ANXA5 était associée au sexe masculin ($p = 0.035$), à une classe élevée d'évaluation de la dyspnée ($p = 0.001$) et à la réactivité des IgG anti-vimentine ($p = 0.040$). Nous n'avons pas pu identifier de rôle pathogène des IgG anti-annexine A5 sur le temps de fibrinoformation.

Conclusion

La réactivité des auto-anticorps IgG anti-Annexine A5 pourrait représenter un biomarqueur prédictif de la survenue d'une HTAP associée au LES ou à la CM. Des études prospectives sont nécessaires pour confirmer cette hypothèse.

II.1.3 – Article original 1

Anti-fibroblast and anti-endothelial cell autoantibodies are associated with pulmonary arterial hypertension (PAH) in patients with connective tissue diseases (CTD)

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Abstract

Objective: Pulmonary arterial hypertension (PAH) is a rare disease that may be associated with connective tissue diseases (CTD). Anti-fibroblast (AFA) and anti-endothelial cell autoantibodies (AECA) have been identified in idiopathic and systemic sclerosis (SSc)-associated PAH. The aim of study was to identify autoantibodies specific to PAH associated with systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD) and primary Sjögren's syndrome (SS) and identify their target antigens.

Methods: Sera were collected from 86 patients with CTD excluding SSc, including 32 with PAH (PAH+) and 54 without PAH (PAH-). AFA and AECA were identified using one (1D) and two dimensions (2D) immunoblots and proteomics. ELISA tests using human recombinant proteins were used to confirm PAH-associated IgG reactivities.

Results: PAH+ patients had similar IgG AFA and AECA reactivities in 56.2% and 40.6% of the cases in 1D immunoblots, respectively. In 2D immunoblots, serum IgG pools of patients with SLE (n=14), MCTD (n=10), SS (n=9) and 14 HCs (n=1) recognized respectively 273±79, 205±77, 109±11 and 109 protein spots in fibroblasts and 189±48, 146±30, 88±33 and 190 protein spots in endothelial cell extracts, respectively. Serum IgG from PAH+ patients recognized 39 fibroblast and 34 endothelial cell protein spots that were not recognized by IgG from PAH- patients, including Annexin A5 (ANXA5). Anti-ANXA5 IgG reactivity was significantly higher in PAH+ compared to PAH- patients with SLE (33% vs 0%, p=0.009) and MCTD (73% vs 0%, p<0.001).

Conclusion: Anti-ANXA5 IgG autoantibody reactivity might serve as a predictive biomarker for PAH associated with SLE or MCTD.

Keywords: pulmonary arterial hypertension, connective tissue disease, fibroblast, endothelial cell, autoantibody, annexin A5, 14-3-3 ε.

Introduction

Pulmonary arterial hypertension (PAH) is a rare disorder, corresponding to group 1 pulmonary hypertension (PH), characterized by progressive increase of pulmonary artery pressure and pulmonary vascular resistance, leading to right-heart failure and increased morbimortality. PAH comprises idiopathic PAH (IPAH), heritable PAH (HPAH) due to identified or unidentified genetic mutations, PAH associated with toxins or drug exposure and various conditions including human immunodeficiency virus (HIV) infection, portal hypertension and connective tissue diseases (CTD), which contribute to 15% of group 1 PH in France, representing the second most frequent cause of PAH (Humbert et al., 2022). In Europe and North America, systemic sclerosis (SSc) represents the first cause of CTD-associated PAH (CTD-PAH) (almost 75%), followed by systemic lupus erythematosus (SLE) (9-19%), mixed connective tissue disease (MCTD) (8-9%) and other CTD including Sjögren's syndrome (SS) (1%) (Chung et al., 2010; Escribano-Subias et al., 2012; Humbert et al., 2006; McGoon et al., 2013). In contrast, in Asia, SLE is the leading cause of CTD-PAH, representing approximately 57% in Taiwan (Lin et al., 2020), 38 to 49% in China (Hao et al., 2014; Zhang et al., 2011), and 29% in Japan (Shirai et al., 2012), whereas SSc contribute to only 6% of CTD-PAH (Hao et al., 2014).

CTD is a heterogeneous group of systemic autoimmune diseases characterized by deregulations of innate and adaptive immune systems, which may share multiple clinical features. Pathophysiology of PAH is complex and multifactorial, involving endothelial cell dysfunction, accumulation of fibroblasts and vascular smooth muscle cells (VSMC) in media and adventitia, along with vasoconstriction, cell proliferation, thrombosis and apoptosis, all contributing to arteriolar vascular remodeling (Humbert et al., 2019).

Inflammatory and immune mechanisms seem to play a central role in PAH pathophysiology (Huertas et al., 2020). Indeed, macrophages, dendritic cells, lymphocytes and plasma cells are present in the adventitia of pulmonary arterioles, with expression of many proinflammatory cytokines (Humbert et al., 1995; Savai et al., 2012). In addition, autoantibodies targeting vessel wall components including anti-fibroblast antibodies (AFA), anti-endothelial cell antibodies (AECA) and anti-vascular smooth muscle cell (VSMC) antibodies have been identified in patients with IPAH and SSc-PAH with a potential pathogenic role for some of them (Bussone et al., 2012; Dib et al., 2012; Tamby et al., 2006; Terrier et al., 2008).

Aside from pathophysiological issues, it is now well documented that SLE-PAH and MCTD-PAH may benefit from immunosuppressants, defining a group of patients potentially "responsive" to immunosuppressants, unlike "non-responsive" SSc-PAH patients (Humbert et al., 2022; Jais et al., 2008; Sanchez et al., 2006). In this setting, although it has not yet been studied to our knowledge, we speculate that autoantibodies directed at vessel wall components could also be identified in patients with these CTD-PAH and help to define a subgroup of patients that could get benefit from early immunosuppression.

Thus, the aim of the study was to identify specific PAH-related AFA and AECA associated with SLE, MCTD and SS and characterize their target antigens.

Patients and methods

Patients and IgG sources

Patients and healthy controls (HCs) were included in the French multicenter Auto-HTAP study (NTC01208792) between June 2010 and July 2014. Serum samples from 256 patients were

collected, aliquoted and stored at -80°C until use. Among them, 86 had a CTD excluding SSc (35 had SLE, 26 had MCTD, and 25 had SS, among them 32/86 with PAH [PAH+] and 54/86 disease-matched controls without PAH [PAH-]), 46 had SSc (23 PAH+ and 23 PAH-), 34 had IPAH, 17 had HPAH (mutations in bone morphogenetic protein receptor type 2 (BMP2) n=15), in filamin A (n=1), and potassium channel subfamily K member 3 (KCNK3) (n=1), 36 had portal hypertension (Hervé et al., 1998), and 37 had congenital heart disease (CHD) (Simonneau et al., 2004) (**Supplemental Figure 1 & Supplemental Table 1**). Serum samples from 150 healthy blood donors used as healthy controls (HCs) were also collected.

PAH was defined on right-heart catheterization by increased mean pulmonary artery pressure (mPAP) above 25 mmHg, a pulmonary artery wedge pressure \leq 15 mmHg and pulmonary vascular resistance (PVR) above 3 Wood units (Galiè et al., 2016). Patients with CTD fulfilled the validated classification criteria for SSc (Masi et al., 1980), SLE (Hochberg, 1997), MCTD (Kasukawa, 1999; Sharp et al., 1972, p. 1972) and SS (Vitali et al., 2002).

All 86 serums from SLE, MCTD and SS patients were tested using 1D-, 2D-immunoblots and ELISA. Other patients (H/IPAH, SSc, and others diseases associated with PAH) were analyzed using ELISA in order to study autoantibody reactivity against the main target antigen identified in 2D-immunoblot and shared between SLE-PAH, MCTD-PAH and SS-PAH. Serum samples were used individually for 1-D immunoblotting and ELISA, and in pools of three (or 2 if the number of patients was too low to make a pool of three) into the same disease subset and according to sample count, sex and based on 1D-immunoblot results for the 2-D immunoblotting. Finally, in 2D-immunoblots, serum pools were tested as follows: 14 for SLE patients (6 PAH+ and 8 PAH-), 10 for MCTD patients (5 PAH+ and 5 PAH-) and 9 for SS patients (2 PAH+ and 7 PAH-). Pools of

sera from patients with or without PAH were compared within the same disease, and with a pool of sera from 14 healthy controls as validated in previous studies (Terrier et al., 2008).

Cells culture and protein extracts

Sv40-immortalized human pulmonary microvascular endothelial cells (HPMEC) and hTERT-immortalized human lung fibroblasts (both from ATCC Manassas, Virginia, USA) were cultured in 175-cm² flask with Endothelial Cell Growth Medium MV kit (PromoCell, Heidelberg, Germany) and Dulbecco's modified Eagle's medium (Gibco BRL Invitrogen, Paisley, UK) respectively, supplemented with 10% heat-inactivated fetal calf serum for fibroblast, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO₂.

1-dimensional immunoblot (1-D Immunoblot)

Protein extracts were prepared in 125 mM Tris/HCl pH 6.8 containing 4% SDS, 1.45 M 2-mercaptoethanol, 1 tablet of protease inhibitor cocktail mini-EDTA free per 10 ml extraction solution (Roche, Basel, Swiss) on ice and sonicated 4 x 30 secs, as detailed in previous work (Dib et al., 2012).

Protein quantification involved the Bradford method. Equal amounts of loading buffer with solubilized proteins (145µl per gel corresponding to 70µg of protein extract per gel) were subjected to 10% polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad Laboratories, Hercules, CA, USA), transferred onto 0.45 µm nitrocellulose membranes by using a semidry electroblotter (model A; Ancos, Hojby, Denmark), blocked with phosphate buffer saline (PBS) Tween 20 polysorbate 0.2% (VWR international SAS, France) at +4°C overnight, incubated for 4 h using a Cassette Miniblot System (Immunelect Inc., Cambridge, MA, USA) at room temperature, with the

sera from patients or HCs at a 1:100 dilution for reactivity with fibroblast and HPMEC proteins extracts. The membranes were then extensively washed with PBS Tween 0.2% buffer and incubated for 1 h with an anti-Fc γ chain-specific secondary goat anti-human IgG antibodies coupled to alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) at a 1:60,000 dilution. Immunoreactivities were revealed with NBT/BCIP (Sigma-Aldrich). A high-resolution acquisition of a densitometric image of the immunoreactivity profiles was performed with the Fusion Fx image acquisition device (Vilber, Collégien, France).

2-dimensional Electrophoresis, Electro-transfer and 2-D Immunoblotting

All instrumentation and related reagents were from Bio-Rad Laboratories, Hercules, CA, USA except when specified. Adapted and updated from previous protocol using large 2-D gels, all experiments were performed using small 2D gels (Guilpain et al., 2007; Servettaz et al., 2008). 1D-electrophoresis corresponded to the isoelectrofocalization on IPGphor3 IEF system (Cytivia, Marlborough, Massachusetts, USA) of 7 cm immobilized pH gradient IPG strip with 3-11 range isoelectric point pI (VWR), previously rehydrated overnight with 125 μ L of HPMEC and fibroblast protein extracts mixed in rehydration buffer (8M Urea, 2M Thiourea, 4% CHAPS, DeStreak Reagent 0.5%) at a final concentration of 70 μ g protein extract per strip (Görg et al., 2000), followed by the second dimension-electrophoresis on 7 x 9 cm mini-protean TGX stain free gels with acrylamide gradient of 7–18% allowed for studying a wide range of antigens of 10–260 kDa, in a migration buffer (25 mM Tris, 192 mM, Glycine 0.1% (w/v) SDS pH 8.3), transferred on 45 μ m nitrocellulose membrane in +4°C transfer buffer (3% Tris, 14.4% Glycine, 0.05% SDS) with a constant electric current of 30 mA per membrane. The quality of gel electrophoresis, and electro-transfer on nitrocellulose membrane was verified with the densitometer GS-800. Membranes

were blocked with phosphate buffer saline (PBS) Tween 20 polysorbate 0.2% (VWR) at +4°C overnight, thereafter incubated at room temperature on a rocking chair for 4 h with the pooled sera from patients or HCs at a 1:100 dilution, washed with PBS Tween 0.2% buffer, and incubated for 1 h with an anti-Fc γ chain-specific secondary goat anti-human IgG antibodies coupled to alkaline phosphatase (Sigma-Aldrich) at a 1:60,000 dilution. Immunoreactivities were revealed in the same previous manner with NBT/BCIP and high-resolution acquisition with the Fusion Fx image acquisition device (Vilber).

Image analysis of 2D-immunoblotting

Images of gels and membranes were first standardized in terms of angulation and cropping using *Image Lab* software (Bio-Rad). These were then analyzed using an algorithm in Python coding, based on the Laplacian of Gaussian method, in order to quantify the protein spots. The detection of a spot was done according to an intensity threshold defined at 0.025 and a radius between 2 and 4 pixels. These thresholds were determined in comparison with the identification performance of a protein spot with the naked eye by two experienced scientists to avoid false positives. The comparison between the different subgroups was carried out optically. Immunoblots performed with sera from patients with PAH (PAH+) were compared to immunoblots with sera from patients with the same underlying disease without PAH (PAH-), and those from HCs, on *Image J* software, in order to identify the immunoreactivity spots specifically associated with PAH.

Antigen identification by Mass Spectrometry

A pitting gel was made in the same manner as described previously, with 70 µg per gel of fibroblast or HPMEC protein extract, washed and then incubated with a solution of Coomassie blue for 48 h at +4°C with gentle shaking, then scanned on the densitometer GS-800. After labeling of PAH-specific immunoreactivity spots present for at least 2 different pools of serum (PAH+), these were picked manually, identified, washed, dehydrated, incubated with modified trypsin (Promega, USA; 12.5 ng/µl in 40 mM of ammonium bicarbonate ABC and 10% of acetonitrile ACN, pH 8.0), then processed by mass spectrometry. Tandem mass spectrometry analyzes were performed using an Ultimate 3000 rapid separation liquid chromatography (RSLC) system connected to a mass spectrometer (Q Exactive Orbitrap liquid chromatography-high resolution mass spectrometry LC-HRMS, Thermo Fisher, Waltham, Massachusetts, USA) with ionization of Electrospray type.

The spectral processing was carried out using version 2.5 of the *Mascot* software (Matrix science, London, United Kingdom) by querying the SwissProt database in humans (February 2021; 564,638 sequences; 203,519,613 residues), by allowing 1 cut site missed by trypsin, as well as indicating the carbamidomethylation of cysteines (following the addition of iodoacetamide), and the oxidation of methionines in variable modifications on the sought peptides. Among the positive identifications based on a Mascot score above the significance threshold $p < 5\%$, we selected proteins identified with at least 2 peptides with an ionic score > 25 for each of them, and an emPAI > 1 . The emPAI is a score that provides an indicative value of the comparative abundance of the identified proteins (Ishihama et al., 2005).

Protein network

To generate and to gain insight into the biological pathways and networks that are involved in our datasets, the lists of target proteins were loaded into the *Ingenuity Pathway Analysis* software (Qiagen Redwood City, California, USA). Target proteins were proteins recognized on 2D-immunoblot from fibroblast and HPMEC extracts, and mapped to corresponding genes in the Ingenuity Pathway Knowledge database. The interactions between proteins were built illustrating direct or indirect chemical interactions, activations or inhibitions between them.

Enzyme linked immunosorbent assay (ELISA)

Target antigens that were recognized by more than 2 serum pools in PAH+ patients in 2D-immunoblotting results, were tested in ELISA for the identification of autoantibodies associated with CTD excluding SSc and specific to the PAH (CTD-PAH). Human recombinant proteins included annexin A5 (ANXA5, Tebu-bio SAS, Le Perray en Yvelines, France), human 14-3-3 ϵ (14-3-3 ϵ , Tebu-bio), human vimentin (Preprotech, Cranbury, New Jersey, USA), human triosephosphate isomerase (TPI, Tebu-bio), human galectin-1 (Gal-1, Preprotech), human elongation factor 2 (EF2, Cloud clone, Katy, Texas, USA). Recombinant proteins were coated overnight at +4°C on 96-wells Polysorb Nunc plates (Thermo Fisher) at the concentration of 1 $\mu\text{g}/\text{mL}$, saturated with 200 μl per well of PBS – bovine serum albumin BSA 1% buffer for 90 min at room temperature, and after washed, incubated with 100 μL of individual patient serum at a 1:100 dilution in PBS BSA 1% Tween 0.05% buffer for 1h at room temperature and specific primary antibody in 2 wells per plates as positive control and for equilibration between plates (rabbit anti-human ANXA5 IgG Ab at a 1:5,000 dilution [Elabscience Biotechnology Inc, USA], rabbit anti-14-3-3 ϵ polyclonal Ab at a

1:5,000 dilution [Elabscience], rabbit anti-vimentin polyclonal Ab at a 1:20,000 dilution, mouse anti-human TPI Ab at a 1:2,000 dilution [Abnova, Taipei City, Neihu district, Taiwan], rabbit anti-human galectin-1 Ab at a 1:2,000 dilution [Preprotech], and rabbit anti-human EF2 Ab at a 1:5,000 dilution [Abnova]). The plates were then extensively washed with PBS Tween 0.2% buffer and incubated for 1 h with a secondary antibody coupled with HRP (Secondary goat anti-rabbit Ab at a 1:2,000 dilution, goat anti-mouse Ab at a 1:6,000 dilution, and rabbit anti-human IgG Ab at a 1:6,000 dilution (Agilent technologies, Santa Clara, CA, USA, for all secondary Ab).

Immunoreactivity was finally revealed with 100 μ L TMB ('3,3',5,5' tétraméthylbenzidine) deposited for 8 minutes at room temperature and away from light. The reaction was stopped with 50 μ L of 2N H₂SO₄ in each well.

The absorbance was measured at 450 nm wavelength with the Clariostar device (BMG Labtech, Ortenberg, Germany) making it possible to obtain the optical density (OD) of each well. A serum was considered positive for the presence of antibodies when the mean OD of the duplicate was greater than the mean + 2 standard derivation of the OD of all the HCs.

Statistical analysis

Continuous variables were expressed as median (interquartile range) or mean \pm standard derivation and were compared with the Mann-Whitney U (Wilcoxon) test, according to the PAH status. Categorical variables, expressed as total and proportion, were compared by the Fisher exact test. Analyses were performed using *R* software, version 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria), and *Graph-Pad Prism 8* software (La Jolla, CA, USA). All statistical tests were performed at the significance threshold of 5%.

Results

Baseline characteristics

The clinical and hemodynamic characteristics of the 86 patients with SLE, MCTD and SS (**Figure 1**) including 32 PAH+ and 54 PAH- (35 SLE, 26 MCTD and 25 SS) patients were compared in **Table 1**. There was no difference between the two groups, except for oral anticoagulants (50% of PAH+ vs none of PAH- patients), endothelin receptor antagonists (65.6% of PAH+ vs none of PAH- patients) and phosphodiesterase-5 inhibitor (62.5% vs none of PAH- patients) ($p < 0.001$ for all).

Immunoblotting of serum IgG reactivities

Based on 1D-immunoblot experiments performed with protein extracts of fibroblasts and endothelial cells, we identified serum IgG reactivity patterns associated with PAH+ and not present in patients without PAH- and HCs. Thus, 18/32 patients (56.2%) had similar AFA reactivities and 13/32 (40.6%) had similar AECA reactivities, allowing to pool serums to perform the 2D-immunoblot experiments (**Supplemental Figure 2**).

In 2D-immunoblot, taken together, serum IgG reactivities from pools of sera from patients and healthy controls were directed at 196 ± 90 protein spots on fibroblast extracts, and 141 ± 55 protein spots on endothelial cell extract, most of them at an isoelectric point (pI) ranging from 4 to 9 and a molecular weight ranging from 25 to 140 kDa. On fibroblast protein extract, serum IgG from pools of sera recognized 273 ± 79 protein spots for SLE, 205 ± 77 for MCTD and 109 ± 11 for SS patients, and 109 protein spots for the pool of 14 HCs (**Supplemental Table 2**).

Thirty-nine protein spots specifically recognized by serum IgG from the PAH+ patients were identified in the fibroblast protein extract, and 34 in the endothelial cell protein extract. The target antigens identified by mass spectrometry are listed in **Tables 2 and 3**. In the fibroblast extract, the identified target antigens recognized by more than 2 PAH+ serum pools and shared between SLE, MCTD and SS with PAH were Annexin A5 (ANXA5) (38.5% of pools), 14-3-3 ϵ (30.8%), Vimentin (23.1%), Triosephosphate isomerase protein (TPI) (46.1%), Galectin-1 (Gal-1) (15.4%) and Elongation factor 2 (EF2) (53.8%, **Table 4**). Annexin A5 protein was recognized as a target antigen by 4/5 serum IgG pools of MCTD-PAH (80%), 1/6 of SLE-PAH serum IgG pools (16.7%) and none of the SS-PAH pools. 14-3-3 ϵ protein was identified as target antigen by 50% of SLE-PAH serum IgG pools, 20% of MCTD-PAH and none in SS serum IgG pools. Annexin A5, TPI and EF2 proteins were identified as three target antigens in both fibroblast and endothelial cell protein extracts (**Tables 2 & 3**).

Using *Ingenuity*, target antigen networks from fibroblast and HPMEC extracts were constructed to evaluate the interaction of these antigens, and their implication in shared cellular processes. Many proteins of the networks are implicated in the cellular processes of inflammation, apoptosis, cell death, or autoimmunity (**Supplemental Figure 3**).

Confirmation by ELISA of serum IgG reactivities

We used selected human recombinant proteins identified by mass spectrometry and suspected to be shared between SLE, MCTD and SS and not recognized by serum IgG pools of PAH- patients and HCs; serum IgG were tested in ELISA for autoantibody reactivities., IgG autoantibody reactivities directed against ANXA5, 14-3-3 ϵ , TPI, Vimentin, and EF2 were more

frequent in patients with SLE, MCTD and SS with or without associated PAH compared to HCs (**Supplemental Table 3 and Supplemental Figure 4**).

In the 86 SLE, MCTD and SS patients, IgG autoantibody reactivities directed against ANXA5 were significantly greater in PAH+ patients (14/32, 44%) compared to 0/54 in PAH- patients ($p<0.001$), particularly in SLE patients (33% in PAH+ versus 0% in PAH-, $p=0.009$), and in MCTD patients (PAH+ 73% versus 0% of PAH-, $p<0.001$). Regarding the 14-3-3 ϵ protein, seven patients with PAH+ associated with SLE had a higher IgG autoantibody reactivity than SLE patients without PAH (46.7% in PAH+ versus 10% in PAH-, $p=0.02$) (**Table 5 and Figure 2**). Concerning the other 4 proteins (TPI, Vimentin, Gal-1, and EF2), IgG reactivities did not differ significantly between PAH+ and PAH- patients (**Table 5**).

We then tested IgG autoantibody reactivity against ANXA5 in the 256 patients. IgG autoantibody reactivities, the most represented reactivities in SLE, MCTD and SS patients with PAH, were detected in 57/141 (40.4%) of PAH+ patients compared to 18/115 (15.7%) in PAH- patients ($p<0.001$, **Figure 3**). In contrast to SLE, MCTD and SS patients, IgG autoantibody reactivity against ANXA5 from 170 other control patients (other patients with or without PAH) did not differ significantly whatever the PAH status. No difference was found between IPAH and HPAH (positive anti-ANXA5 antibodies in 35% each) (**Figure 3 and Supplemental table 4**).

Discussion

In this study, we identified that serum IgG from patients with SLE, MCTD and SS reacted against proteins from human pulmonary vessel fibroblast and endothelial cell that were specific to PAH compared to healthy controls and patients without PAH. We identified by 2D-immunoblot and mass spectrometry target antigens participating in endothelial cells and fibroblasts functions, including cellular proliferation, apoptosis, inflammation or vascular remodeling. Finally, we confirmed the binding of serum IgG from patients with PAH to AFA and ACEA to ANXA5 and 14-3-3 ϵ proteins, which to our knowledge was not previously described in patients with PAH.

It is now well accepted that inflammation plays an important role in the pathogenesis of idiopathic PAH, illustrating by an increase of pro-inflammatory cytokines and chemokines synthesis from endothelial cells, fibroblasts and vascular smooth muscle cells (Cracowski et al., 2014; Humbert et al., 1995). Alongside remodeling to the intima and the media, the adventitia plays a major role in which significant remodeling involving many immune cells has been demonstrated. In vascular lesions, dendritic cells (DCs) and CD8⁺ cytotoxic and CD4⁺ helper T-cells and few B-cells are present and participate to the vessel wall remodeling (Savai et al., 2012). Most importantly in the hypothesis of an antigen-mediated immune response is the demonstration of architectural organization into ectopic tertiary lymphoid follicles in the pulmonary vasculature during IPAH (Perros et al., 2012). Structures resembling lymphoid follicles were identified in SSc-PAH (Dumoitier et al., 2017), in which B-cells would be activated by DCs and T-cells, suggesting a local autoimmune response.

In our previous works, AFA were identified in 40% of sera from patients with IPAH and 30% of sera from SSc-PAH patients (Tamby et al., 2006). AECA were also identified in the serum of

patients with SSc-PAH, SSc without PAH and IPAH (Dib et al., 2012). Anti-VSMC antibodies were detected in 25% of IPAH patients and 75% of SSc-PAH patients (Bussone et al., 2012). Their target antigens were implicated in cellular processes, such as the regulation of cytoskeletal function, oxidative stress and cell energy metabolism (Bussone et al., 2012; Terrier et al., 2008). Several antigens identified in the present work had already been identified in previous studies that we conducted in SSc-PAH or IPAH, such as calmodulin-1, far upstream element-binding protein 2, alpha-enolase, or stress-induced-phosphoprotein 1 (Bussone et al., 2012; Terrier et al., 2008). We sought to test new antigens, shared by various CTD-PAH excluding SSc, which could correspond to future biomarkers.

We identified anti-ANXA5 IgG autoantibodies in one third of SLE-PAH patients and more than two-thirds of MCTD-PAH patients, and anti-14-3-3 ϵ autoantibodies in nearly half of patients with SLE-PAH. Our results highlight IgG autoantibody reactivity against ANXA5 in patients with CTD-PAH allowing to distinguish them from patients without PAH. Screening for the anti-ANXA5 IgG antibody reactivity in the 170 other patients of the Auto-HTAP population did not reveal a similar discrimination. Anti-ANXA5 IgG antibody reactivity in patients with SLE, MCTD and SS might help to predict the occurrence of PAH during follow-up.

The ANXA5 protein constitutes, with others annexins, a pool of ubiquitous cytoplasmic proteins implicated in signal transduction. ANXA5 inhibits prothrombin activation and participates in the prevention of thrombus formation. ANXA5 may also have an anti-apoptotic and anti-inflammatory effect. Moreover, it could contribute to reduce vascular remodeling and endothelial cells dysfunction (Ewing et al., 2011). In the monocrotaline rat model of PAH, a beneficial effect of the injection of mesenchymal stem cell-derived microvesicles loaded with

ANXA5 was observed on mPAP (Chen et al., 2014). ANXA5 protein might play an important role in vascular remodeling during PAH. Although their exact pathogenic effect has not been clearly identified, these autoantibodies could block the fibrinof ormation-inhibiting action of annexin A5. Anti-ANXA5 antibodies have been detected in sera from patients with SLE, SSc, antiphospholipid syndrome (APS), and recurrent spontaneous abortions (Esposito et al., 2005; Rand et al., 2004). Antiphospholipid antibodies interfere with the binding of ANXA5 to anionic phospholipid in endothelial cell membrane, activate partial thromboplastin time reagent and prothrombin time reagent and therefore reduces the anticoagulant properties of the protein (Rand et al., 1998). In SSc patients, anti-ANXA5 antibodies were identified in 15% of the patients, and associated with increased frequency of digital necrosis and PAH (Horimoto et al., 2020). In the present study, we did not see any significant difference between IgG antibody reactivities of SSc-PAH patients and SSc patients without PAH.

The 14-3-3 proteins correspond to a group of proteins that are implicated in the regulation of inflammation, such as IKK/NF κ B or JAK/STAT pathways (Munier et al., 2021, pp. 14-3-3). These proteins are also found in the extracellular space (Kaplan et al., 2017). During apoptosis, cytosolic 14-3-3 proteins are released into the extracellular environment including plasma, which may contribute to explain its immunogenicity (Kaplan et al., 2017, p. 14). Another hypothesis is that a number of cytoplasmic proteins could be expressed at the cell surface, in favor of cell proliferation and vascular remodeling, and become immunogenic. Other mechanisms contributing to the occurrence of antibodies directed against 14-3-3 proteins could be discussed, including molecular mimicry triggered by an infectious agent and/or autoantigen oxidization as a consequence of free radicals as reported in other autoimmune disorders such as SSc (Servettaz et al., 2009).

Our study has some limitations. Thus, the number of patients included is limited for each of the CTD studied. However, PAH is a rare disease in which CTD is the second most common cause. Patients from tertiary care hospitals affiliated with the reference center for PAH management were included. Analysis of results comparing patients with PAH associated with SLE, MCTD or SS to patients without PAH with SLE, MCTD or SS is probably limitative. Moreover, the patients studied are not incident patients, and therefore they are heterogeneous, with variable durations of follow-up and variable ongoing treatment. This limitation is inherent to life cohort. For 2D experiments, we used pools of 3 sera. We agree that it does not allow us to make sure that we do not miss a reactivity specific to one individual in the pool of 3, but it would not have been possible to perform one 2D gel / membrane per patient taking into account the complexity of the technical approach. But that is an assumed risk, because it probably only counteracts minor reactivities, and the major and therefore useful reactivities are preserved. This technique of serum pools has also been used and validated on other populations such as idiopathic or SSc-associated PAH (Bussone et al., 2012; Dib et al., 2012; Terrier et al., 2008). The use of glucocorticoids and immunosuppressants in a number of patients may also have attenuated potential autoantibody reactivity. the proportion of patients treated with glucocorticoids was equivalent between those with and those without PAH. We identified target antigens but failed to demonstrate a pathogenic effect. This should be done in future work.

Our study also has several strengths. We included a respectable number of patients, given the low prevalence of CTD-PAH in Europe, in a prospective and multicenter study. We had detailed clinical data for each patient, which were therefore well phenotyped. The diagnosis of PAH was based on a right heart catheterization for all patients. We studied two protein extracts, coming

from two cells strongly implicated in the pathogenesis of PAH. We did not study the pulmonary VSMC, which is another cell involved in remodeling. 2D-electrophoresis on small gel combined with immunoblotting and modern techniques of mass spectrometry allow the identification of an exhaustive panel of antigens. The use of an optical method under Image J, assisted by the upstream identification of spots in 2D-immunoblot by the Python algorithm fulfilled this objective. Beyond the identification of antigenic targets, we have confirmed several IgG autoantibody reactivities, taking a step towards the development of a screening technique in routine care in order to predict the occurrence of PAH in the follow-up of CTD patients. Screening for these autoantibodies in a prospective cohort would validate their prognostic value.

Serum IgG anti-Annexin A5 and anti-14-3-3 ϵ antibody reactivities could represent predictive biomarkers for the development of PAH associated with SLE or MCTD. Prospective studies are needed to confirm this hypothesis.

Disclosure

The authors declare no conflict of interest.

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Legends to Figures.

Figure 1: Flow chart.

For each disease subset, patients with pulmonary arterial hypertension (PAH+) were compared to disease-matched control patients without PAH (PAH-) and to healthy controls. Connective tissue diseases (CTD); mixed connective tissue diseases (MCTD); Sjögren's syndrome (SS); systemic lupus erythematosus (SLE); systemic sclerosis (SSc). For each subset of patients, N indicates the number of patients included.

Figure 2: Autoantibody reactivity against Annexin A5 and 14-3-3 ϵ in patients with SLE, MCTD and SS according to pulmonary arterial hypertension (PAH) status.

Serum IgG reactivities of patients with systemic lupus erythematosus (SLE) (**A, D**), mixed connective tissue disease (MCTD) (**B, E**), or Sjögren's syndrome (Sjogren) (**C, F**) with PAH (PAH+) (●) or without PAH (PAH-) (●), were tested by ELISA against annexin A5 (upper panels) and 14-3-3 ϵ (lower panels). Each individual is represented by a single dot. Intensity of reactivity is quantified by optical density (O.D) measured in arbitrary units.

Figure 3: Autoantibody reactivity against Annexin A5 in Auto-HTAP patients and controls.

Panel A. Serum IgG reactivities of all 256 patients with PAH (PAH+) (●) or without PAH (PAH-) (●), were tested by ELISA against annexin A5 without distinguishing disease subsets. Panel B. Serum IgG reactivities of 265 Auto-HTAP patients were tested by disease subset (SLE (●), MCTD (●), Sjögren's syndrome (●), SSc (●), Portal HP (●), CHD (■), IPAH (●), HPAH (●)) without distinguishing those with or without PAH, and 150 HCs (▼). Each individual is represented by a single dot. Intensity of reactivity is quantified by optical density (O.D) measured in arbitrary units.

Supplemental Figure 1: Flow chart of overall Auto-HTAP population.

Legend Fig S1.

For each disease subset, patients with pulmonary arterial hypertension (PAH+) were compared to disease-matched control patients without PAH (PAH-) and to healthy controls, except for idiopathic PAH (IPAH) and heritable PAH (HPAH) which were compared to each other. Congenital heart disease (CHD); mixed connective tissue diseases (MCTD); Sjögren's syndrome (SS); systemic lupus erythematosus (SLE); systemic sclerosis (SSc). For each subset of patients, N indicates the number of patients included.

Supplemental Figure 2: 1D-immunoblot of serum IgG from patients with SLE, MCTD and SS with or without pulmonary arterial hypertension.

Legend: 1-D immunoblot of serum IgG with protein extracts of human lung fibroblasts. Serum IgG from patients with pulmonary arterial hypertension (PAH) related to systemic lupus erythematosus (SLE-PAH), PAH related to mixed connective tissue disease (MCTD-PAH), SLE and MCTD without PAH and healthy controls (HC) were immunoblotted at 70 µg of soluble extract of human lung fibroblasts transferred onto 0.45 µm nitrocellulose membrane. Normal human polyclonal IgG (intravenous immunoglobulins (IVIg)) and secondary anti-Fc gamma antibody tested alone were used as positive and negative controls, respectively. Phosphate buffered saline with Tween 20 polysorbate 0.2% (PBS).

Supplemental Figure 3: Target antigens networks.

Legend Fig S3.

Networks generated by Ingenuity pathway analysis with human proteins identified as specific target antigens recognized by serum IgG of patients with SLE, MCTD and SS and associated with pulmonary arterial hypertension (PAH). **A:** target antigens in human pulmonary fibroblast protein extract; **B:** target antigens in human pulmonary microvascular endothelial cells protein extract. Solid lines indicate direct interaction between proteins. Many proteins of the networks are implicated in the cellular process of inflammation, apoptosis, cell death, or auto-immune disorders.

Supplemental Figure 4: Autoantibody reactivity against 6 target antigens in patients with SLE, MCTD and SS compared to healthy controls.

Legend Fig S3.

Serum IgG reactivities of patients with SLE, MCTD and SS (○) and healthy controls (▼), were tested by ELISA against annexin A5 protein (A), 14-3-3 ε (B), triosephosphate isomerase (TPI) (C), vimentin (D), galectin-1 (Gal-1) (E), and elongation factor 2 (EF-2) (F) proteins. Each individual is represented by a single dot. Intensity of reactivity is quantified by optical density (O.D) measured in arbitrary units. Antibody (Ab).

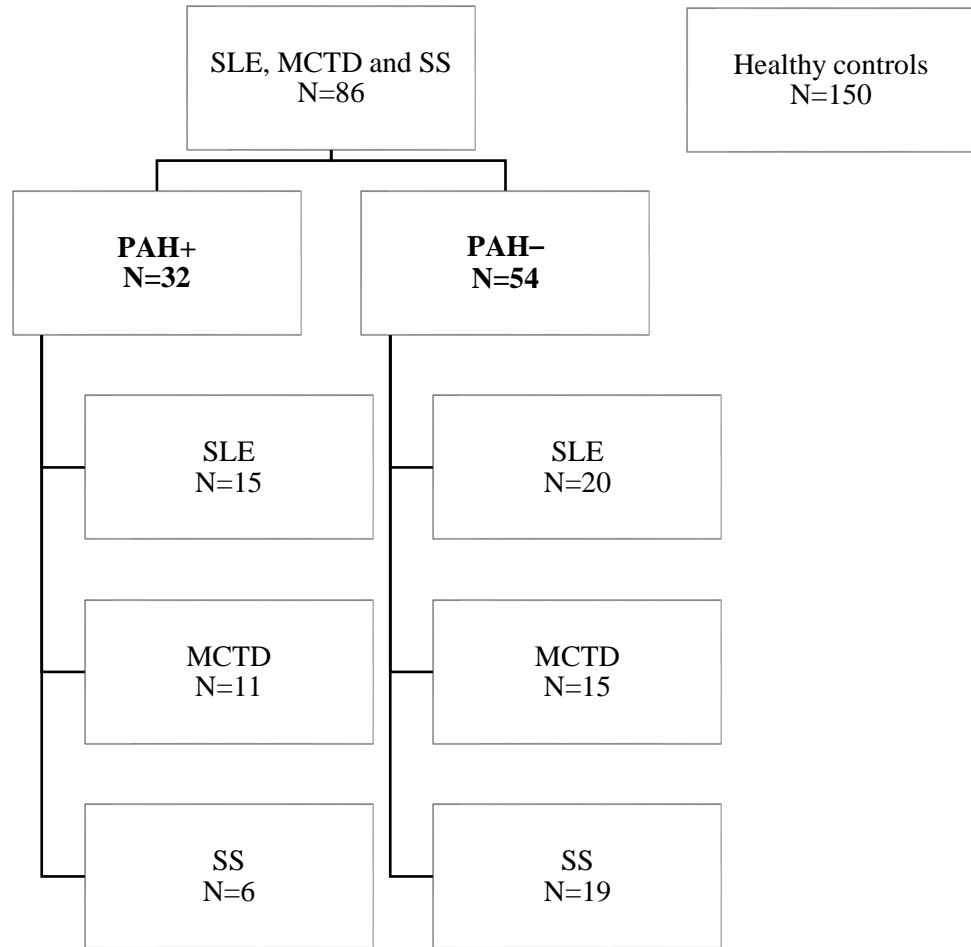


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For each disease subset, patients with pulmonary arterial hypertension (PAH+) were compared to disease-matched control patients without PAH (PAH-) and to healthy controls. Mixed connective tissue diseases (MCTD); Sjögren's syndrome (SS); systemic lupus erythematosus (SLE). For each subset of patients, N indicates the number of patients included.

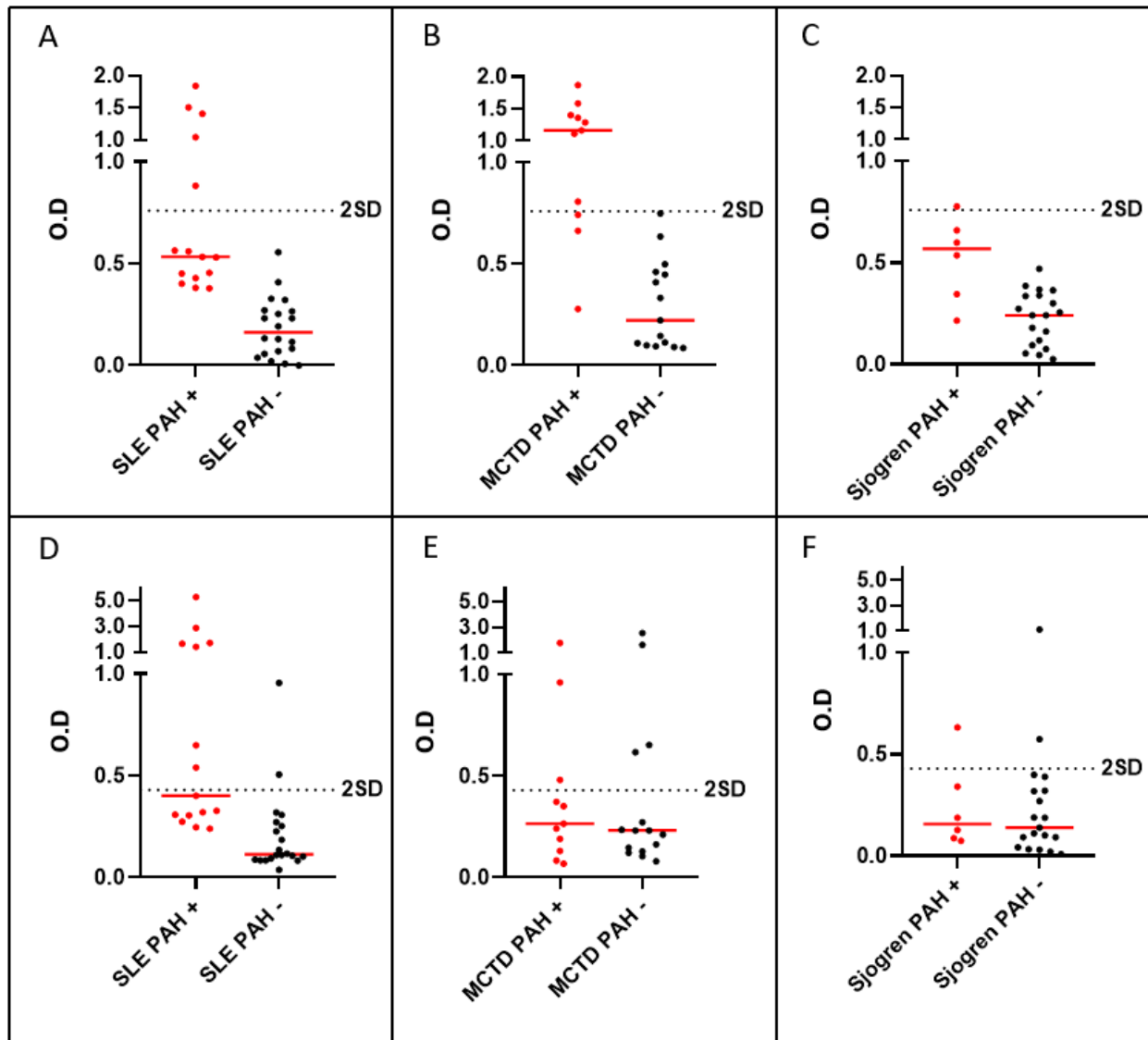


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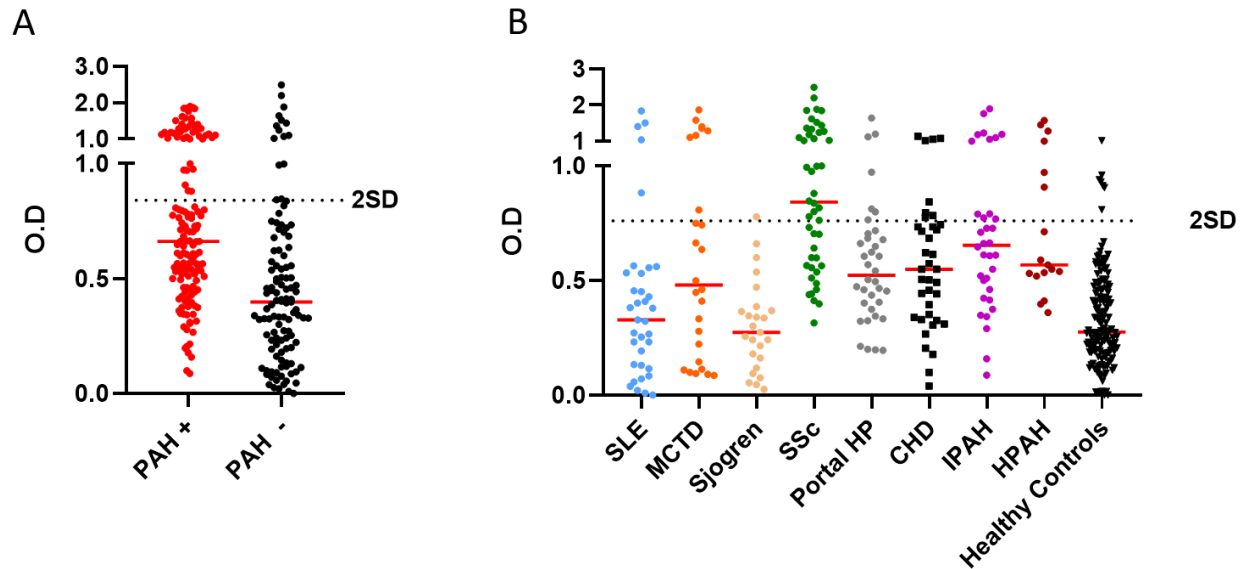
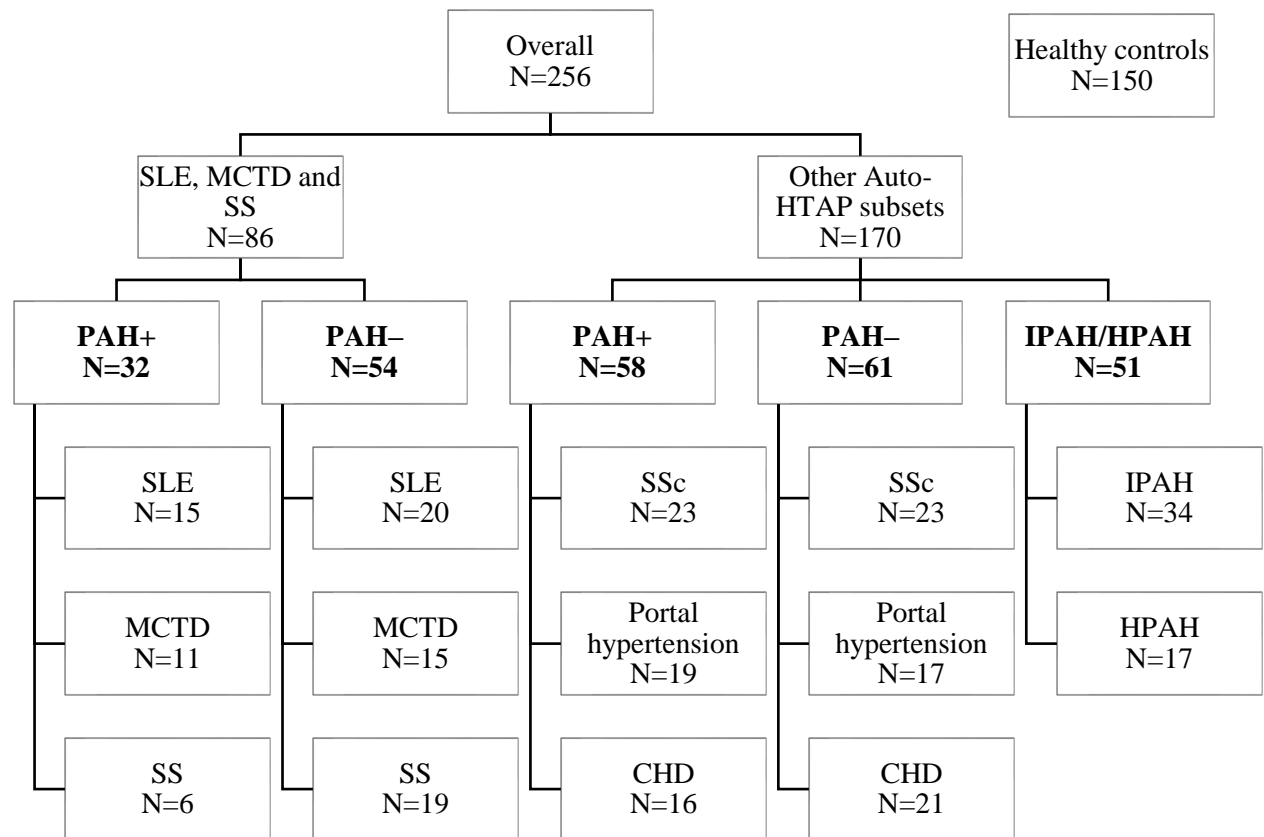


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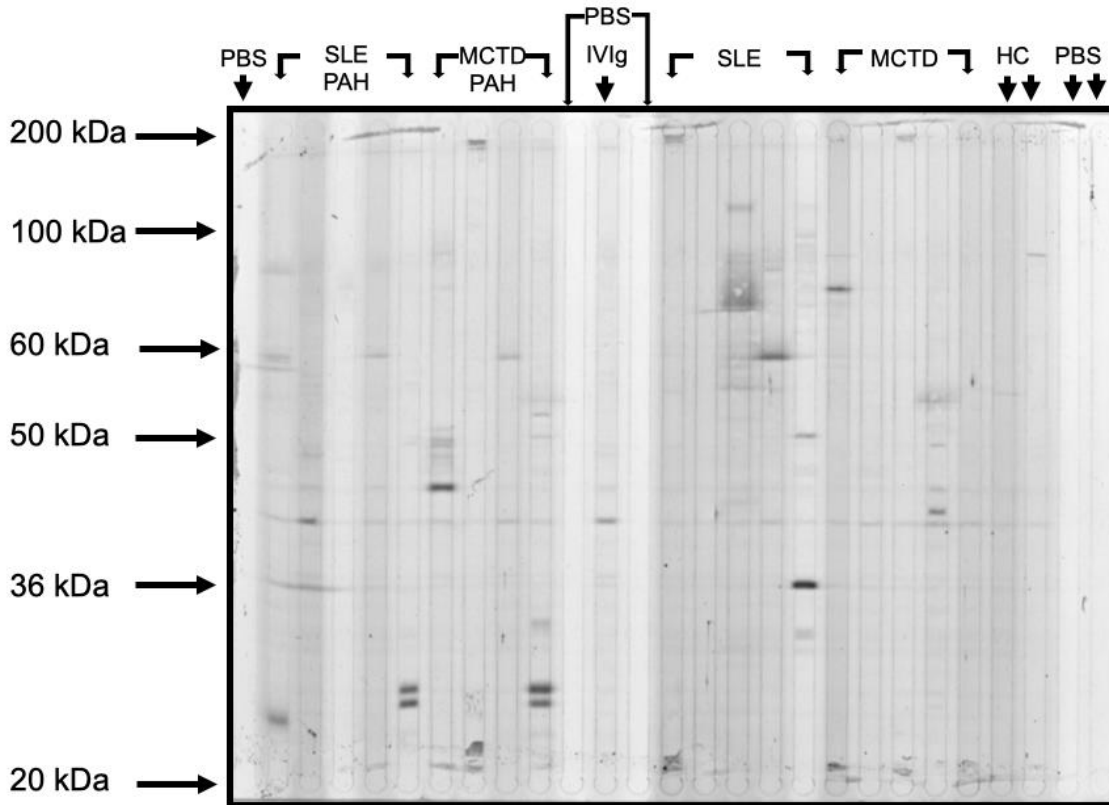


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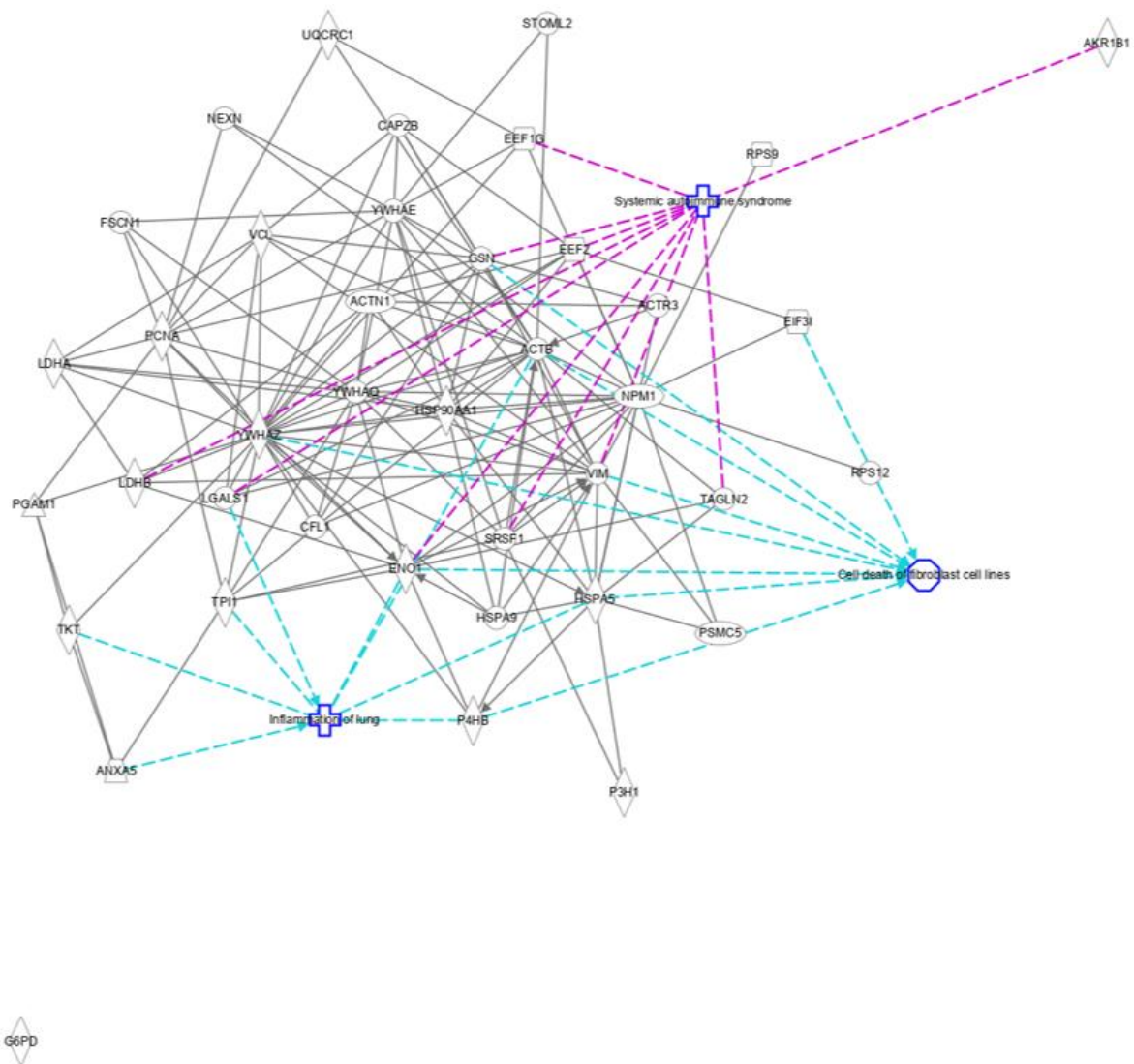


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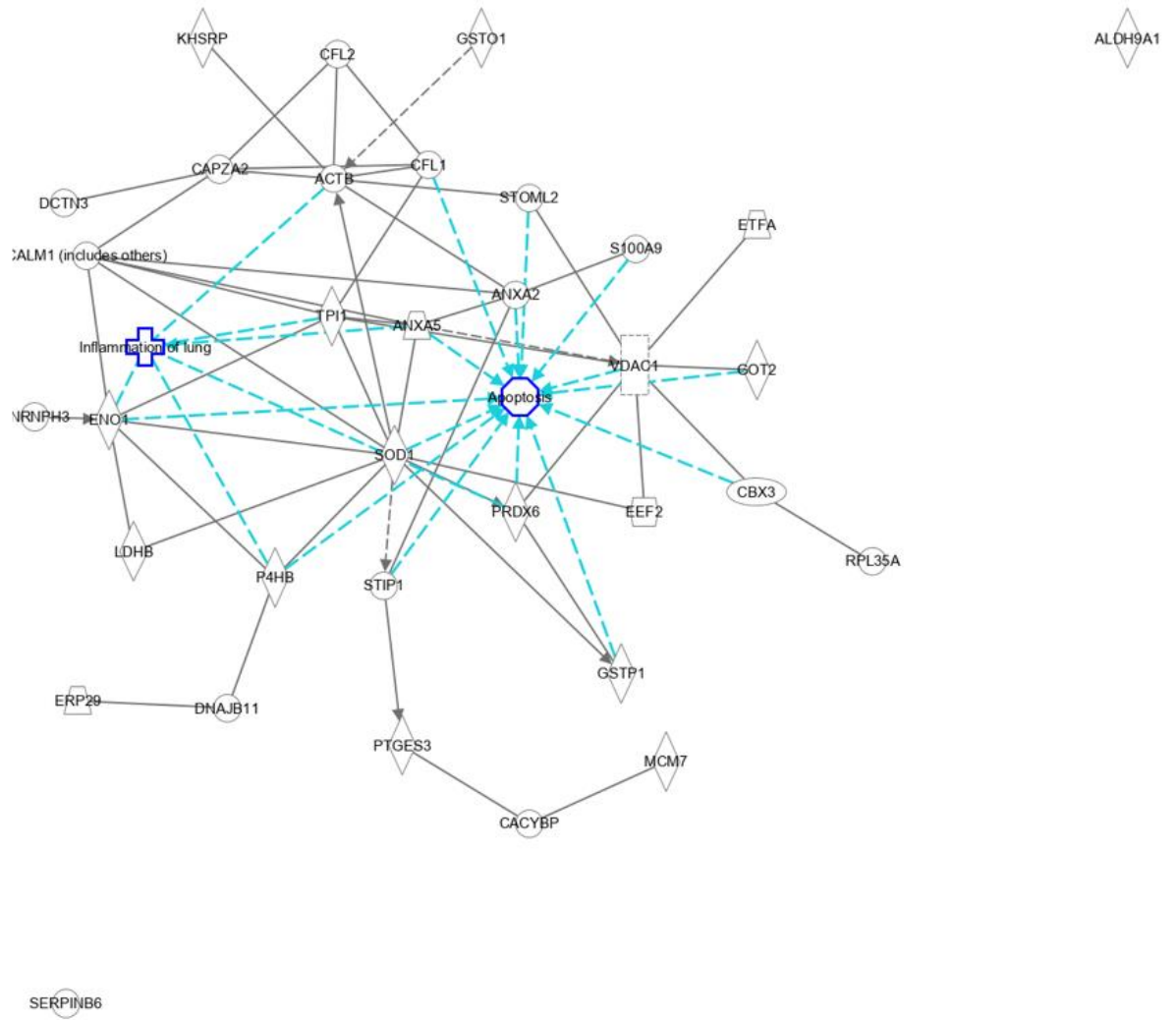
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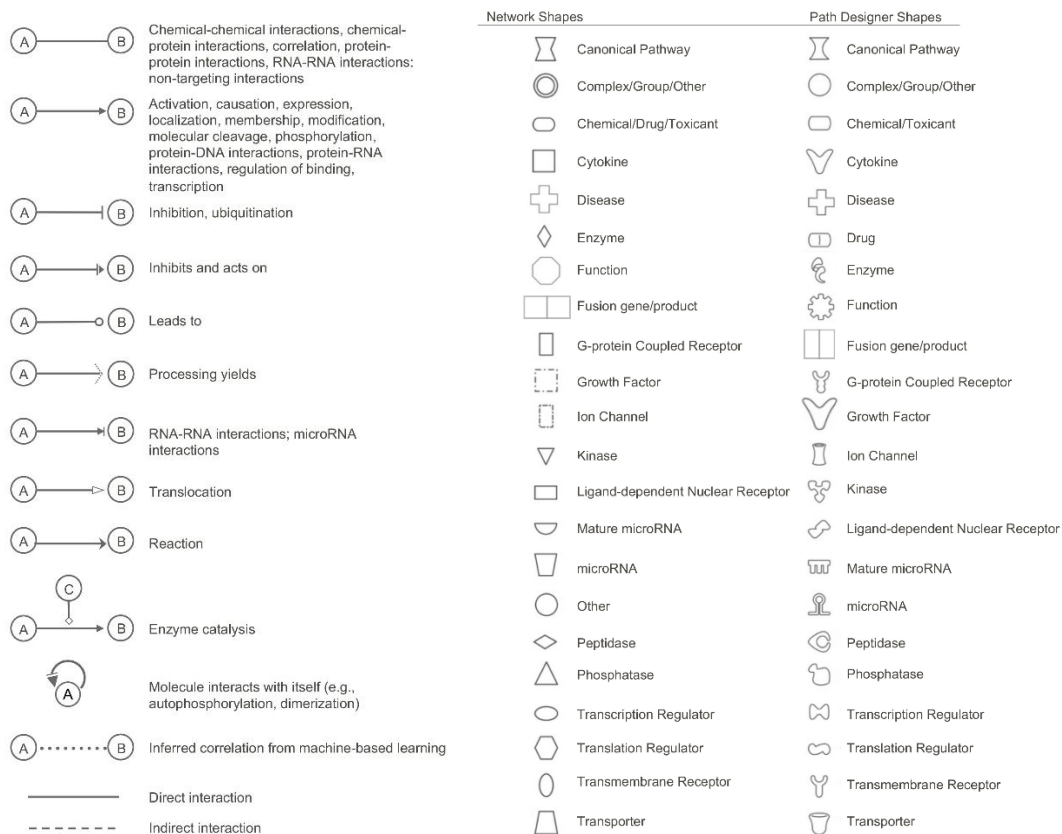
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A



B

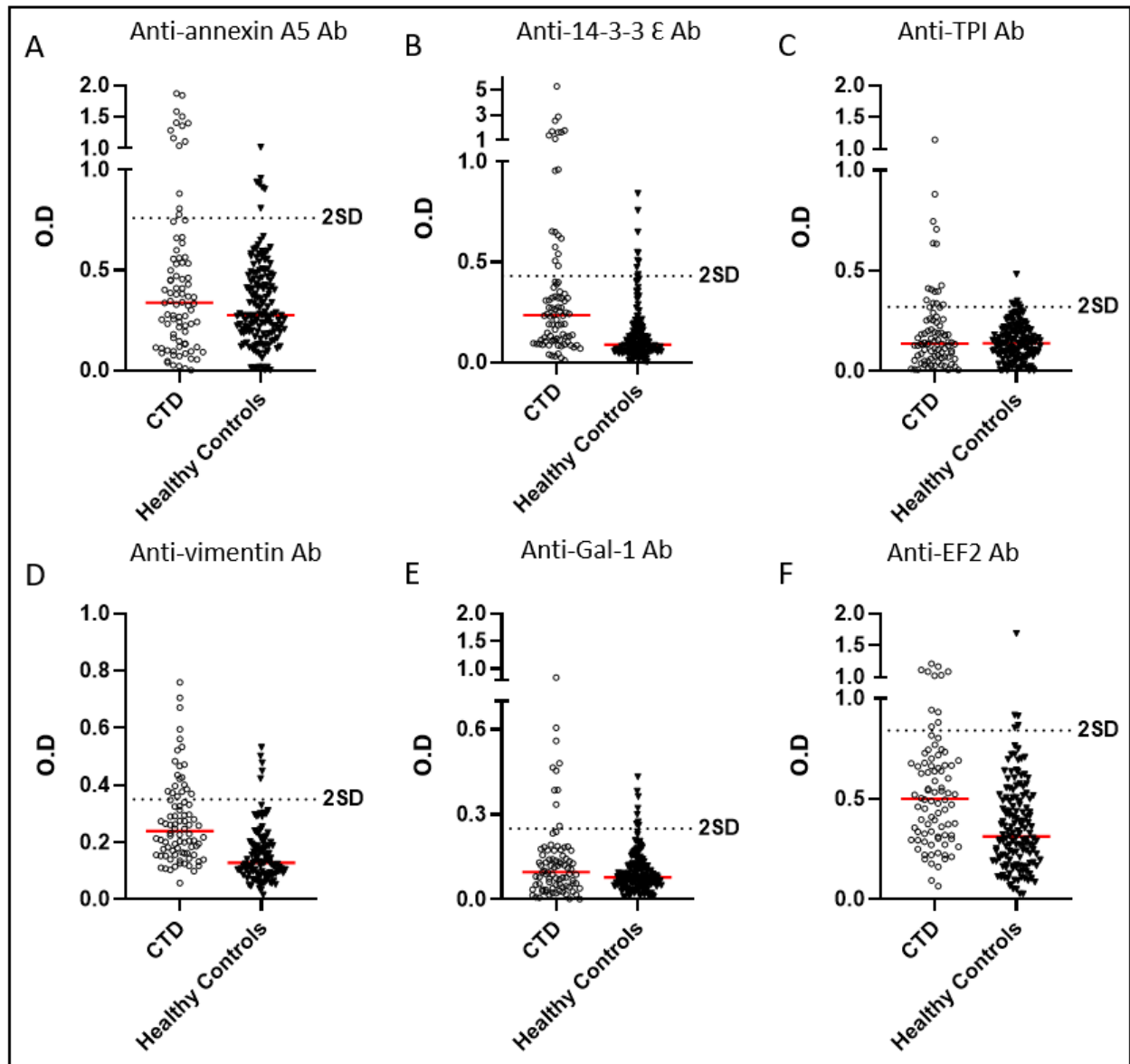




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Table 1: Baseline characteristics of SLE, MCTD and SS patients with systemic sclerosis with or without pulmonary arterial hypertension (PAH).

	n	Overall, N = 86¹	PAH-, N = 54¹	PAH+, N = 32¹	p-value²
Gender	86				0.290
Female		77 (89.5%)	50 (92.6%)	27 (84.4%)	
Age (Y)	86	50 (38-60)	52 (40-62)	49 (36-57)	0.260
Disease	86				0.270
SLE		35 (40.7%)	20 (37.0%)	15 (46.9%)	
MCTD		26 (30.2%)	15 (27.8%)	11 (34.4%)	
SS		25 (29.1%)	19 (35.2%)	6 (18.8%)	
PAH follow-up (Y)	32	4 (1-8)	-	4 (1-8)	
RHC					
mPAP (mmHg)	32	43 (33-50)	-	43 (33-50)	
PCWP (mmHg)	32	8 (5-11)	-	8 (5-11)	
PFTs					
FVC (%)	26/32	81 (65-100)	-	81 (65-100)	
DLCO (%)	24/32	54 (43-64)	-	54 (43-64)	
TLC (%)	26/32	86 (78-98)	-	86 (78-98)	
Treatments					
Oral anticoagulant	86	16 (18.6%)	0 (0%)	16 (50.0%)	0.020
Epoprostenol	86	4 (4.6%)	0 (0%)	4 (12.5%)	0.560
ERA	86	21 (24.4%)	0 (0%)	21 (65.6%)	<0.001
PDE5i	86	20 (23.3%)	0 (0%)	20 (62.5%)	<0.001
Glucocorticoids	86	48 (55.8%)	28 (51.9%)	20 (62.5%)	0.340
Azathioprine	86	11 (12.8%)	8 (14.8%)	3 (9.4%)	0.530
MMF	86	11 (12.8%)	5 (9.3%)	6 (18.8%)	0.320
Cyclophosphamide	86	5 (5.8%)	3 (5.6%)	2 (6.2%)	0.999
Methotrexate	86	6 (7.0%)	5 (9.3%)	1 (3.1%)	0.410

¹N (%); Quantitative data were expressed as median and interquartile range (IQR); Categorical data were expressed by number / total available and percentage (%).

²Categorical data were compared by Fisher's exact test; Quantitative data were compared by Wilcoxon rank sum test.

Transfer coefficient for carbon monoxide in the lung (DLCO); endothelin receptor antagonist (ERA); forced vital capacity (FVC); mixed connective tissue disease (MCTD); mycophenolate mofetil (MMF); mean pulmonary artery pressure (mPAP); number of patients in each subset (N); number of patients with available data (n); presence of pulmonary arterial hypertension (PAH+) or without PAH (PAH-); pulmonary capillary wedge pressure (PCWP); phosphodiesterase-5 inhibitor (PDE5i); pulmonary function test (PFTs); Sjögren's syndrome (SS); right heart catheterization (RHC); systemic lupus erythematosus (SLE); total lung capacity (TLC); years (Y).

Table 2: Mass spectrometry data of human pulmonary fibroblast proteins recognized by PAH+ and not by PAH- and healthy control serum immunoglobulin G.

Protein ID on gel	Protein	SwissProt accession number	emPAI score	Theoretical/Estimated MW (kD)	Theoretical/Estimated pI	Num. of significant matches	Num. of significant sequences
1	Heat shock protein 90-alpha	HS90A_HUMAN	5.58	80/84	4.9/4.2	145	50
2	Vimentin	VIME_HUMAN	62.4	55/53	5.0/4.6	668	62
3	Proliferating cell nuclear antigen (PCNA)	PCNA_HUMAN	4.23	33/28	4.5/4.2	24	12
4	14-3-3 protein zeta	1433T_HUMAN	9.53	28/27	4.7/4.3	39	18
5	14-3-3 protein epsilon	1433E_HUMAN	30.55	30/29	4.6/4.4	139	25
6	Protein disulfide-isomerase	PDIA1_HUMAN	11.74	60/57	4.7/4.8	361	50
7	Nucleophosmin	NPM_HUMAN	26.09	35/32	4.6/5	175	28
8	Elongation factor 2	EF2_HUMAN	3.77	85/95	6.4/5	82	43
9	Endoplasmic reticulum chaperone	BIP_HUMAN	7.71	70/72	5.0/5	213	52
10	Annexin A5	ANXA5_HUMAN	29.26	32/35	4.9/5	480	38
11	Galectin-1	LEG1_HUMAN	15.74	12/14	5.3/5	174	14
12	Prolyl 3-hydroxylase	P3H1_HUMAN	4.22	80/83	5.0/5.2	157	36
13	Nexilin	NEXN_HUMAN	5.98	75/80	5.3/5.4	101	47
14	Alpha-actinin-1	ACTN1_HUMAN	4.37	85/102	5.2/5.4	157	52
15	Actin, cytoplasmic 1	ACTB_HUMAN	7.83	70/41	5.2/5.4	179	24
16	Annexin A6	ANXA6_HUMAN	4.54	70/75	5.4/5.6	73	38
17	Stomatin-like protein 2	STML2_HUMAN	1.83	40/38	6.8/5.6	50	12
18	Serine/arginine-rich splicing factor 1	SRSF1_HUMAN	1.15	35/27	10./5.6	19	6

19	Cytochrome b-c1 complex subunit 1, mitochondrial	QCR1_HUMAN	4.12	50/52	5.9/5.7	170	26
20	Eukaryotic translation initiation factor 3 subunit I	EIF3I_HUMAN	3	38/36	5.3/5.7	52	14
21	Gelsolin	GELS_HUMAN	2.53	80/85	5.9/6	125	35
22	Actin-related protein 3	ARP3_HUMAN	3.13	50/47	5.6/6	88	21
23	F-actin-capping protein subunit b	CAPZB_HUMAN	11.76	33/31	5.3/6	105	22
24	L-lactate dehydrogenase B	LDHB_HUMAN	4.74	35/36	5.7/6	153	18
25	40S ribosomal protein S9	RS9_HUMAN	2.21	25/22	10./6.2	13	10
26	Vinculin	VINC_HUMAN	16.22	100/123	5.5/6.5	384	99
27	Triosephosphate isomerase	TPIS_HUMAN	3.73	27/30	7.4/6.5	58	17
28	Elongation factor 1-gamma	EF1G_HUMAN	5.05	50/50	6.2/6.6	119	26
29	Phosphoglycerate mutase 1	PGAM1_HUMAN	8.14	28/28	6.6/6.6	96	19
30	40S ribosomal protein S12	RS12_HUMAN	2.46	15/14	6.8/6.6	13	7
31	Alpha-enolase	ENOA_HUMAN	1.48	50/47	7.0/6.7	22	16
32	Glucose-6-phosphate 1-dehydrogenase	G6PD_HUMAN	10.6	60/59	6.3/6.9	236	40
33	Aldo-keto reductase family 1 member B1	ALDR_HUMAN	4.96	35/35	6.5/7	95	22
34	Fascin	FSCN1_HUMAN	3.61	60/54	6.8/7	93	30
35	26S proteasome regulatory subunit 8	PRS8_HUMAN	3.34	40/45	7.1/7.9	34	20
36	Transgelin-2	TAGL2_HUMAN	8.28	22/22	8.4/7.9	27	13
37	Transketolase	TKT_HUMAN	6.39	70/67	7.5/7.9	163	38
38	Cofilin-1	COF1_HUMAN	37.01	18/18	8.2/8	191	21
39	L-lactate dehydrogenase A	LDHA_HUMAN	11.54	33/36	8.4/8.2	107	23

emPAI = Exponentially modified protein abundance index; kDa = kilodalton; MW = molecular weight; PAH = pulmonary arterial hypertension; pI = isoelectric point;

The emPAI is a score that provides an indicative value of the comparative abundance of the identified proteins.

Table 3: Mass spectrometry data of human microvascular endothelial cell proteins recognized by PAH+ and not by PAH- and healthy control serum immunoglobulin G.

Protein ID on gel	Protein	SwissProt accession number	emPAI score	Theoretical/Estimated MW (kD)	Theoretical/Estimated pI	Num. of significant matches	Num. of significant sequences
1	60S ribosomal protein L35a	RL35A_HUMAN	1.77	12/15	11.0/11	5	5
2	Calmodulin-1	CALM1_HUMAN	6.41	16/20	4.0/4.1	38	11
3	Cofilin-1	COF1_HUMAN	18.23	18/22	8.2/8.8	132	21
4	Cofilin-2	COF2_HUMAN	1.65	18/24	7.6/8	10	8
5	Superoxide dismutase	SODC_HUMAN	1.66	15/23	5.7/6.1	15	6
6	Protein S100-A9	S10A9_HUMAN	1.64	13/23	5.7/5.5	6	4
7	Dynactin subunit 3	DCTN3_HUMAN	2.06	21/25	5.3/5.6	16	9
8	Chromobox protein homolog 3	CBX3_HUMAN	2.12	20/26	5.2/5.5	13	8
9	Prostaglandin E synthase 3	TEBP_HUMAN	1.66	18/25	4.3/4.1	16	7
10	Calcyclin-binding protein	CYBP_HUMAN	4.03	26/34	8.2/8.5	32	13
11	Glutathione S-transferase P	GSTP1_HUMAN	3.86	23/28	5.4/6	36	13
12	Triosephosphate isomerase	TPIS_HUMAN	10.96	26/33	6.4/6.8	98	24
13	Peroxiredoxin-6	PRDX6_HUMAN	9.23	25/32	6/7	88	19
14	Endoplasmic reticulum resident protein 29	ERP29_HUMAN	2	28/34	6.7/6.2	25	10
15	Glutathione S-transferase omega-1	GSTO1_HUMAN	2.49	27/36	6.2/6.2	62	12
16	Electron transfer flavoprotein subunit alpha, mitochondrial	ETFA_HUMAN	2.37	35/37	8.6/8.8	30	14
17	Annexin A2	ANXA2_HUMAN	2.72	38/37	7.5/7.7	21	18

18	L-lactate dehydrogenase B	LDHB_HUMAN	6.02	36/40	5.7/6.3	37	13
19	F-actin-capping protein subunit alpha-2	CAZA2_HUMAN	3.23	32/40	5.5/6.2	15	10
20	Annexin A5	ANXA5_HUMAN	26.34	35/40	4.9/8.5	65	21
21	Heterogeneous nuclear ribonucleoprotein H3	HNRH3_HUMAN	3.71	36/40	6.3/7	22	10
22	Aspartate aminotransferase, mitochondrial	AATM_HUMAN	3.08	47/45	9.1/10	28	12
23	Alpha-enolase	ENOA_HUMAN	1.48	47/50	7.0/9.5	9	9
24	Voltage-dependent anion-selective channel protein-1	VDAC1_HUMAN	1.53	30/50	8.6/9	7	6
25	DnaJ homolog subfamily B member 11	DJB11_HUMAN	2.65	40/47	5.8/6.6	20	9
26	Actin, cytoplasmic 1	ACTB_HUMAN	4.56	41/47	5.2/6	39	12
27	Stomatin-like protein 2, mitochondrial	STML2_HUMAN	6.23	38/47	6.8/5.9	29	14
28	Serpin B6	SPB6_HUMAN	6.53	42/45	5.1/5.7	25	12
29	4-trimethylaminobutyraldehyde dehydrogenase	AL9A1_HUMAN	8.22	53/55	5.6/6.2	31	18
30	Protein disulfide-isomerase	PDIA1_HUMAN	17.9	57/65	4.7/4.9	117	32
31	DNA replication licensing factor	MCM7_HUMAN	5.98	81/95	6.0/6.8	46	26
32	Stress-induced-phosphoprotein 1	STIP1_HUMAN	10.5	62/75	6.4/7.2	46	26
33	Far upstream element-binding protein 2	FUBP2_HUMAN	3.22	73/95	6.8/7.6	27	18
34	Elongation factor 2	EF2_HUMAN	5.11	95/100	6.4/7.4	50	28

emPAI = Exponentially modified protein abundance index; kDa = kilodalton; MW = molecular weight; PAH = pulmonary arterial hypertension; pI = isoelectric point;

The emPAI is a score that provides an indicative value of the comparative abundance of the identified proteins.

Table 4: Target antigens recognized and shared by serum IgG pools of SLE, MCTD and SS patients with pulmonary arterial hypertension (PAH) in the fibroblast protein extract.

	Overall, N=13 pools	SLE, N=6 pools	MCTD, N=5 pools	SS, N=2 pools
Annexin A5	5/13 (38.5%)	1/6 (16.7%)	4/5 (80.0%)	0/2
14-3-3 epsilon	4/13 (30.8%)	3/6 (50.0%)	1/5 (20.0%)	0/2
Vimentin	3/13 (23.1%)	1/6 (16.7%)	1/5 (20.0%)	1/2 (50.0%)
Triosephosphate isomerase	6/13 (46.2%)	4/6 (66.7%)	2/5 (40.0%)	0/2
Galectin-1	2/13 (15.4%)	1/6 (16.7%)	1/5 (20.0%)	0/2
Elongation factor 2	7/13 (53.8%)	3/6 (50.0%)	3/5 (60.0%)	1/2 (50.0%)

Data are expressed as positive pools count / all PAH+ pools (%). Mixed connective tissue disease (MCTD); number of serum pool in each subset of disease with PAH (N); Sjögren's syndrome (SS); systemic lupus erythematosus (SLE).

Table 5: IgG autoantibody reactivity against 6 target autoantigens in patients with SLE, MCTD and SS patients according to pulmonary arterial hypertension (PAH) status.

	SLE			MCTD			SS		
	PAH-, N = 20	PAH+, N = 15	p-value	PAH-, N = 15	PAH+, N = 11	p-value	PAH-, N = 19	PAH+, N = 6	p-value
Anti-Annexin A5 Ab	0 (0%)	5 (33.3%)	0.009	0 (0%)	8 (72.7%)	<0.001	0 (0%)	1 (16.7%)	0.24
Anti-14-3-3 epsilon Ab	2 (10.0%)	7 (46.7%)	0.02	4 (26.7%)	3 (27.3%)	0.99	2 (10.5%)	1 (16.7%)	0.99
Anti-Triosephosphate isomerase Ab	1 (5.0%)	3 (20.0%)	0.29	4 (26.7%)	3 (27.3%)	0.99	3 (15.8%)	1 (16.7%)	0.99
Anti-Vimentin Ab	4 (20.0%)	4 (26.7%)	0.70	7 (46.7%)	4 (36.4%)	0.70	2 (10.5%)	1 (16.7%)	0.99
Anti-Galectin-1 Ab	2 (10.0%)	1 (6.7%)	0.99	2 (13.3%)	2 (18.2%)	0.99	3 (15.8%)	0 (0%)	0.55
Anti-Elongation factor 2 Ab	0 (0.0%)	3 (20.0%)	0.07	2 (13.3%)	0 (0%)	0.49	5 (26.3%)	1 (16.7%)	0.99

Presence of autoantibody was defined by an optic density (O.D.) above 2 standard derivations in healthy controls, and compared as categorical data by Fisher's exact test. Data are expressed as positive count (%). Lines in bold correspond to a p-value <0.05.

Antibody (Ab); mixed connective tissue disease (MCTD); number of patients in each subset (N); pulmonary arterial hypertension (PAH): presence (PAH+) or not (PAH-); Sjögren's syndrome (SS); systemic lupus erythematosus (SLE).

Table S1: Baseline characteristics in 170 auto-HTAP patients

	n	Overall, N = 170¹	PAH–, N = 61¹	PAH+, N = 109¹	p-value²
Gender	170				0.06
Female		108 (63.5%)	33 (54.1%)	75 (68.8%)	
Age (Y)	170	54 (44-67)	53 (42-68)	54 (46-66)	0.80
Disease	170				
SSc		46 (27.1%)	23 (37.7%)	23 (21.1%)	
Portal hypertension		36 (21.2%)	17 (27.9%)	19 (17.4%)	
CHD		37 (21.8%)	21 (34.4%)	16 (14.7%)	
Idiopathic PAH		34 (20.0%)	-	34 (31.2%)	
Heritable PAH		17 (10.0%)	-	17 (15.6%)	
PAH follow-up (Y)	109	3 (2-8)	-	3 (2-8)	
ANA	116	63/116 (54.3%)	28/38 (73.7%)	35/78 (44.9%)	0.003
RHC					
mPAP (mmHg)	109	48 (39-57)	-	48 (39-57)	
PCWP (mmHg)	109	9 (6-11)	-	9 (6-11)	
PFTs					
FVC (%) *	104	92 (81-104)	102 (78-103)	92 (82-104)	0.93
DLCO (%) #	92	59 (47-73)	68 (62-81)	58 (46-72)	0.16
TLC (%) †	104	96 (84-104)	98 (80-103)	96 (85-104)	0.83
Treatments					
Oral anticoagulant	170	69 (40.6%)	1 (1.6%)	68 (62.4%)	0.003
Epoprostenol	170	24 (14.1%)	0 (0.0%)	24 (22.0%)	0.02
ERA	170	76 (44.7%)	1 (1.6%)	75 (68.8%)	<0.001
PDE5i	170	60 (35.3%)	0 (0.0%)	60 (55.0%)	<0.001
Glucocorticoids	170	17 (10.0%)	13 (21.3%)	4 (3.7%)	<0.001
Azathioprine	170	1 (0.6%)	1 (1.6%)	0 (0.0%)	0.36
MMF	170	7 (4.1%)	5 (8.2%)	2 (1.8%)	0.10
Cyclophosphamide	170	1 (0.6%)	0 (0.0%)	1 (0.9%)	0.999

Methotrexate 170 2 (1.2%) 2 (3.3%) 0 (0.0%) 0.13

¹N (%); Quantitative data were expressed as median and interquartile range (IQR); Categorical data were expressed by number and percentage (%), and in case of missing data by number / total available and percentage (%).

²Categorical data were compared by Fisher’s exact test; Quantitative data were compared by Wilcoxon rank sum test.

PAH– group included disease-matched control patients without PAH.

*Data were available for 104 patients (missing value in 14 PAH+ patients, and 52 in PAH– patients).

#Data were available for 92 patients (missing value in 25 PAH+ patients, and 53 in PAH– patients).

‡Data were available for 104 patients (missing value in 14 PAH+ patients, and 52 in PAH– patients).

Antinuclear antibodies (ANA); congenital heart disease (CHD); transfer coefficient for carbon monoxide in the lung (DLCO); endothelin receptor antagonist (ERA); forced vital capacity (FVC); mycophenolate mofetil (MMF); mean pulmonary artery pressure (mPAP), number of patients in each subset (N); number of patients with available data (n); pulmonary arterial hypertension (PAH): presence (PAH+) or not (PAH–); pulmonary capillary wedge pressure (PCWP); phosphodiesterase-5 inhibitor (PDE5i); pulmonary function tests (PFTs); right heart catheterization (RHC); systemic sclerosis (SSc); total lung capacity (TLC); years (Y).

Supplemental Table 2: protein spots associated with PAH+ status in 2D-immunoblot

	SLE			MCTD			SS			HCs
	Overall ¹	PAH+ ¹	PAH– ¹	Overall ¹	PAH+ ¹	PAH– ¹	Overall ¹	PAH+ ¹	PAH– ¹	
Fibroblast protein extract	273±79	217±61	329±48	205±77	261±65	150±30	109±11	117±3	101±11	109 [#]
HPMEC protein extract	189±48	227±0	150±10	146±30	156±15	136±47	88±33	105±42	70±17	190 [#]

Healthy controls (HCs); Human pulmonary microvascular endothelial cells (HPMEC); mixed connective tissue disease (MCTD); pulmonary arterial hypertension (PAH) : presence (+) or not (–); Sjögren’s syndrome (SS); systemic lupus erythematosus (SLE).

¹mean±standard derivation

[#]Only one membrane of one pool of 14 sera for HCs

Supplemental Table 3: IgG autoantibody reactivity against 6 target antigens in patients with SLE, MCTD and SS compared to healthy controls

	N	Overall, N = 236	CTD, N = 86	HCs, N = 150	p-value
Anti-Annexin A5 Ab	236	21 (8.9%)	14 (16.3%)	7 (4.7%)	0.003
Anti-14-3-3 epsilon Ab	236	28 (11.9%)	19 (22.1%)	9 (6.0%)	<0.001
Anti-Triosephosphate isomerase Ab	236	21 (8.9%)	15 (17.4%)	6 (4.0%)	<0.001
Anti-Vimentin Ab	236	27 (13.6%)	22 (25.6%)	5 (4.4%)	<0.001
Anti-Galectin-1 Ab	236	18 (7.6%)	10 (11.6%)	8 (5.3%)	0.080
Anti-Elongation factor 2 Ab	236	16 (6.9%)	11 (12.8%)	5 (3.4%)	0.007

Presence of autoantibody was defined by an optic density (O.D.) above 2 standard derivations in healthy controls, and compared as categorical data by Fisher's exact test. Data are expressed as positive count (%). Bold correspond to a p-value <0.05. Antibody (Ab); connective tissue disease (CTD); healthy controls (HCs).

Supplemental Table 4: IgG autoantibody reactivity against Annexin A5 target autoantigen in 170 Auto-HTAP patients and controls

	SSc N = 46			Portal HT N = 36		
	PAH- , N = 23	PAH+ , N = 23	p-value	PAH- , N = 17	PAH+ , N = 19	p-value
Anti-Annexin A5 Ab	15 (65.2%)	13 (56.5%)	0.55	1 (5.9%)	6 (31.6%)	0.09

	CHD N = 37			IPAH vs HPAH N = 51		
	PAH- , N = 21	PAH+ , N = 16	p-value	HPAH , N = 17	IPAH , N = 34	p-value
Anti-Annexin A5 Ab	2 (9.5%)	6 (37.5%)	0.06	6 (35.3%)	12 (35.3%)	0.99

Presence of autoantibody was defined by an optic density (O.D.) above 2 standard derivations in healthy controls, and compared as categorical data by Fisher's exact test. Data are expressed as positive count (%). Lines in bold correspond to a p-value <0.05.

Antibody (Ab); congenital heart disease (CHD); heritable PAH (HPAH); idiopathic PAH (IPAH); number of patients in each subset (N); pulmonary arterial hypertension (PAH): presence (PAH+) or not (PAH-); portal hypertension (Portal HT); systemic sclerosis (SSc).

II.1.4 – Impact clinique de la réactivité des auto-anticorps anti-Annexine A5 et anti-14-3-

3 ε

Nous avons exploré en complément l'association entre la réactivité des auto-anticorps anti-annexine A5 (ANXA5) et anti-14-3-3 ε identifiés et les caractéristiques cliniques des patients via des analyses exploratoires (test exact de Fisher pour les variables catégorielles et test de rang de Wilcoxon-Mann-Whitney pour les variables quantitatives).

Nous avons d'abord analysé les associations avec une réactivité des auto-anticorps anti-ANXA5 au sein des patients ayant une connectivité. Ces résultats sont résumés dans le **Tableau 3**. Nous avons confirmé que la réactivité anti-ANXA5 était associée à la présence d'une HTAP (100% vs 25%) et associée à la connectivité sous-jacente puisque plus de 50% des patients avec cette réactivité étaient atteints d'une connectivité mixte et 1/3 d'un LES. Les Ac anti-ANXA5 étaient plus fréquemment détectés chez les hommes (29% vs 7%, $p=0.035$). Ils étaient associés à une évaluation de la dyspnée de classes III ou IV chez plus de la moitié des patients (versus 15% sans réactivité anti-ANXA5, $p=0.001$), malgré l'absence de différence statistiquement significative concernant les données hémodynamiques du cathétérisme cardiaque droit au diagnostic de l'HTAP ou des paramètres fonctionnels respiratoires. Aucun patient ayant une réactivité anti-ANXA5 n'avait de syndrome des antiphospholipides associé. L'association retrouvée avec la détection d'auto-anticorps anti-ribonucléoprotéine (RNP) est probablement liée à la forte proportion de patients avec une connectivité mixte (57%). La réactivité des Ac anti-ANXA5 était par ailleurs associée à la réactivité des Ac anti-vimentine (50% vs 21%, $p=0.040$).

Tableau 3 : Données cliniques en fonction de la réactivité des auto-anticorps anti-ANXA5.

Variables	N	Total N = 86 ¹	Ac anti-ANXA5 négatifs N = 72 ¹	Ac anti-ANXA5 positifs N = 14 ¹	p-value²
HTAP	86	32/86 (37%)	18/72 (25%)	14/14 (100%)	<0.001
Age (années)	86	50 (38-60)	52 (39-62)	46 (30-55)	0.127
Connectivite	86				0.035
Connectivite mixte		26/86 (30%)	18/72 (25%)	8/14 (57%)	
Lupus érythémateux systémique		35/86 (41%)	30/72 (42%)	5/14 (36%)	
Syndrome de Sjögren		25/86 (29%)	24/72 (33%)	1/14 (7.1%)	
Sexe féminin	86	77/86 (90%)	67/72 (93%)	10/14 (71%)	0.035
Suivi de l'HTAP (années)	32	4 (1-8)	4 (1-8)	4 (1-8)	0.645
Classe de Dyspnée	83				0.001
I		42/83 (51%)	40/69 (58%)	2/14 (14%)	
II		22/83 (27%)	18/69 (26%)	4/14 (29%)	
III		18/83 (22%)	10/69 (14%)	8/14 (57%)	
IV		1/83 (1.2%)	1/69 (1.4%)	0/14 (0%)	
Échocardiographie					
PAP systolique (mmHg)	32	66 (42-80)	63 (36-77)	66 (43-81)	0.745
Cathétérisme cardiaque droit					
PAP moyenne (mmHg)	32	43 (33-50)	43 (35-51)	41 (33-47)	0.531
PCP (mmHg)	32	8 (5-11)	8 (6-10)	9 (5-11)	0.826
Index cardiaque (L/min/m ²)	32	3.10 (2.35-3.90)	3.10 (2.30-3.50)	3.25 (2.55-4.40)	0.371
Débit cardiaque (L/min)	32	5.10 (4.18-6.08)	4.60 (4.15-5.60)	5.80 (4.70-6.30)	0.282
Épreuves fonctionnelles respiratoires					
CPT%	26	86 (78-98)	84 (69-94)	88 (84-98)	0.367
CVF%	26	81 (65-100)	88 (74-103)	70 (64-97)	0.247
DLCO%	24	54 (43-64)	60 (48-65)	43 (34-58)	0.147
Immunologie					
Ac antinucléaires (ANA)	83	83/83 (100%)	69/69 (100%)	14/14 (100%)	
Ac anti-ADN natif	78	40/78 (51%)	35/64 (55%)	5/14 (36%)	0.198

Variables	N	Total	Ac anti-ANXA5 négatifs	Ac anti-ANXA5 positifs	p-value ²
		N = 86 ¹	N = 72 ¹	N = 14 ¹	
Ac anti-SSA/Ro	73	29/73 (40%)	25/62 (40%)	4/11 (36%)	0.999
Ac anti-SSB/La	73	11/73 (15%)	10/62 (16%)	1/11 (9.1%)	0.999
Ac anti-Sm	73	7/73 (9.6%)	5/62 (8.1%)	2/11 (18%)	0.283
Ac anti-RNP	76	31/76 (41%)	21/65 (32%)	10/11 (91%)	<0.001
Autres auto-anticorps					
Ac anti-14-3-3 ε	86	19/86 (22%)	15/72 (21%)	4/14 (29%)	0.500
Ac anti-TPI	86	15/86 (17%)	10/72 (14%)	5/14 (36%)	0.063
Ac anti-Vimentine	86	22/86 (26%)	15/72 (21%)	7/14 (50%)	0.040
Ac anti-Galectine-1	86	10/86 (12%)	7/72 (9.7%)	3/14 (21%)	0.356
Ac anti-EEF2	86	11/86 (13%)	10/72 (14%)	1/14 (7.1%)	0.683
Ac multiples (≥2)	86	14/86 (16%)	7/72 (9.7%)	7/14 (50%)	0.001
Traitement spécifique HTAP	86	23/86 (27%)	12/72 (17%)	11/14 (79%)	<0.001
Traitements combinés	86	17/86 (20%)	9/72 (12%)	8/14 (57%)	<0.001
Corticoïdes	86	48/86 (56%)	40/72 (56%)	8/14 (57%)	0.913
Immunosuppresseurs					
Azathioprine	86	11/86 (13%)	11/72 (15%)	0/14 (0%)	0.198
Mycophénolate mofétil	86	11/86 (13%)	8/72 (11%)	3/14 (21%)	0.377
Cyclophosphamide	86	5/86 (5.8%)	5/72 (6.9%)	0/14 (0%)	0.586
Rituximab	86	2/86 (2.3%)	2/72 (2.8%)	0/14 (0%)	0.999
Méthotrexate	86	6/86 (7.0%)	6/72 (8.3%)	0/14 (0%)	0.583

¹n/N (%) ; Médiane (25%-75%) ;

²Variables catégorielles : test exact de Fisher ; Variables quantitatives : test de rang de Wilcoxon ;

Ac : anticorps ; Classe de Dyspnée MRC (medical research council) ; CVF% : pourcentage (%) de la théorique de la capacité vitale fonctionnelle ; CPT% : % de la théorique de la capacité pulmonaire totale ; DLCO% : % de la théorique de la capacité de diffusion du monoxyde de carbone ; facteur d'élongation 2 (EEF2) ; HTAP : hypertension artérielle pulmonaire ; PAP : pression artérielle pulmonaire ; PCP : pression capillaire pulmonaire ; RNP : ribonucléoprotéine ; Sm : antigène Smith ; SSA/B : sicca syndrome A/B ; TPI : triphosphate isomérase.

Ensuite, nous avons exploré l'impact clinique de la réactivité des auto-anticorps anti-14-3-3 ε chez les patients atteints d'un LES (**Tableau 4**). Nous avons restreint cette analyse à la population de LES car c'était la seule connectivite au cours de laquelle la réactivité des Ac anti-

14-3-3 ϵ était discriminante envers la présence d'une HTAP. Il n'a pas été mis en évidence d'association particulière entre les caractéristiques cliniques des patients et la réactivité anti-14-3-3 ϵ excepté son association avec la présence d'une HTAP ($p=0.022$), et l'association avec la des auto-anticorps anti-triosephosphate isomérase (44% vs 0%, $p=0.002$), anti-vimentine (56% vs 12%, $p=0.015$), et anti-facteur d'élongation 2 (33% vs 0%, $p=0.013$). Il n'a pas été identifié d'association avec les paramètres hémodynamiques ou des explorations fonctionnelles respiratoires au diagnostic.

Tableau 4 : Données cliniques en fonction de la réactivité des IgG anti-14-3-3 ϵ .

Variabes	N	Total N = 35 ¹	Ac anti-14-3-3 ϵ négatifs N = 26 ¹	Ac anti-14-3-3 ϵ positifs N = 9 ¹	p-value²
HTAP	35	15/35 (43%)	8/26 (31%)	7/9 (78%)	0.022
Age (années)	35	44 (36-50)	44 (36-51)	46 (36-49)	0.940
Sexe féminin	35	28/35 (80%)	20/26 (77%)	8/9 (89%)	0.648
Suivi de l'HTAP (années)	15	5 (1-10)	6 (4-11)	4 (1-8)	0.520
Classe de Dyspnée	32				0.999
I		20/32 (62%)	15/23 (65%)	5/9 (56%)	
II		6/32 (19%)	4/23 (17%)	2/9 (22%)	
III		6/32 (19%)	4/23 (17%)	2/9 (22%)	
IV		0	0	0	
Échocardiographie					
PAP systolique (mmHg)	13	60 (38-70)	62 (56-66)	43 (34-78)	0.945
Cathétérisme cardiaque droit					
PAP moyenne (mmHg)	15	44 (38-48)	44 (38-48)	44 (34-48)	0.772
PCP (mmHg)	15	7 (1-9)	4 (3-7)	9 (7-9)	0.093
Index cardiaque (L/min/m ²)	15	3.00 (2.25-3.20)	2.70 (2.33-3.38)	3.05 (2.40-3.18)	0.948
Débit cardiaque (L/min)	15	4.50 (3.67-5.50)	4.45 (3.67-5.25)	4.85 (3.90-5.50)	0.932

Variables	N	Total N = 35 ¹	Ac anti-14-3-3 ε négatifs N = 26 ¹	Ac anti-14-3-3 ε positifs N = 9 ¹	p-value ²
Épreuves fonctionnelles respiratoires					
CPT%	11	86 (84-94)	85 (84-86)	90 (80-95)	0.792
CVF%	11	90 (78-101)	80 (77-98)	96 (84-106)	0.360
DLCO%	11	53 (44-66)	64 (43-66)	50 (46-62)	0.927
Immunologie					
Ac antinucléaires (ANA)	34	34/34 (100%)	25/25 (100%)	9/9 (100%)	
Ac anti-DNA natif	34	29/34 (85%)	21/25 (84%)	8/9 (89%)	0.999
Ac anti-SSA/Ro	27	9/27 (33%)	8/19 (42%)	1/8 (12%)	0.201
Ac anti-SSB/La	27	1/27 (3.7%)	1/19 (5.3%)	0/8 (0%)	0.999
Ac anti-Sm	27	4/27 (15%)	0/19 (0%)	4/8 (50%)	0.004
Ac anti-RNP	27	6/27 (22%)	3/19 (16%)	3/8 (38%)	0.319
Autres auto-anticorps					
Ac anti-Annexin A5	35	5/35 (14%)	3/26 (12%)	2/9 (22%)	0.586
Ac anti-TPI	35	4/35 (11%)	0/26 (0%)	4/9 (44%)	0.002
Ac anti-Vimentine	35	8/35 (23%)	3/26 (12%)	5/9 (56%)	0.015
Ac anti-Galectine-1	35	3/35 (8.6%)	1/26 (3.8%)	2/9 (22%)	0.156
Ac anti-EEF2	35	3/35 (8.6%)	0/26 (0%)	3/9 (33%)	0.013
Ac multiples (≥2)	35	5/35 (14%)	0/26 (0%)	5/9 (56%)	<0.001
Traitement spécifique HTAP	35	9/35 (26%)	5/26 (19%)	4/9 (44%)	0.192
Traitements combinés	35	7/35 (20%)	4/26 (15%)	3/9 (33%)	0.340
Corticoïdes	35	23/35 (66%)	16/26 (62%)	7/9 (78%)	0.450
Immunosuppresseurs					
Azathioprine	35	6/35 (17%)	3/26 (12%)	3/9 (33%)	0.162
Mycophénolate mofétil	35	6/35 (17%)	3/26 (12%)	3/9 (33%)	0.162
Cyclophosphamide	35	3/35 (8.6%)	3/26 (12%)	0/9 (0%)	0.553
Rituximab	0	0	0	0	
Méthotrexate	35	1/35 (2.9%)	1/26 (3.8%)	0/9 (0%)	0.999

Variables	N	Total	Ac anti-14-3-3 ε négatifs	Ac anti-14-3-3 ε positifs	p-value ²
		N = 35 ¹	N = 26 ¹	N = 9 ¹	

¹n/N (%); Médiane (25%-75%)

²Variables catégorielles : test exact de Fisher ; Variables quantitatives : test de rang de Wilcoxon.

Ac : anticorps ; Classe de Dyspnée MRC (medical research council) ; CVF% : pourcentage (%) de la théorique de la capacité vitale fonctionnelle ; CPT% : % de la théorique de la capacité pulmonaire totale ; DLCO% : % de la théorique de la capacité de diffusion du monoxyde de carbone ; facteur d'élongation 2 (EEF2) ; HTAP : hypertension artérielle pulmonaire ; PAP : pression artérielle pulmonaire ; PCP : pression capillaire pulmonaire ; RNP : ribonucléoprotéine ; Sm : antigène de Smith ; SSA/B : sicca syndrome A/B ; TPI : triphosphate isomérase.

II.1.5 – Rôle pathogène des IgG purifiées sur le temps de fibrinoformation

Dans le dernier volet de cette étude et en complément, nous avons exploré le potentiel pathogénique des IgG purifiées de patients atteints de CM et de LES ayant une réactivité des auto-anticorps anti-ANXA5 sur le temps de fibrinoformation afin d'explorer leur potentiel effet sur ce processus biologique. Ces expériences ont été réalisées en collaboration et sur la plateforme d'analyse du service d'Hématologie Biologique du Pr Michaela FONTENAY de l'hôpital Cochin, avec le Dr Jérôme DUCHEMIN. La fibrinoformation correspond à la transformation du fibrinogène soluble en fibrine insoluble grâce à la thrombine (**Figure 6**). La thrombine provoque la formation de monomères, qui s'agrègent pour former des polymères.

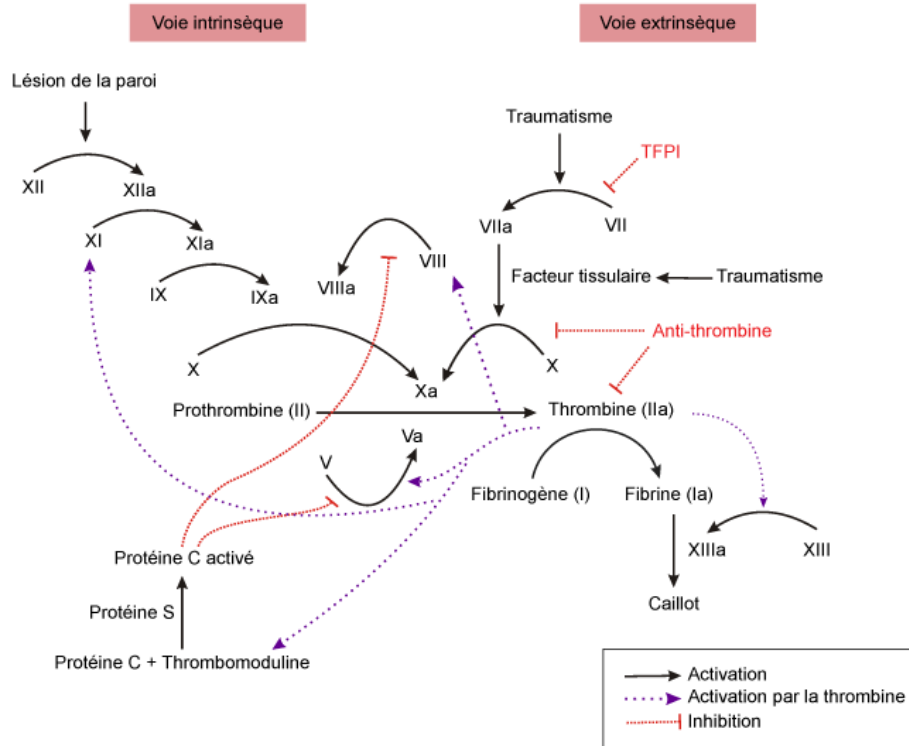


Figure 6 : Cascade de la coagulation (Pitte M et al. 2019)

TFPI, inhibiteur de la voie du facteur tissulaire.

Les principaux points de méthodes relatifs à l'étude du rôle pathogène des IgG purifiées étaient :

a. Collecte de sérums de patients atteints de CM (n=7) dont 4 patients avec HTAP et réactivité IgG anti-ANXA5, et 3 sans HTAP et sans réactivité IgG anti-ANXA5, de LES (n=7) dont 4 patients avec HTAP et réactivité IgG anti-ANXA5, et 3 sans HTAP et sans réactivité IgG anti-ANXA5, et de témoins sains (n=5).

b. Purification des IgG à partir des sérums de chaque groupe sur une colonne de protéine G Sépharose, concentrées en boudin de dialyse pendant 72 heures ; quantification des IgG

purifiées par spectrophotométrie à 260 nm et 280 nm. Vérification de la pureté des fractions d'IgG purifiées par électrophorèse sur gel de polyacrylamide en une dimension.

c. Test de fibrinoformation (temps de fibrinoformation) :

- Incubation d'une solution d'IgG purifiées en concentration croissantes (0.2, 0.4, 0.6, 0.8 et 1 mg/mL) d'un patient atteint d'une HTAP associée à une connectivite mixte présentant une forte réactivité anti-ANXA5 avec du plasma normal à 37°C pendant 30 minutes sur automate STAR evolution 2 (Stago®) sur la plateforme du service d'Hématologie Biologie de l'hôpital Cochin, pour réaliser une courbe dose-réponse du temps de fibrinoformation,
- Préparation des dilutions à la concentration de 1 mg/mL d'IgG purifiées dans du PBS des patients avec ou sans réactivité anti-ANXA5, d'IgG purifiées de témoins sains, d'une solution du tampon PBS sans IgG purifiées et d'une solution d'immunoglobulines polyvalentes humaines (Clairyg® 50 mg/mL),
- Mesure du temps de fibrinoformation après incubation de l'ensemble des solutions d'IgG purifiées à la concentration constante de 1 mg/mL avec du plasma normal à 37°C pendant 30 minutes automate STAR evolution 2 afin de rechercher de potentielles différences entre les groupes (positivité versus négativité de la réactivité anti-ANXA5).

La répartition des échantillons tests était la suivante :

- IgG purifiées de patients atteints de CM associée à une HTAP et avec réactivité anti-ANXA5 (n=4),
- IgG purifiées de patients atteints de CM non associée à une HTAP et sans réactivité anti-ANXA5 (n=3),

- IgG purifiées de patients atteints de LES associé à une HTAP et avec réactivité anti-ANXA5 (n=4),
- IgG purifiées de patients atteints de LES non associé à une HTAP et sans réactivité anti-ANXA5 (n=3),
- IgG purifiées de témoins sains (n=5),
- Immunoglobulines polyvalentes humaines (n=1).

Mesure du temps de fibrinof formation pour chaque échantillon et comparaison des résultats entre les groupes de patients (avec et sans réactivité anti-ANXA5) et les témoins sains. Les résultats étaient présentés par leur médiane et leur écart interquartile, et comparés par le test de Kruskal-Wallis. L'impact de la variation des autres cofacteurs (thrombine et fibrinogène) sur le temps de fibrinof formation a été vérifié.

L'analyse du temps de fibrinof formation à différentes concentrations d'IgG purifiées a révélé un effet dose-réponse significatif sur le sérum testé (HTAP associée à une CM avec réactivité anti-ANXA5). Nous avons observé une augmentation d'allure linéaire du temps de fibrinof formation en corrélation avec l'augmentation de la concentration d'IgG purifiées de l'échantillon provenant d'un patient atteint d'une HTAP associée à une CM avec une forte réactivité anti-ANXA5 (**Figure 7**). Cette observation suggère que les IgG purifiées du sérum de ce patient ont un impact sur la formation de fibrine, avec des effets proportionnels à leur concentration. Cet échantillon a été sélectionné pour les analyses préliminaires de calibration du temps de fibrinof formation et recherche d'une dose-réponse car ce patient avait une HTAP, une forte réactivité IgG anti-ANXA5, et nous disposions pour ce patient d'une grande quantité d'IgG purifiées. En revanche, aucune prolongation significative du temps de fibrinof formation

n'a été notée avec l'augmentation de la concentration dans les échantillons d'immunoglobulines polyvalentes humaines (IgIV) ni dans les IgG purifiées provenant d'un témoin sain sans réactivité anti-ANXA5. Cette absence d'effet dose-réponse dans ces deux autres groupes contrôles suggère que l'effet observé est spécifiquement lié aux IgG purifiées de ce patient avec une HTAP-CM et une réactivité des anticorps anti-ANXA5.

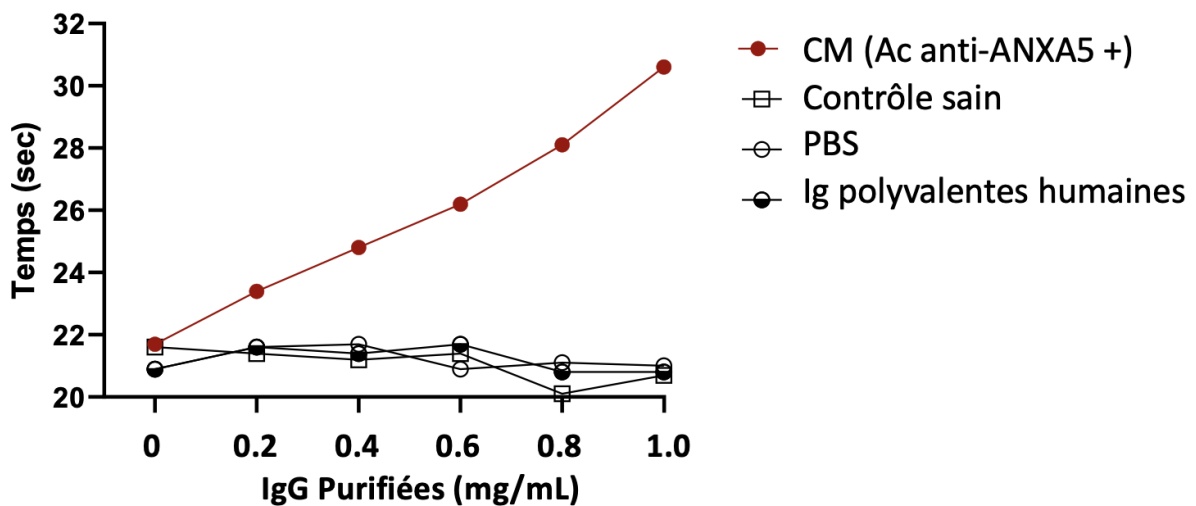


Figure 7 : Temps de fibrinoformation en présence de concentrations croissantes d'IgG purifiées.

Légende : CM, connectivite mixte ; PBS, phosphate buffered saline ; sec, secondes.

Nous avons ensuite évalué l'impact de l'ensemble des échantillons d'IgG purifiées sur le temps de fibrinoformation. Nous n'avons pas mis en évidence de différence entre le temps médian de fibrinoformation entre les groupes d'IgG purifiées de patients avec HTAP présentant une réactivité anti-ANXA5 (temps médian de fibrinoformation de 20.2 sec, IQR 20.0-20.3), ceux des patients sans HTAP et sans réactivité anti-ANXA5 (20.2 sec, IQR 19.9-20.4) et les témoins sains (19.9 sec, IQR 19.9-20.0, $p=0.292$) (**Figure 8**). L'analyse en sous-groupe en fonction de la connectivite ne montrait pas non plus de différence significative. Contrairement à notre hypothèse, nous n'avons pas démontré d'impact des IgG purifiées sur le temps de

fibrinof ormation. La réactivité IgG anti-ANXA5 ne semble pas influencer le temps de fibrinof ormation dans les conditions de notre expérience.

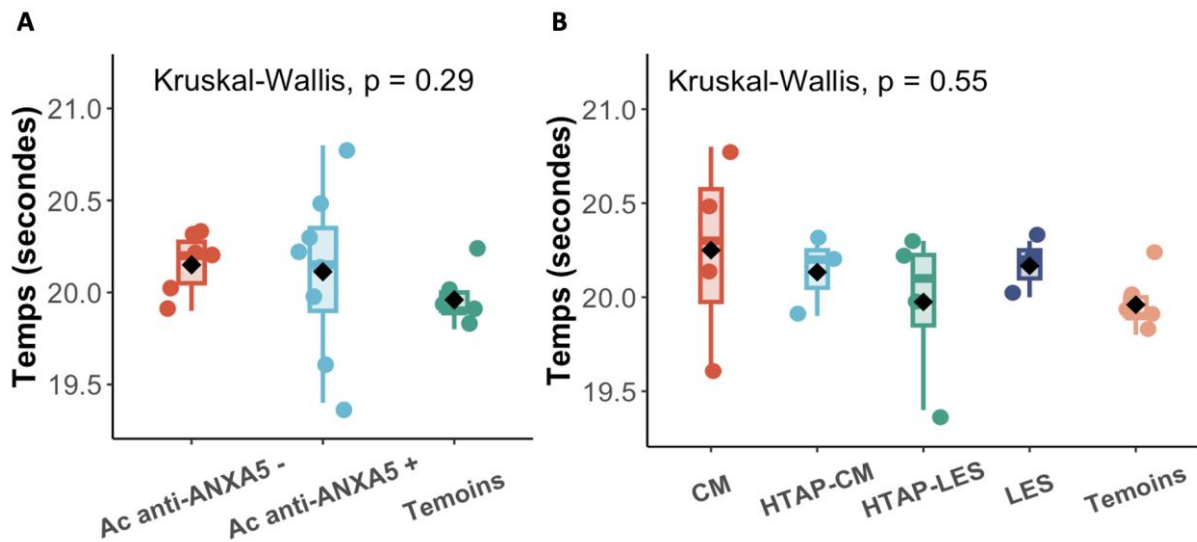


Figure 8 : Temps de fibrinof ormation en présence d'IgG purifiées de patients avec HTAP et réactivité anti-ANXA5 comparés à ceux sans HTAP et sans réactivité anti-ANXA5.

Légende : Temps de fibrinof ormation après incubation avec les IgG purifiées (1 mg/mL) issues de patients avec et sans réactivité des Ac anti-ANXA5 ($n=14$) et chez les témoins sains ($n=5$) (Panneau A), et au sein des sous-groupes de connectivites (avec HTAP et réactivité anti-ANXA5 versus sans HTAP et sans réactivité anti-ANXA5) (Panneau B). ANXA5, annexine A5 ; Ac anti-ANXA5 +, réactivité IgG anti-ANXA5 ; Ac anti-ANXA5 -, absence de réactivité IgG anti-ANXA5 ; CM, connectivite mixte ; HTAP, hypertension artérielle pulmonaire ; HTAP- CM, HTAP associée à la connectivite mixte ; HTAP-LES, HTAP associée au lupus érythémateux systémique ; LES, lupus érythémateux systémique ; Témoins, témoins sains.

II.2 – Auto-anticorps dans l’HTAP associée à l’infection par le virus de l’immunodéficience humaine (VIH)

II.2.1 – Introduction

Dans un deuxième article, nous nous sommes intéressés à une autre forme d’HTAP potentiellement réversible, l’HTAP associée à l’infection par le VIH (HTAP-VIH).

En parallèle de l’existence d’une inflammation chronique, des mécanismes d’auto-immunité semblent jouer un rôle dans la pathogénie de l’HTAP-VIH, permettant de faire l’hypothèse qu’il pourrait également exister des auto-anticorps dirigés contre les composants de la paroi vasculaire chez les patients ayant une HTAP-VIH. La physiopathologie de l’HTAP-VIH est complexe et méconnue. Certaines protéines virales semblent cibler l’endothélium vasculaire pulmonaire et avoir la capacité d’induire de la prolifération cellulaire, de l’activation endothéliale, de l’apoptose et la synthèse de cytokines pro-inflammatoires, mais il n’a pas été démontré d’infection de l’endothélium vasculaire pulmonaire par le virus du VIH. L’activation immunitaire chronique associée à l’infection par le VIH et l’épuisement immunitaire progressif induisent une diminution des lymphocytes T CD4⁺ auxiliaires et une altération des mécanismes de contrôle des lymphocytes B auto réactifs pouvant être à l’origine d’auto-anticorps spécifiques. Nous avons donc fait l’hypothèse que par analogie au groupe de connectivites (LES, CM et SS) pour lesquels nous avons détecté des AFA et AECA et identifié leurs cibles antigéniques, il pourrait exister également des auto-anticorps spécifiques de HTAP-VIH.

II.2.2 – Résumé

Introduction

L'HTAP associée au virus de l'immunodéficience humaine (VIH) (HTAP-VIH) est une complication grave au cours de l'infection par le VIH et associée à un mauvais pronostic. Sa physiopathologie est complexe et mal comprise. La détection d'auto-anticorps ciblant les cellules endothéliales vasculaires pulmonaires et les fibroblastes n'a jamais été testée dans l'HTAP-VIH à notre connaissance. Notre objectif était d'identifier les auto-anticorps spécifiques de l'HTAP-VIH et de caractériser leurs cibles antigéniques.

Méthodes

Les sérums de 40 patients VIH dont 20 associés à une HTAP sans autre cause que le VIH (HTAP-VIH) ont été incubés avec des extraits protéiques de fibroblaste et de cellule endothéliale vasculaire pulmonaire. L'identification de spots de réactivité a été effectuée par immunoblot 2D en comparant des pools de 3 sérums de patients VIH (avec ou sans HTAP associée) et d'un pool de sérums de 14 témoins sains. Les réactivités auto-anticorps ont été confirmées par technique ELISA sur sérums individuels incubés avec des protéines recombinantes humaines après identification des cibles antigéniques par spectrométrie de masse, et comparées à 150 sérums de témoins sains.

Résultats

En immunoblot 2D, 13 spots de réactivité ont été identifiés sur l'extrait de fibroblastes et 17 sur l'extrait endothélial pulmonaire présents uniquement chez les patients ayant une HTAP-VIH. L'annexine A5, la protéine 14-3-3 ϵ , la vimentine, et l'élongation facteur 2 ont été identifiées comme les cibles antigéniques des auto-anticorps détectés partagées par au moins 2 pools de sérums d'HTAP-VIH. En ELISA, la réactivité anti-ANXA5 était significativement plus

fréquemment détectée dans les sérums des patients HTAP-VIH (55%) comparés à ceux sans HTAP (0%, $p < 0.001$). La réactivité anti-14-3-3 ϵ était elle aussi plus fréquente dans le groupe HTAP-VIH (35% versus 5%, $p = 0.040$). La positivité des anticorps anti-ANXA5 était associée à l'âge ($p = 0.040$) et à un âge plus élevé au moment du diagnostic du VIH ($p = 0.020$).

Conclusion

Les réactivités des anticorps sériques IgG anti-Annexine A5 et anti-14-3-3 ϵ pourraient être utilisées comme marqueur prédictif de la survenue d'une HTAP au cours de l'infection par le VIH. Cependant, ces données doivent être confirmées par la réalisation d'études sur un nombre plus important de patients et par la réalisation d'études longitudinales chez des patients prélevés en amont de la survenue d'une HTAP.

II.2.3 – Article original 2

Anti-fibroblast and anti-endothelial cell autoantibodies are present in the serum of patients with Pulmonary Arterial Hypertension related to Human Immunodeficiency Virus infection (HIV-PAH): A Brief Report

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Abstract

Introduction

Human immunodeficiency virus (HIV)-related pulmonary arterial hypertension (HIV-PAH) is a severe complication. Its pathophysiology is complex and poorly understood. We aimed to investigate autoantibodies targeting pulmonary vascular endothelial cells and fibroblasts in HIV-PAH patients and identify their target antigens.

Methods

Sera from 40 HIV patients, including 20 with PAH, were tested for reactivity with protein extracts of pulmonary vascular endothelial cells and fibroblasts. Serum pools of three patients with (PAH+) or without PAH (PAH-) and a unique pool of sera from 14 healthy controls were tested using 2D immunoblot. Target antigens were identified by mass spectrometry. Serum IgG reactivities were confirmed by ELISA using recombinant human proteins and compared to 150 serums from healthy controls.

Results

Thirteen and 17 protein spots were recognized by IgG in pools of sera from PAH+ patients but not PAH- patients in fibroblast and endothelial cell protein extracts, respectively. Target antigens identified by mass spectrometry were annexin A5 (ANXA5), 14-3-3 ϵ , vimentin, and elongation factor 2, were recognized by IgG from at least 2 serum pools of PAH+ and not by PAH- patients. Anti-ANXA5 and anti-14-3-3 ϵ antibodies were significantly more frequently detected in sera of PAH+ patients than in those without PAH (55% vs 0%, $p < 0.001$) and (35% versus 5%, $p = 0.040$), respectively. The positivity of anti-ANXA5 antibodies was associated with older age ($p = 0.040$) and an older age at the time of the HIV diagnosis ($p = 0.020$).

Conclusion

Serum IgG antibodies directed toward ANXA5 and 14-3-3 ϵ are present in the serum from HIV-PAH patients.

Keywords: pulmonary arterial hypertension, Human Immunodeficiency Virus, fibroblast, endothelial cell, autoantibody, annexin A5 protein, 14-3-3 ϵ protein.

Introduction

Pulmonary arterial hypertension (PAH) is a rare and complex disease, with a poor prognosis (Humbert et al., 2022). Human immunodeficiency virus (HIV)-associated pulmonary arterial hypertension (HIV-PAH) is a severe cardiovascular complication. The HIV-PAH confers a poor prognosis whatever the stage of the infectious disease (Nunes et al., 2003). The contemporary use of highly active antiretroviral treatment (HAART) has led to a massive reduction in mortality, with an increase in the long-term survival of HIV patients (Quinn, 2008). This survival is accompanied by an increase of acquired immunodeficiency syndrome (AIDS)-unrelated chronic conditions such as HIV-PAH (Boccaro, 2008).

According to the WHO at the end of 2022, there were approximately 39 million people living with the HIV in the entire world. The prevalence of HIV-PAH is probably 0.5% of HIV patients (Sitbon et al., 2008). Histological lesions typically encountered in PAH are found in more than 75% of HIV-PAH patients (Mehta et al., 2000), which are indistinguishable from lesions found in non-infectious causes of idiopathic PAH, particularly resembling to plexogenic pulmonary arteriopathy (Petitpretz et al., 1994).

Some HIV features, including a chronic immune activation and inflammation, are probably implicated in HIV-PAH pathogenesis, increasing 1000-fold the risk for developing PAH compared to general population. However, the pathophysiology remains unclear. Autoantibodies targeting vessel wall cells such as anti-endothelial cell antibodies (AECA), anti-fibroblast antibodies (AFA),

and antibodies directed at vascular smooth muscle cells have been identified in idiopathic PAH or systemic sclerosis-associated PAH (Bussone et al., 2012; Dib et al., 2012; Tamby et al., 2005; Terrier et al., 2008). To our knowledge there is no data available regarding the identification of AECA and AFA in HIV-PAH patients. Early detection of HIV patients at risk of developing PAH is crucial. The aim of the study was to identify specific PAH-related AFA and AECA associated with HIV-PAH and characterize their antigen targets.

Patients and methods

All patients and healthy controls (HCs) were included in a French prospective multicenter Auto-HTAP study (NTC01208792). Serum IgG samples from 40 HIV patients were collected between June 2010 and July 2014, aliquoted and stored at -80°C until use. Among them, 20 were HIV-PAH (PAH+), and other 20 without PAH (PAH-). Among PAH+ patients, HIV infection was the only cause of PAH, without evidence for porto-pulmonary hypertension. Serum IgG samples from 150 healthy blood donors used as healthy controls (HCs) were also collected. PAH was defined according to the third World Symposium on Pulmonary Arterial Hypertension, reference at the time, on right-heart catheterization by increased mean pulmonary artery pressure (mPAP) above 25 mmHg, pulmonary artery wedge pressure below 15 mmHg and pulmonary vascular resistance above 3 Wood (Galiè et al., 2009). Patients with HIV infection were identified with a positive ELISA test confirmed by western blot.

Cells culture of human lung fibroblast and human pulmonary microvascular endothelial cell (HPMEC), protein extracts, 2D-immunoblot, mass spectrometry and ELISA protocols are described in the appendix. Serum samples were used individually for ELISA, and in pools of three

(or 2 if the number of patients was too low to make a pool of three) according to sample count, sex and PAH status for the 2-D immunoblotting. Finally, in 2D-immunoblot, serum pools were tested as follows: 7 for HIV patients associated with PAH (7 PAH+), 7 for HIV patients without PAH (7 PAH-). Pools of sera from PAH+ patients were compared with pools of sera from PAH- patients, and with a pool of sera from 14 healthy controls as validated in previous studies (Terrier et al., 2008). Antigen targets were identified by rapid separation liquid chromatography combined with a mass spectrometer and interrogation of the SwissProt protein database. For ELISA, human recombinant proteins used were annexin A5 (ANXA5, Tebu-bio SAS, Le Perray en Yvelines, France), 14-3-3 ϵ (14-3-3 ϵ , Tebu-bio), vimentin (Preprotech, Cranbury, New Jersey, USA), elongation factor 2 (EF2, Cloud clone, Katy, Texas, USA). IgG autoantibody reactivity corresponding to a positive detection was defined as an optic density (O.D.) above the threshold of 2 standard deviations over the mean value obtained in healthy controls.

Continuous variables were expressed as median (interquartile range, IQR) and were compared with the Mann-Whitney test, according to the PAH status (PAH+ vs PAH-) or between the overall HIV patients and HCs, or in the IgG antibody reactivity (presence vs absence). Categorical variables, expressed as total and proportion, were compared by the Fisher exact test. Analyses were performed using *R* software, version 4.1.1 (R Foundation; <http://www.R-project.org>, and *Graph-Pad Prism 8* software (La Jolla, CA, USA). All statistical tests were performed at the significance threshold of 5%.

Results

Baseline characteristics

Clinical and hemodynamic characteristics of the 40 HIV patients including 20 PAH+ and 20 PAH– patients are detailed in **Table 1**. No difference was found regarding gender, age, HIV features such as HIV viral load and peripheral CD4⁺ count. In contrast, PAH+ patients had a higher proportion of HCV co-infection (50.0% vs 20.0%), HBV co-infection (30.0% vs 5.0%) and substance addiction (55.0% vs 5.5%, $p < 0.05$ for all). Ninety percent of PAH+ patients were treated with a specific medication of PAH, including 8/18 (44.4%) with a combination therapy.

Immunoblotting of serum IgG reactivities

In 2D-immunoblot, serum IgG from pools of HIV patient sera and healthy controls bound to 119 ± 42 protein spots on membranes of human lung fibroblast extract, and 157 ± 30 protein spots on membranes of HPMEC extract. On fibroblast protein extract, serum IgG from pooled sera bound 119 protein spots for 14 HCs, 149 ± 8 for PAH+, and 95 ± 63 for PAH–. On HPMEC protein extract, serum IgG from pooled sera bound to 190 protein spots for HCs, 172 ± 6 for PAH+, and 125 ± 2 for PAH–. Using mass spectrometry, 13 and 17 protein spots specifically recognized by serum IgG from the PAH+ patients were identified in fibroblast and HPMEC protein extracts, respectively (**Supplemental Table 1**). Annexin A5 (ANXA5), 14-3-3 ϵ , vimentin and elongation factor 2 (EF2) protein spots were recognized by serum IgG from at least two sera pools of PAH+ patients (2/7 pools for each protein spot) and not identified in PAH– patients and HCs. Among protein spots identified by mass spectrometry, ANXA5 and EF2 protein spots were shared by fibroblast and HPMEC protein extracts.

Confirmation by ELISA of serum IgG reactivities

Serum IgG reactivities were tested against ANXA5, 14-3-3 ϵ , vimentin and EF2 recombinant proteins. Anti-ANXA5, anti-14-3-3 ϵ , anti-vimentin and anti-EF2 IgG antibody reactivities were significantly all more frequently detected in HIV patients compared with HCs (**Supplemental Table 2 & Figure 1**). Among HIV patients, IgG autoantibody reactivities against ANXA5 was significantly more frequently detected in PAH+ (11/20, 55.0%) than in PAH- (0/20) ($p < 0.001$). IgG autoantibody reactivities directed against 14-3-3 ϵ was also more frequently detected in PAH+ (7/20, 35.0%) than in PAH- (1/20, 5.0%) ($p = 0.040$). IgG reactivities directed against vimentin and EF2 did not differ significantly between PAH+ and PAH- patients (**Table 2 & Figure 1**).

HIV patients with anti-ANXA5 IgG autoantibodies were older (median 50 IQR [48-55] years) than those without anti-ANXA5 IgG autoantibodies (47 [38-51] years, $p = 0.040$) at the time of blood sampling, without difference in HIV disease duration (13 [10-20] vs 18 [12-25] years, $p = 0.270$). There were no difference in clinical features associated with anti-ANXA5 or anti-14-3-3 ϵ anti-ANXA5 IgG autoantibodies (**Supplemental Table 3**).

Discussion

Using a 2D-immunoblotting technique we identified IgG reactivities directed at human lung fibroblast and pulmonary microvascular endothelial cell protein extracts in patients with HIV-PAH. Among target antigens of these antibodies, ANXA5, 14-3-3 ϵ , Vimentin, and EF2 were identified. Using an ELISA test with human recombinant antigens, we confirmed the positivity of

IgG autoantibody reactivities directed at ANXA5 and 14-3-3 ϵ in HIV-PAH patients significantly more frequently than in HIV patients without PAH.

The pathogenesis of HIV-related PAH is unclear and involve many different parameters including direct pathogenic effects of HIV proteins, substance addiction, chronic inflammation and autoimmunity. Direct infection of the pulmonary artery with HIV has not been documented to our knowledge (Mette et al., 1992), therefore unable to explain an immune reaction triggered by antigen presentation. Some viral proteins, such as the viral protein Gp120, HIV transactivator of transcription (TAT) or protein negative factor (Nef) targets the human lung endothelium, inducing proliferation, activation and apoptosis, and synthesis of inflammatory cytokine (IL-6) and the vasoconstrictive endothelin-1 (Tcherakian et al., 2013). The HIV-related PAH pathogenesis remains unclear and a multi-hits hypothesis is advanced including chronic inflammation associated to the gut CD4⁺ T-cells depletion, inducing lipopolysaccharides translocation and IL-6 overproduction, and immunodepression related to decrease of blood CD4⁺ T-cells. In agreement with the literature (Baker et al., 2011), we did not find any association of the PAH status and HIV viral load or CD4⁺ count. A recent meta-analysis seems to identify a lower CD4⁺ T-cell count, a detectable viral load and the absence of HAART as risk factors associated with PAH in HIV patients (Liu et al., 2022). Our results seem to be in agreement with these data (66.7% vs 29.4%), although they do not reach statistical significance.

Annexin A5 proteins express anti-apoptosis and anti-inflammatory properties, inhibits prothrombin activation and is also implicated in the prevention of vascular remodeling (Ewing et al., 2011). Anti-ANX5 antibodies are encountered in various autoimmune disorders such as antiphospholipid syndrome or systemic sclerosis, and associated in this setting with occurrence

of PAH as in other connective tissue diseases, and counteract the anticoagulant properties of the ANXA5 protein (Rand et al., 1998).

During the course of HIV infection, a progressive decrease in CD4⁺ T-cells is accompanied by dysfunction of the B-cell compartment with the recognition of autoantigens. The chronic underlying immune activation and progressive immune exhaustion leads to the decrease of regulatory CD4⁺ T-cells and the alteration of these self-reactive B-cell control mechanisms (Stahl et al., 2005). The development of autoantibodies targeting ANXA5 and 14-3-3 ϵ during HIV- PAH could be related to these mechanisms. Many autoimmune manifestations are associated with HIV infection, such as immune thrombocytopenia, systemic vasculitis, diffuse immune lymphocytic syndrome, antiphospholipid syndrome or anti-cardiolipin or anti- β 2PG1 autoantibodies reactivity or systemic lupus erythematosus (Zandman-Goddard & Shoenfeld, 2002). In a cohort of HIV patients, 23% tested positive for antinuclear antibody, 17% for antineutrophil cytoplasm antibody (ANCA) or 17% for anti-glomerular basal membrane antibody (Savige et al., 1994). A potential pathogenic mechanism contributing to the occurrence of anti-ANXA5 or 14-3-3 ϵ autoantibody might be molecular mimicry triggered by HIV or autoantigen oxidation alongside the HIV-related chronic inflammation, as described in the systemic sclerosis (Servettaz et al., 2007).

The limitations of our study are the limited number of patients, the lack of knowledge of HAART treatment, but the precise data collected made it possible to find observations described in the literature as the preferential association of PAH in HIV patients with a concomitant addiction to a substance. In contrast, our study displays certain strengths, such as the prospective character of the collection, PAH diagnosis based on right heart catheterization, the use of 2D-

immunoblot on pools of 3 sera already validated (Tamby et al., 2005; Terrier et al., 2008), and the confirmation of suspected reactivities on all the sera in ELISA.

Serum IgG anti-Annexin A5 and anti-14-3-3 ϵ antibody reactivities could represent a potential predictive tool for the occurrence of PAH during HIV infection.

Disclosure

The authors declare no conflict of interest.

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Figures.

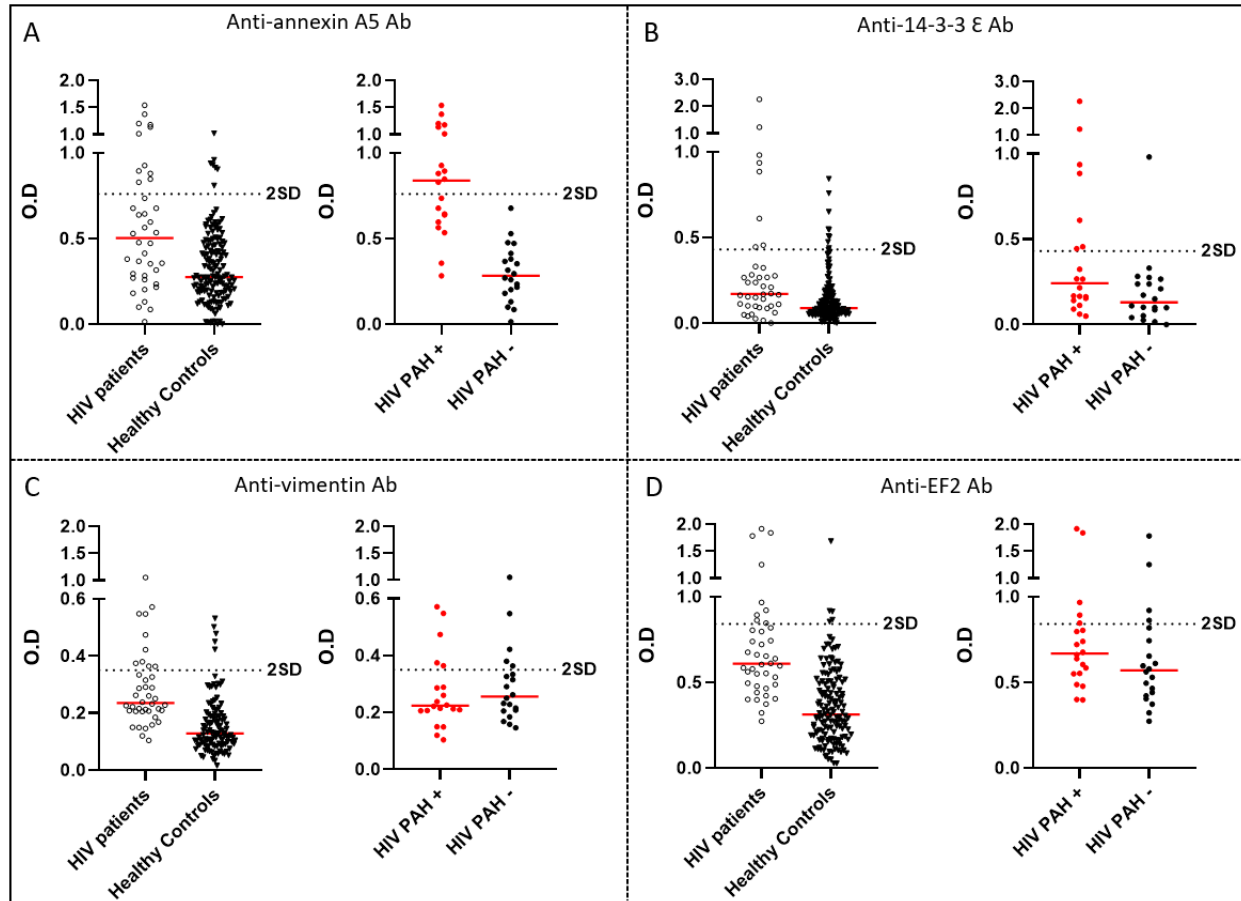
Figure 1: Autoantibody reactivity against Annexin A5, 14-3-3 ϵ , vimentin and elongation factor 2.

Serum IgG reactivities of patients with human immunodeficiency virus (HIV) (○) and healthy controls (▼), and within HIV patients with pulmonary arterial hypertension (PAH+) (●) or without PAH (PAH-) (●), were tested by ELISA against annexin A5 (panel A), 14-3-3 ϵ (panel B), vimentin (panel C), and elongation factor 2 (EF2) (panel D) proteins. Each individual is represented by a single dot. IgG autoantibody reactivity corresponding to a positive detection was defined as an optic density (O.D.) above the threshold of 2 standard deviations over the mean value obtained in healthy controls. Intensity of reactivity is quantified by optical density (O.D) measured in arbitrary units (AU). Antibody (Ab).

Supplemental Figure 1: 2D-Immunoblot of pooled serum IgG from HIV patients with or without PAH with protein extracts of human lung fibroblasts.

Pooled serum IgG (at a 1:100 dilution) from HIV patients without PAH (panel A) and patients with pulmonary arterial hypertension related to HIV infection (panel B) were immunoblotted at 70 μ g of soluble extract of human lung fibroblasts transferred onto 0.45 μ m nitrocellulose membrane after 1D-dimension via immobilized pH gradient IPG strip isoelectrofocalization with 3-11 range isoelectric point pI electrophoresis (horizontally) followed by 2D-electrophoresis on 7 x 9 cm mini-protean TGX stain free gels with acrylamide gradient of 7–18% allowed for studying a wide range of antigens of 10–260 kDa (vertically). Detection of immunoreactivity was performed by incubation with anti-Fc γ chain-specific secondary goat anti-human IgG antibody (at a 1:60,000 dilution) coupled to alkaline phosphatase, and revealed using the NBT/BCIP method.

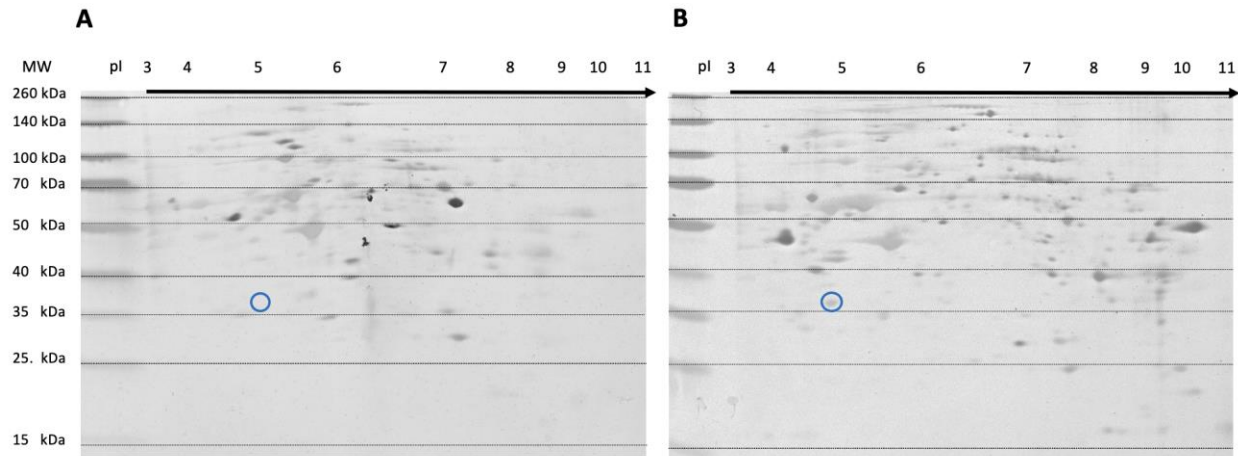
Figure 1: Autoantibody reactivities directed against Annexin A5, 14-3-3 ϵ , vimentin and elongation factor 2.



Legend Fig1.

Serum IgG reactivities of human immunodeficiency virus (HIV) infected patients (o) and healthy controls (\blacktriangledown) (left panel), and within HIV infected patients with pulmonary arterial hypertension (PAH +) (\bullet) or without PAH (PAH -) (\bullet) (right panel), were tested by Enzyme linked immunosorbent assay (ELISA) against annexin A5 (panel A), 14-3-3 ϵ (panel B), vimentin (panel C), and elongation factor 2 (EF2) (panel D) proteins. Each individual is represented by a single dot. IgG autoantibody reactivity corresponding to a positive detection was defined as an optic density (O.D.) above the threshold of 2 standard deviations over the mean value obtained in healthy

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antibody (at a 1:60,000 dilution) coupled to alkaline phosphatase, and revealed using the NBT/BCIP method. The circle marked the geographic region of the spot corresponding to the annexin-A5 protein. The circle (○) highlighted the geographic region of the spot corresponding to the annexin-A5 protein. This spot was absent on the immunoblot incubated with pooled serum IgG from HIV patients without PAH (panel A), but present on the immunoblot incubated with pooled serum IgG from HIV patients with PAH (panel B).

Table 1: Characteristics of HIV infected patients with pulmonary arterial hypertension (PAH+) or without PAH (PAH-)

	n	Overall, N = 40¹	PAH-, N = 20¹	PAH+, N = 20¹	p-value²
Gender	40				0.750
Female		23 (57.5%)	12 (60.0%)	11 (55.0%)	
Age (Y)	40	48 (41-52)	48 (37-51)	49 (43-52)	0.360
PAH disease duration (Y)	20	6 (3-8)	-	6 (3-8)	-
HIV features					
HIV disease duration (Y)	40	16 (10-22)	13 (9-19)	19 (12-24)	0.210
Age at HIV diagnosis (Y)	40	29 (26-38)	27 (23-37)	30 (26-24)	0.660
HIV viral load detectable	20	7/20 (35.0%)	5/17 (29.4%)	2/3 (66.7%)	0.270
HIV viral load, copies/mL	20	0 (0-55)	0 (0-51)	22 (11-2500)	0.320
HIV viral load > 400 copies/mL	20	3/20 (15.0%)	2/17 (11.8%)	1/3 (33.3%)	0.400
CD4 ⁺ count/mm ³	27	636 (388-739)	652 (319-757)	480 (400-702)	0.960
CD4 ⁺ < 200/mm ³	27	3/27 (11.1%)	2/18 (11.1%)	1/9 (11.1%)	0.999
CD4 ⁺ /CD8 ⁺ ratio	20	0.8 (0.6-1.2)	0.8 (0.5-1.2)	1.1 (1.0-1.2)	0.380
Comorbidity					
HBV co-infection	40	7 (17.5%)	1 (5.0%)	6 (30.0%)	0.040
HCV co-infection	40	14 (35.0%)	4 (20.0%)	10 (50.0%)	0.030
Substance addiction	40	28 (70.0%)	17 (5.0%)	11 (55.0%)	0.030
RHC					
mPAP (mmHg)	20	43 (36-49)	-	43 (36-49)	
PCWP (mmHg)	20	7 (5-9)	-	7 (5-9)	
PFTs					
FVC%	20	92 (74-100)	-	92 (74-100)	
DLCO%	13/20	59 (55-68)	-	59 (55-68)	

	n	Overall, N = 40¹	PAH-, N = 20¹	PAH+, N = 20¹	p-value²
TLC%	20	94 (85-105)	-	94 (85-105)	
Treatments					
Oral anticoagulant	40	6 (15.0%)	0 (0%)	6 (30.0%)	0.999
Specific medication of PAH	40	18 (45.0%)	-	18 (90.0%)	
Monotherapy	40	10 (25.0%)	-	10/18 (55.6%)	
Combination therapy	40	8 (20.0%)	-	8/18 (44.4%)	
Epoprostenol	40	2 (5.0%)	-	2/18 (11.1%)	
ERA	40	16 (40.0%)	-	16/18 (88.9%)	
PDE5i	40	7 (17.5%)	-	7/18 (38.9%)	

¹N (%); Quantitative data were expressed as median and interquartile range (IQR); Categorical data were expressed by number / total available and percentage (%).

²Categorical data were compared by Fisher's exact test; Quantitative data were compared by Mann-Whitney test.

Bold correspond to a p-value <0.05.

Antibody (Ab); Transfer coefficient for carbon monoxide in the lung % predicted (DLCO%); Elongation factor 2 (EF2); Enzyme linked immunosorbent assay (ELISA); Endothelin receptor antagonist (ERA); Forced vital capacity % predicted (FVC%), hepatitis B virus (HBV); hepatitis C virus (HCV); human immunodeficiency virus (HIV); number of patients in each subset (N); number of patients with available data (n); mean pulmonary arterial pressure (mPAP), patient with pulmonary arterial hypertension (PAH+) or without PAH (PAH-); Pulmonary capillary wedge pressure (PCWP); Phosphodiesterase-5 inhibitor (PDE5i); Right heart catheterization (RHC); Total lung capacity % predicted (TLC%).

Table 2: IgG autoantibody reactivities directed against Annexin A5, 14-3-3 ϵ , vimentin and elongation factor 2 in human immunodeficiency virus (HIV) patients with (PAH+) and without PAH (PAH-).

	n	Overall, N = 40¹	PAH-, N = 20¹	PAH+, N = 20¹	p- value²
Anti-annexin A5 Ab	40	11 (27.5%)	0 (0%)	11 (55.0%)	<0.001
Anti-14-3-3 ϵ Ab	40	8 (20.0%)	1 (5.0%)	7 (35.0%)	0.040
Anti-EF2 Ab	40	10 (25.0%)	4 (20.0%)	5 (25.0%)	0.999
Anti-vimentin Ab	40	9 (22.5%)	5 (25.0%)	5 (25.0%)	0.999

¹N (%): Categorical data were expressed by number / total available and percentage (%).

²Categorical data were compared by Fisher's exact test.

Positivity of autoantibody was defined by an optic density (O.D.) above 2 standard derivations in healthy controls.

Antibody (Ab); Elongation factor 2 (EF2); number of patients in each subset (N); number of patients with available data (n); presence of pulmonary arterial hypertension (PAH+); absence of PAH (PAH-); Bold correspond to a p-value <0.05.

Supplemental Table 1: Identification by mass spectrometry of proteins from human pulmonary fibroblast (A) and human pulmonary microvascular endothelial cell (B) specifically recognized by serum IgG from HIV infected patients with pulmonary arterial hypertension (PAH+) but not by HIV infected patients without PAH (PAH-) or healthy control.

A- Human fibroblast protein extract

Protein ID on gel	Protein	SwissProt accession number	emPAI score	Theoretical/Estimated MW (kD)	Theoretical/Estimated pI	Num. of significant matches	Num. of significant sequences
1	Heat shock protein 90-alpha	HS90A_HUMAN	5.58	80/84	4.9/4.2	145	50
2	Vimentin	VIME_HUMAN	62.4	55/53	5.0/4.6	668	62
3	14-3-3 protein zeta	1433T_HUMAN	9.53	28/27	4.7/4.3	39	18
4	14-3-3 protein epsilon	1433E_HUMAN	30.55	30/29	4.6/4.4	139	25
5	Elongation factor 2	EF2_HUMAN	3.77	85/95	6.4/5	82	43
6	Annexin A5	ANXA5_HUMAN	29.26	32/35	4.9/5	480	38
7	Prolyl 3-hydroxylase	P3H1_HUMAN	4.22	80/83	5.0/5.2	157	36
8	Annexin A6	ANXA6_HUMAN	4.54	70/75	5.4/5.6	73	38
9	Vinculin	VINC_HUMAN	16.22	100/123	5.5/6.5	384	99
10	Elongation factor 1-gamma	EF1G_HUMAN	5.05	50/50	6.2/6.6	119	26
11	Phosphoglycerate mutase 1	PGAM1_HUMAN	8.14	28/28	6.6/6.6	96	19
12	Alpha-enolase	ENOA_HUMAN	1.48	50/47	7.0/6.7	22	16
13	Aldo-keto reductase family 1 member B1	ALDR_HUMAN	4.96	35/35	6.5/7	95	22

B- Human microvascular endothelial cell protein extract

Protein ID on gel	Protein	SwissProt accession number	emPAI score	Theoretical/Estimated MW (kD)	Theoretical/Estimated pI	Num. of significant matches	Num. of significant sequences
1	60S ribosomal protein L35a	RL35A_HUMAN	1.77	12/15	11.0/11	5	5
2	Superoxide dismutase	SODC_HUMAN	1.66	15/23	5.7/6.1	15	6
3	Dynactin subunit 3	DCTN3_HUMAN	2.06	21/25	5.3/5.6	16	9
4	Chromobox protein homolog 3	CBX3_HUMAN	2.12	20/26	5.2/5.5	13	8
5	Calcyclin-binding protein	CYBP_HUMAN	4.03	26/34	8.2/8.5	32	13
6	Glutathione S-transferase P	GSTP1_HUMAN	3.86	23/28	5.4/6	36	13
7	Triosephosphate isomerase	TPIS_HUMAN	10.96	26/33	6.4/6.8	98	24
8	Peroxiredoxin-6	PRDX6_HUMAN	9.23	25/32	6/7	88	19
9	Annexin A2	ANXA2_HUMAN	2.72	38/37	7.5/7.7	21	18
10	Annexin A5	ANXA5_HUMAN	26.34	35/40	4.9/8.5	65	21
11	Heterogeneous nuclear ribonucleoprotein H3	HNRH3_HUMAN	3.71	36/40	6.3/7	22	10
12	Alpha-enolase	ENOA_HUMAN	1.48	47/50	7.0/9.5	9	9
13	Serpin B6	SPB6_HUMAN	6.53	42/45	5.1/5.7	25	12
14	4-trimethylaminobutyraldehyde dehydrogenase	AL9A1_HUMAN	8.22	53/55	5.6/6.2	31	18
15	Stress-induced-phosphoprotein 1	STIP1_HUMAN	10.5	62/75	6.4/7.2	46	26
16	Far upstream element-binding protein 2	FUBP2_HUMAN	3.22	73/95	6.8/7.6	27	18
17	Elongation factor 2	EF2_HUMAN	5.11	95/100	6.4/7.4	50	28

emPAI = Exponentially modified protein abundance index; ID: identification; kDa = kilodalton; MW = molecular weight; pI = isoelectric point;

Supplemental Table 2: IgG autoantibody reactivities directed against Annexin A5, 14-3-3 ϵ , vimentin and elongation factor 2 in healthy controls and patients with human immunodeficiency virus (HIV).

	n	Overall, N = 190¹	Healthy controls, N = 150¹	HIV, N = 40¹	p- value²
Anti-Annexin A5 Ab	190	18 (9.5%)	7 (4.7%)	11 (27.5%)	<0.001
Anti-14-3-3 ϵ Ab	190	17 (8.9%)	9 (6.0%)	8 (20.0%)	<0.001
Anti-EF 2 Ab	190	14 (7.5%)	5 (3.4%)	10 (25.0%)	<0.001
Anti-Vimentin Ab	190	15 (9.8%)	5 (4.4%)	9 (22.5%)	<0.001

¹N (%): Categorical data were expressed by number / total available and percentage (%).

²Categorical data were compared by Fisher's exact test.

Positivity of autoantibody was defined by an optic density (O.D.) above 2 standard derivations in healthy controls

Antibody (Ab); Elongation factor 2 (EF2); human immunodeficiency virus (HIV); number of patients in each subset (N); number of patients with available data (n); Bold correspond to a p-value <0.05.

Supplemental Table 3: Characteristics of HIV patients according to the IgG autoantibody reactivities against ANXA5 or 14-3-3 ϵ antigens

	n	Anti-ANXA5 autoantibody reactivity			Anti-14-3-3 ϵ autoantibody reactivity		
		Negative, N = 29 ¹	Positive, N = 11 ¹	p-value ²	Negative, N = 32 ¹	Positive, N = 8 ¹	p-value ²
Presence of PAH		9 (31.0%)	11 (100.0%)	<0.001	13 (40.6%)	7 (87.5%)	0.040
Gender	40			0.480			
Female		18 (62.1%)	5 (45.5%)		16 (50.0%)	7 (87.5%)	0.110
Age (Y)	40	47 (38-51)	50 (48-55)	0.040	48 (40-52)	46 (42-50)	0.660
PAH follow-up (Y)	20	7 (4-8)	4 (2-8)	0.490	4 (2-8)	7 (6-9)	0.380
HIV features							
HIV disease duration (Y)	40	13 (10-20)	18 (12-25)	0.270	17 (12-23)	10 (10-14)	0.110
Age at HIV diagnosis (Y)	40	27 (24-29)	36 (31-40)	0.020	30 (26-38)	28 (24-36)	0.490
HIV viral load detectable	20	6/18 (33.3%)	1/2 (50.0%)	0.999	6/18 (33.3%)	1/2 (50.0%)	0.999
HIV viral load, copies/mL	20	0 (0-63)	11 (6-17)	0.320	0 (0-44)	2490 (1245- 3734)	0.510
CD4 ⁺ count, /mm ³	27	636 (346- 739)	586 (454- 770)	0.780	582 (313- 698)	702 (406- 776)	0.380
CD4 ⁺ /CD8 ⁺ ratio	20	0.8 (0.6-1.2)	0.9 (0.9-0.9)	0.860	0.8 (0.6-1.2)	0.6 (0.6-0.6)	0.490
Comorbidity							
HBV co-infection	40	5 (17.2%)	2 (18.1%)	0.999	5 (15.6%)	2 (25.0%)	0.590
HCV co-infection	40	10 (34.5%)	4 (36.4%)	0.999	11 (34.4%)	3 (37.5%)	0.690
Substance addiction	40	7 (24.1%)	3 (27.3%)	0.999	8 (25.0%)	2 (25.0%)	0.999
RHC							

mPAP (mmHg)	20	42 (29-47)	44 (36-50)	0.460	40 (36-45)	46 (32-54)	0.610
PCWP (mmHg)	20	8 (6-9)	7 (5-8)	0.340	7 (5-8)	8 (6-9)	0.470
PFTs							
FVC%	20	98 (92-104)	78 (72-96)	0.260	95 (72-102)	87 (82-97)	>0.999
DLCO%	13/20	59 (55-66)	59 (47-78)	0.999	58 (55-65)	68 (60-78)	0.500
TLC%	20	105 (95-109)	92 (83-97)	0.130	92 (81-100)	104 (104-112)	0.170
ELISA[#]							
Anti-Annexin A5 Ab	40	-	-	-	9 (28.1%)	2 (25.0%)	0.999
Anti-14-3-3 ϵ Ab	40	6 (20.7%)	2 (18.2%)	0.999	-	-	-
Anti-Vimentin Ab	40	7 (24.1%)	31 (27.3%)	0.999	7 (21.9%)	3 (37.5%)	0.390
Anti-EF2 Ab	40	7 (24.1%)	2 (18.2%)	0.999	5 (15.6%)	4 (50.0%)	0.060

¹N (%); Quantitative data were expressed as median and interquartile range (IQR); Categorical data were expressed by number / total available and percentage (%).

²Categorical data were compared by Fisher's exact test; Quantitative data were compared by Mann-Whitney test.

[#]Presence of autoantibody was defined by an optic density (O.D.) above 2 standard derivations in healthy controls.

Bold correspond to a p-value <0.05.

Antibody (Ab); Transfer coefficient for carbon monoxide in the lung % predicted (DLCO%); Elongation factor 2 (EF2); Enzyme linked immunosorbent assay (ELISA); Endothelin receptor antagonist (ERA); Forced vital capacity % predicted (FVC%), hepatitis B virus (HBV); hepatitis C virus (HCV); human immunodeficiency virus (HIV); number of patients in each subset (N); number of patients with available data (n); mean pulmonary arterial pressure (mPAP), pulmonary arterial hypertension (PAH); Pulmonary capillary wedge pressure (PCWP); Phosphodiesterase-5 inhibitor (PDE5i); Right heart catheterization (RHC); Total lung capacity % predicted (TLC%).

Partie III – Discussion générale et perspectives

Les travaux antérieurs du laboratoire ont identifié des IgG anti-fibroblastes (AFA) et des IgG anti-cellules endothéliales vasculaires pulmonaires (AECA) dans l'HTAP idiopathique et associée à la sclérodémie systémique (ScS) (Dib et al., 2012; Tamby et al., 2005, 2006; Terrier et al., 2008). Dans le présent travail nous rapportons la détection d'auto-anticorps dirigés contre des antigènes provenant d'extraits protéiques de fibroblastes et de cellules endothéliales microvasculaires pulmonaires humaines dans les sérums de patients atteints d'HTAP associée à des connectivites telles que le LES ou la CM ou dans l'HTAP associée à l'infection par le VIH. L'identification de ces AFA et AECA dans ces populations d'HTAP associée aux connectivites autres que la ScS et associée au VIH est un résultat intéressant car il s'agit d'HTAP potentiellement réversibles sous traitement. Dans notre travail, nous avons identifié les cibles antigéniques des AFA et AECA spécifiquement associées à l'HTAP. Les 6 antigènes potentiels identifiés étaient l'annexine A5 (ANXA5), la 14-3-3 ϵ , la triosephosphate isomérase, la vimentine, la galectine-1 et le facteur 2 d'élongation.

Tout d'abord, nous avons mis en évidence une association significative entre la réactivité IgG anti-ANXA5 et l'HTAP associée au LES et la CM. Ces auto-anticorps anti-ANXA5 pourraient constituer, en plus des autres facteurs prédictifs identifiés de l'HTAP, un biomarqueur prédictif du développement de l'HTAP chez les patients atteints de ces connectivites (Chaigne et al., 2023; Hachulla et al., 2018). De plus, cette réactivité semble spécifique de ces sous-groupes de connectivites associée à une HTAP (LES et CM), puisqu'elle n'est pas retrouvée dans les HTAP associées à la ScS ou à d'autres pathologies compliquées d'une HTAP comme les cardiopathies congénitales ou les hypertensions porto-pulmonaires. La réactivité IgG anti-ANXA5 a été par ailleurs associée à d'autres paramètres cliniques, tels que le sexe masculin et une dyspnée plus

sévère, ce qui souligne leur potentiel intérêt dans la caractérisation de la sévérité de l'HTAP. Ceci nécessite cependant d'être étudié de manière prospective dans des cohortes de patients atteints de connectives avec ou sans HTAP.

Les annexines sont une famille de protéines se liant aux phospholipides chargés négativement et aux bicouches des membranes. Leurs fonctions ne sont pas totalement élucidées. L'ANXA5 est présente à la surface membranaire des cellules endothéliales humaines (Rand et al., 1997) et possède de puissantes propriétés anticoagulantes liées à son affinité importante pour les phospholipides anioniques membranaires et à sa capacité à déplacer les facteurs de coagulation des surfaces phospholipidiques. Les anticorps anti-phospholipides ont la capacité d'inhiber la liaison entre l'ANXA5 et les phospholipides membranaires, inhibant donc son activité anticoagulante (Ferreira et al., 2020; Rand et al., 1997). Indépendamment de sa fonction antithrombotique, l'ANXA5 a une action anti-inflammatoire et anti-apoptotique (Ewing et al., 2011).

Les anticorps anti-ANXA5 ont été détectés dans plusieurs connectivites comme le LES ou la ScS. Au cours du LES, ils ont été détectés chez 19% des patients et leur détection est associée à la survenue d'événement thrombotique artériels et veineux (Kaburaki et al., 1997; Satoh et al., 1999), à des fausses couches récurrentes ou des morts fœtales in-utero (Kaburaki et al., 1997; Nojima et al., 2001; Sater et al., 2011) ainsi qu'au syndrome des anti-phospholipides (SAPL). Ces anticorps ont des propriétés similaires à l'anticoagulant circulant de type lupique en allongeant le temps de céphaline activé (Nakamura et al., 1995). Les anticorps anti-ANXA5 font partie des auto-anticorps non conventionnels décrits dans le SAPL, dont la prévalence est évaluée à 67% dans les SAPL dit séropositifs (remplissant les critères biologiques avec la présence d'au moins un

anticorps anti-phospholipide parmi l'anticoagulant circulant de type lupique, les anticorps anti-cardiolipine et anti- β -2-glycoprotéine de type I) ; et évaluée à 43% dans les SAPL dits séronégatifs (X. Liu et al., 2022). Il n'a jamais été rapporté d'association entre la réactivité des anticorps anti-ANXA5 et l'HTAP au cours du LES. Au cours de la ScS la détection des anticorps anti-ANXA5 a été rapportée chez environ 15% des patients ScS. Dans la ScS, leur détection a été identifiée comme étant associée à la survenue d'ischémie digitale (Horimoto et al., 2020; Sugiura & Muro, 1999). Dans cette petite étude observationnelle portant sur 70 patients ScS, l'identification d'IgG anti-ANXA5 était aussi associée à la détection d'une HTAP (27.3% versus 6.8%, $p=0.048$) (Horimoto et al., 2020).

D'autres mécanismes contribuant à l'apparition d'anticorps anti-ANXA5 pourraient être discutés, parmi lesquels le mimétisme moléculaire avec un agent infectieux et/ou l'oxydation de l'auto-antigène par des radicaux libres, comme cela a été rapporté dans la ScS (Servettaz et al., 2009). Bien que les mécanismes conduisant à l'apparition des anticorps anti-ANXA5 n'aient pas été complètement élucidés, il a été proposé que dans le contexte d'une apoptose accrue que l'ANXA5 extracellulaire/membranaire ou des corps apoptotiques pourrait constituer un stimulus antigénique et entraîner la production d'anticorps anti-ANXA5 sans que cela préjuge d'un éventuel rôle pathogène.

Dans les expériences que nous avons menées, nous n'avons pas identifié d'effet pathogène des IgG anti-ANXA5. Plusieurs explications à cela sont possibles, parmi lesquelles une altération de la réactivité des IgG liée à la technique de purification utilisée, comme une modification du site de reconnaissance de l'épitope sur les régions variables des IgG en pH acide. Dans des travaux antérieurs une différence de réactivité des immunoglobulines humaines polyvalentes (IgIV)

purifiées a été observée en fonction du protocole d'enrichissement de fraction F(ab')₂ (Bruley-Rosset et al., 2003). Cela a été aussi documenté au cours de l'infection VIH, avec un répertoire des réactivités IgM et IgG modifié par rapport aux individus sains, et entre les patients VIH avec une taux de CD4⁺ faible versus ceux avec un taux de CD4⁺ élevé (Stahl et al., 2005). Une autre explication pourrait être que les patients avaient pour un certain nombre d'entre eux déjà des traitements en particulier des corticoïdes et/ou un immunosuppresseur qui peuvent diminuer la réactivité des autoanticorps. Ainsi, environ 50% de nos patients étaient sous corticoïdes au moment du prélèvement, mais il y avait autant de patients sous corticoïdes parmi les patients ayant des IgG anti-ANXA5 et ceux n'en ayant pas. De la même manière, il y avait autant de patients sous immunosuppresseurs parmi les patients ayant des IgG anti-ANXA5 et ceux n'en ayant pas. Enfin, aucun patient ayant une réactivité anti-ANXA5 n'avait un SAPL associé.

Le test de fibrinofomation que nous avons utilisé n'est peut-être pas le bon test de coagulation pour explorer le rôle pathogène des IgG anti-ANXA5. Ce test *in vitro* est facile à mettre en place pour tester l'impact des IgG purifiées sur la coagulation. Certaines études mesuraient l'incorporation de l'ANXA5 dans les bicouches phospholipides sur des cultures cellulaires trophoblastiques avec mesure de temps de coagulation par ellipsométrie (Rand et al., 1997). Malheureusement cette technique n'est plus disponible sur la plateforme d'Hématologie Biologique. Bien que le rôle pathogène direct des IgG anti-ANXA5 sur le temps de fibrinofomation n'ait pas été démontré, ces auto-anticorps pourraient jouer un rôle pathogène dans la survenue de l'HTAP via d'autres mécanismes comme l'inflammation, la prolifération ou l'activation des fibroblastes ou des cellules endothéliales, ou la transition endothelio-mesenchymateuse par analogie à d'autres situations cliniques impliquant le système immunitaire

adaptatif comme le rejet de transplantation rénale médié par les anticorps (Louis et al., 2019; Ranchoux et al., 2015). Enfin, il est possible que rôle le pathogène des IgG anti-ANXA5 soit le fait d'IgM via des anticorps naturels et non d'IgG. La détection des IgG anti-ANXA5 constitue tout de même un potentiel marqueur biologique diagnostique/pronostique de la survenue d'une HTAP.

Ces résultats nous invitent à poursuivre nos expériences et à identifier de nouvelles hypothèses quant aux mécanismes sous-jacents et les facteurs potentiels influençant la fibrinofomation, en tenant compte des complexités de cette réaction biologique. D'autres variables ou composantes pourraient jouer un rôle prépondérant dans le processus de fibrinofomation, qui n'ont pas été prises en compte dans nos expériences et correspondent à de potentiels cofacteurs essentiels des auto-anticorps identifiés. Concernant la différence de résultats entre les deux séries de temps de fibrinofomation testant les IgG purifiées du patients test avec HTAP et IgG anti-ANXA5 ayant fait la démonstration de l'effet dose-réponse, plusieurs hypothèses ont été soulevées dont une variable non identifiée relative à la technique, au plasma de sujet sain utilisé, mais surtout le fait que ce patient a dû être reprélevé entre les deux séries de manipulations car les expériences de calibration avaient consommé la quasi-totalité des IgG purifiées de cet échantillon. Ce patient a été mis sous immunosuppresseur (mycophénolate mofétil) entre les deux recueils de sérums. Cela a pu influencer la quantité des IgG anti-ANXA5 présentes dans son sérum. Il serait intéressant de tester à nouveau le sérum du deuxième prélèvement en ELISA pour la réactivité IgG anti-ANXA5.

Nous avons identifié par ailleurs des IgG anti-14-3-3 ϵ chez les patients ayant une HTAP associée au LES. Cette association soulève la question du rôle potentiel de ces auto-anticorps dans le développement d'une HTAP et de leur potentiel rôle prédictif sur la survenue d'une HTAP. Les

protéines 14-3-3 représentent un groupe de protéines impliquées dans la régulation de l'inflammation, comme les voies IKK/NFκB ou JAK/STAT (Munier et al., 2021, pp. 14-3-3). Ces protéines sont également présentes dans l'espace extracellulaire (Kaplan et al., 2017). Au cours de l'apoptose, les protéines 14-3-3 sont libérées dans l'espace extra-cellulaire et la circulation, ce qui peut contribuer à expliquer leur immunogénicité. Une autre hypothèse est qu'un certain nombre de protéines cytoplasmiques pourraient être exprimées à la surface des cellules, en faveur de la prolifération cellulaire et du remodelage vasculaire, et devenir immunogènes. Ici aussi nous pouvons faire l'hypothèse d'un mimétisme moléculaire avec un agent infectieux ou par oxydation d'auto-antigène comme les protéines 14-3-3. Les autres cibles antigéniques identifiées mais dont la réactivité n'a pas été confirmée comme étant spécifiquement associée à l'HTAP par ELISA comprenaient notamment la triosephosphate isomérase, la vimentine, la galectine-1 ou le facteur d'élongation 2. La synthèse de triosephosphate isomérase ou du facteur d'élongation 2 est augmentée dans les cellules endothéliales sous l'effet de l'hypoxie (Brea et al., 2011; Yamaji et al., 2004). Les mécanismes rendant ces protéines immunogènes sont encore inconnus. Les vimentines sont des filaments intermédiaires de classe III présents dans diverses cellules non épithéliales, mais fortement exprimés dans les fibroblastes et les myofibroblastes, et impliqués dans la stabilisation des ARNm du collagène de type I. L'augmentation de leur synthèse a été observée dans la peau sclérosée de patients sclérodermiques (Corallo et al., 2017). La réactivité IgG anti-14-3-3 ϵ était associée à d'autres auto-anticorps dirigés contre des cibles antigéniques telles que la triosephosphate isomérase, la vimentine et l'élongation facteur 2, suggérant des interactions complexes et nécessitant des études complémentaires. Les fonctions physiologiques de ces protéines et leur rôle dans la machinerie moléculaire de plusieurs cellules

impliquées dans la physiopathologie de l'HTAP tel que la cellule endothéliale ou le fibroblaste ou l'apoptose pourraient suggérer leur implication dans les mécanismes de remodelage vasculaire rencontrés dans l'HTAP, mais cela reste à ce stade très hypothétique, d'autant plus que nous n'avons pas observé de différence de réactivité des auto-anticorps pour ces cibles antigéniques entre les sérums des patients atteints d'HTAP et ceux des patients sans HTAP.

La littérature sur l'effet pathogène des auto-anticorps anti-cellules endothéliales dans les connectivites est limitée et contradictoire (Becker et al., 2014; Dib et al., 2012; Tamby et al., 2005). Il a été mis en évidence que la détection d'anticorps anti-récepteur A de l'endotheline-1 (ET_AR) et de l'angiotensine-1 (AT₁R) dans des sérums de patients HTAP-ScS et des HTAP associées aux autres connectivites était plus fréquente comparée aux patients avec une HTAPI, une HTAP associée à une cardiopathie congénitale ou une HTP chronique thromboembolique (Becker et al., 2014). Ces auto-anticorps ont un effet agoniste en augmentant les capacités de vasoconstriction artériolaire pulmonaire. Dans un modèle murin, le transfert des IgG purifiées des patients ayant une HTAP et des auto-anticorps anti-ET_AR et anti-AT₁R chez des souris saines induit une artériolopathie pulmonaire (Becker et al., 2014; Dib et al., 2012; Tamby et al., 2005). Par ailleurs, des AECA identifiés chez des patients atteints de néphrite lupique ont la capacité à induire l'apoptose des cellules endothéliales, contrairement à des AECA identifiés chez des patients atteints de ScS ou d'HTAPI. Les cibles antigéniques de ces auto-anticorps n'ont pas été identifiées (Arends, Damoiseaux, Duijvestijn, Debrus-Palmans, Vroomen, et al., 2013). La pléiotropie des potentiels effets pathogènes de ces auto-anticorps dépend de leurs cibles antigéniques. Par ailleurs, la capacité des auto-anticorps anti-U1-ribonucléoprotéine (U1-RNP) à activer des cellules endothéliales artérielles pulmonaires humaines via l'induction de la synthèse de plusieurs

molécules d'adhésion a été identifiée (Okawa-Takatsuji et al., 1999). Un tel phénomène a également été identifié en incubant des IgG purifiées issues de patients avec une CM sur des cellules endothéliales de veine ombilicale humaine normale (HUVEC) (Bodolay et al., 2004).

Nous avons également identifié des IgG anti-ANXA5 et anti-14-3-3 ϵ dans l'HTAP associée à l'infection par le VIH. Cette association élargit notre compréhension de l'implication du système immunitaire dans la physiopathologie de cette forme d'HTAP indépendamment des maladies auto-immunes systémiques. Nous n'avons pas mis en évidence d'association significative entre l'HTAP et le taux de CD4⁺ ou la charge virale VIH, contrairement à certaines données de la littérature (Y. Liu et al., 2022). Ce résultat peut être lié à la petite taille de notre effectif et donc à un manque de puissance. L'absence de recueil dans notre base de données d'un traitement par antirétroviral hautement actif (HAART) est aussi une limite à l'interprétation de nos résultats. L'analyse de l'association entre la réactivité des auto-anticorps et la prescription d'un HAART serait intéressante à étudier. Les manifestations auto-immunes sont fréquentes au cours de l'infection par le VIH avec des incidences variables en fonction du stade de l'infection. Elles sont fréquentes au stade d'infection aiguë (stade I, charge virale élevée et une immunité quasi intacte) et au stade de reconstitution immunitaire (stade IV, sous traitement antirétroviral avec une ré augmentation voir normalisation du taux de CD4⁺) (Vega & Espinoza, 2018). Au cours de l'infection par le VIH il existe initialement une cascade inflammatoire aiguë puis une activation immunitaire soutenue avec une diminution progressive des lymphocytes T CD4⁺, une activation polyclonale de lymphocytes T CD4⁺ stimulés par des superantigènes VIH, une inactivation des lymphocytes T régulateurs, une activation polyclonale des lymphocytes B, une hypergammaglobulinémie et la

formation d'auto-anticorps et de complexes immuns (Zandman-Goddard & Shoenfeld, 2002). Au cours de la phase de reconstitution immunitaire, le taux des lymphocytes T CD4⁺ augmente, mais avec la persistance d'un défaut fonctionnel de plusieurs sous-populations (Ascher & Sheppard, 1988; Lange & Lederman, 2003). Les manifestations auto-immunes les plus fréquemment rencontrées au cours de l'infection par le VIH sont les cytopénies auto-immunes, les arthrites séronégatives, le psoriasis, les myopathies inflammatoires, le syndrome d'infiltration lymphocytaire diffus (DILS), les granulomatoses sarcoïdose-like, ou le syndrome de Guillain Barré. Les mécanismes sous-jacents à la production d'auto-anticorps au cours du VIH comprennent le mimétisme moléculaire entre plusieurs épitopes du virus et des protéines de la cellule de l'hôte, comme c'est le cas entre la glycoprotéine GP2b3a des plaquettes et la GP120 du VIH (Bettaieb et al., 1996), ou comme avec un certain nombre de molécules impliquées dans la tolérance du soi (HLA-DR4, HLA-DR2, régions variables des chaînes α/β ou gamma du TCR) (Silvestris et al., 1995) ; ainsi que la stimulation antigénique persistante des lymphocytes B mémoires (Bernasconi et al., 2002). Il existe une certaine homologie entre ces mécanismes auto-immun au cours de l'infection par le VIH et ceux rencontrés dans la physiopathologie de l'HTAP (Hautefort et al., 2015; Huertas et al., 2016). Les données sont cependant discordantes quant au rôle des traitements antirétroviraux comme facteur protecteur de la survenue d'une HTAP chez les patients infectés par le VIH.

Notre travail comporte un certain nombre de limites. Nous avons collecté un grand nombre de sérums de patients avec et sans HTAP, regroupant de nombreuses maladies associées à l'HTAP correspondant un à nombre important de sérums. Mais pris indépendamment, les effectifs des

sous-groupes de maladies sont assez limités du fait de la rareté des pathologies étudiées (maladies auto-immunes systémiques rares et HTAP associée au VIH). Par ailleurs, les patients n'étaient pas des patients incidents mais des patients prévalents, prélevés à des moments différents de la prise en charge de leur HTAP. Ainsi, un certain nombre de patients avaient des traitements, en particulier des corticoïdes et/ou un immunosuppresseur qui peuvent diminuer la réactivité des autoanticorps. Cela a probablement un impact sur le taux des IgG étudiées et donc les résultats de réactivité des AFA et AECA. Cependant, si 50% de nos patients étaient sous corticoïdes au moment du prélèvement, il n'a pas été observé de différence entre les patients avec une réactivité anti-ANXA5 et les patients qui n'en avaient pas. De la même manière, il y avait autant de patients sous immunosuppresseurs parmi les patients ayant des IgG anti-ANXA5 et ceux sans IgG anti-ANXA5. Enfin, aucun patient ayant des IgG anti-ANXA5 n'avait un SAPL associé.

Par ailleurs, il est important d'aborder les techniques utilisées. Dans nos expériences, nous avons utilisé des techniques d'immunoblot en une (1D) et en deux dimensions (2D), ainsi que des tests ELISA sur protéines recombinantes humaines.

Nous avons tout d'abord étudié la présence d'auto-anticorps anti-fibroblastes (AFA) et anti-cellules endothéliales vasculaires pulmonaires (AECA) en immunoblot-1D. Nous n'avons pas étudié la réactivité de ces AFA ou AECA par d'autres approches comme l'ELISA cellulaire, l'immunofluorescence indirecte (IFI) ou la cytométrie en flux. Les travaux antérieurs de notre équipe ont étudié la réactivité des AECA sur des HUVEC non perméabilisées (Dib et al., 2012) ou la réactivité des auto-anticorps anti-cellules musculaires lisses vasculaires perméabilisées (Bussone et al., 2012). Nous n'avons pas effectué de tests d'immunofluorescence indirecte sur des cellules en culture en incubant les sérums de patients atteints d'HTAP associée aux

connectivites autres que la ScS ou associée au VIH afin de confirmer la spécificité de la cible cellulaire de ces autoanticorps.

Nous avons utilisé l'immunoblot-1D comme un outil de dépistage des AFA et AECA. L'immunoblot-1D n'est pas une technique avec un fort potentiel de discrimination antigénique du fait de la présence possible d'un très grand nombre de protéines dans les bandes de réactivité identifiées (Tamby et al., 2005). Du fait du très grand nombre de sérums à tester et de la nécessité de constituer des groupes les plus homogènes possibles de sérums, nous avons utilisé une technique d'immunoblot-1D afin d'identifier les sérums ayant de potentielles réactivités associées à une HTAP. Cela a permis de constituer les pools de sérums à tester en immunoblot-2D.

L'utilisation de pools de sérums peut être une limite de notre travail, car cela peut induire des faux négatifs de réactivité spécifique relative à un individu du pool de trois sérums. Il nous a semblé très difficile de faire un immunoblot-2D pour chaque sérum de patient, compte tenu de la complexité de l'approche technique et du nombre de sérum à tester. Ce risque est assumé par l'objectif de ne pas s'attacher à des réactivités mineures mais de se concentrer sur les réactivités majeures. Cette technique des pools de sérums a été utilisée et validée antérieurement dans des travaux chez des patients atteints d'HTAP idiopathique ou associée à la ScS (Bussone et al., 2012; Dib et al., 2012; Terrier et al., 2008). Les expériences en immunoblot-2D augmentent le potentiel de discrimination protéique (résolution) et correspondaient à une étape de choix pour l'identification des cibles antigéniques. Elle permet de tester un très grand nombre d'antigènes potentiels issus d'un extrait protéique avec une forte résolution grâce à la séparation protéique en fonction du point isoélectrique (1^{ère} dimension), et du poids moléculaire (2^{ème} dimension).

L'innovation principale par rapport aux travaux antérieurs du laboratoire a été le recours à des petits gels stain-free industriels bio-Rad 8.6 × 6.7 cm 8-16 % de gradient permettant une concentration des protéines testées issues de l'extrait sur une surface géographique plus faible par rapport aux grands gels « fait maison » antérieurs. En effet, dans tous les travaux effectués antérieurement dans le laboratoire utilisant une approche d'immunoblot-2D, nous avons utilisé des grands gels. Dans le présent travail, le caractère industriel des gels et leur taille offrent un gain de reproductibilité entre les expériences, permet une économie de matériel biologique (quantité d'extrait protéique, volume de sérum nécessaire), un gain de temps de manipulation et une réduction de manipulations intermédiaires (colorations) ce qui diminue le risque de contamination par des protéines non issues de l'extrait protéique testé. Ces gels permettent de tester un nombre de sérums plus important tout en offrant un gain de temps en termes de manipulation. Par ailleurs leur propriété « *stain-free* » permettait un contrôle qualité en scannant à chaque étape via un imageur, et de supprimer une étape de vérification de migration par technique de coloration au bleu de Coomassie ajoutant des manipulations supplémentaires. Ces gels ont par ailleurs une très grande résolution des spots et ne nécessitent pas de logiciel d'ajustement spatial pour l'utilisation discriminante que nous en avons. Son principal inconvénient, que les gels soient grands ou petits, est la dénaturation d'un certain nombre de protéines comme les protéines membranaires, conséquence de l'utilisation d'agents dénaturants comme l'urée ou le SDS (sodium dodécyl sulfate), dénaturant un grand nombre de protéines, ainsi que leur structure tridimensionnelle important dans la reconnaissance de l'épitope par les régions hypervariables des IgG. On ne peut pas méconnaître une potentielle concentration accentuée des protéines du fait de la taille réduite du gel, et donc un regroupement de plusieurs

protéines sur une même zone géographique identifiées par la spectrométrie de masse. Concernant la technique de spectrométrie de masse, les technologies modernes possèdent un très haut pouvoir d'identification protéique. Cette identification basée sur l'analyse spectrale est assistée par support informatique après interrogation des bases de données protéiques exhaustives. Une des limites de cette technique dans notre problématique était la multitude de protéines candidates correspondant à de potentiels antigènes cibles. Ceci s'explique par le haut potentiel discriminant relatif aux performances de la technologie, mais aussi par le piquage dans le gel sur une zone géographique restreinte sur laquelle plusieurs protéines peuvent avoir migré. Des assistances informatiques existent, tel que l'utilisation de l'indice emPAI, qui représente un score fournissant une valeur indicative de l'abondance comparative des protéines identifiées. Nous avons par ailleurs identifié les antigènes ayant un potentiel rationnel physiopathologique avant de les tester en ELISA après revue de la littérature. Nous avons fait le choix de tester en ELISA les antigènes identifiés et partagés entre plusieurs groupes d'HTAP associée aux connectivites et/ou à l'infection par le VIH, afin d'identifier de potentiel biomarqueurs utiles en pratique clinique. Les ELISA étaient réalisés via des techniques que nous avons mises au point à façon sur protéines recombinantes.

Un certain nombre de perspectives découlent de ce travail. Tout d'abord, afin de renforcer la validité externe de nos résultats, il nous semble essentiel de tester la réactivité IgG anti-ANXA5 sur des cohortes de répliation de patients atteints de LES, de CM ou d'infection par le VIH mieux caractérisés concernant les différentes lignes thérapeutiques reçues. Par ailleurs, une étude prospective sur patients incidents avec un suivi longitudinal serait le design optimal pour juger du

caractère pronostique de l'identification d'une réactivité anti-ANXA5 au début de la maladie sur le développement futur d'une HTAP au cours du suivi des patients. Ces études permettraient de définir la performance prédictive en termes de valeur prédictive négative et de sensibilité de la réactivité de ces auto-anticorps. Par ailleurs, le suivi longitudinal de cette réactivité anti-ANXA5 serait aussi intéressant afin de savoir s'il existe une corrélation temporelle entre la détection de ces auto-anticorps et l'identification de l'HTAP au cours du suivi, ou de sa sévérité. Par ailleurs, l'étude des réseaux protéiques connus impliqués dans l'HTAP via des techniques de protéomique pourrait permettre d'identifier des partenaires protéiques de l'ANXA5 et de mieux appréhender l'interaction entre la détection de ses auto-anticorps et les processus pathophysiologiques connus dans l'HTAP. L'étude de la détection d'anticorps anti-cellules musculaires lisses vasculaires et l'identification de leurs cibles antigéniques dans ses populations de connectivites et d'infection par le VIH n'a pas été réalisée dans ce travail à cause du temps disponible limité pour l'ensemble des expériences (immunoblot 1 et 2D, ELISA dédiée aux cibles antigéniques identifiées). Il nous paraît important que ces analyses puissent être réalisées afin de compléter les données concernant une autre cellule vasculaire impliquée dans la physiopathologie de l'HTAP.

Pour documenter le rôle pathogénique de ces auto-anticorps, on pourrait envisager d'incuber des cultures de fibroblastes et de cellules endothéliales vasculaires pulmonaires avec des IgG purifiées de patients avec HTAP et avec IgG anti-ANXA5 et/ou anti-14-3-3 ϵ pour étudier leur potentiel rôle dans l'activation de la cellule endothéliale (augmentation de synthèse de protéines d'adhésion telles que VCAM-1 ou ICAM) (Arends, Damoiseaux, Duijvestijn, Debrus-Palmans, Vroomen, et al., 2013; Blum et al., 2018), et l'acquisition de marqueurs de transition endothelio-mésenchymateuse comme la perte de marqueurs endothéliaux (CD31, VE-Cadhérine) et à

contrario la synthèse du marqueur α -smooth muscle actine (α -SMA) ou la phospho-vimentine (Ranchoux et al., 2015). Ces mécanismes étant en partie médiés par le facteur de transcription Twist-1, son activité pourra être mesurée par reverse transcriptase PCR quantitative (qRT-PCR). Le TGF- β étant un facteur de croissance impliqué dans la physiopathologie de l'HTAP et stimulant la transition endothelio-mésenchymateuse, il pourra être utilisé comme contrôle positif (Díez et al., 2010). Un immunosuppresseur comme le sirolimus pourra être utilisé comme contrôle négatif (Gao et al., 2011; Ranchoux et al., 2015). Nous pourrions envisager de purifier les IgG sur colonne de chromatographie anti-ANXA5 et/ou l'étude spécifique de l'interaction anticorps-antigène via l'étude spécifique des fragments F(ab')₂ pour accroître la spécificité du mécanisme étudié.

En conclusion, ce travail de recherche apporte un certain nombre d'éléments nouveaux dans l'implication d'auto-anticorps anti-fibroblastes et anti-cellules endothéliales vasculaires pulmonaires associés à l'HTAP associée au LES, à la CM et à l'infection par le VIH. Ces auto-anticorps pourraient avoir un intérêt pronostique pour identifier les patients à risque de développer l'HTAP, ouvrant la voie à des stratégies de dépistage et de traitement précoces dans cette maladie au pronostic sombre. Des recherches additionnelles sont nécessaires pour élucider les mécanismes sous-jacents et déterminer leur rôle précis dans le développement de l'HTAP.

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Annexe 1 : Physiopathologie de la Sclérodermie systémique



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Quarterly Medical Review - Systemic Sclerosis
Pathophysiology of systemic sclerosis



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SUMMARY

Systemic sclerosis (SSc) is a rare connective tissue disease characterized by vascular remodeling, fibroblast activation and extra-cellular matrix production in excess and autoimmunity.

Environmental factors including mainly silica and solvents have been assumed to contribute to the development of SSc, together with genetic factors including gene variants implicated in innate immunity such as IRF5 and STAT4, and epigenetic factors including histone post-translational modifications, DNA hypomethylation, and microRNAs or long- non coding RNAs system were reported to participate in immune activation and fibrosis processes in patients with SSc.

A number of animal models of SSc have been set up over the years, including genetic and induced SSc models. These models, together with data obtained from human SSc patients, contributed to better understand the mechanisms contributing to vasculopathy and fibrosis.

Alongside the pathophysiological process of SSc, several cellular and molecular actors are involved, such as dysregulations in the innate and adaptive immune cells, of the fibroblast, the implication of pro-inflammatory and pro-fibrosing signaling pathways such as the *Wnt*, *TGF-β* pathways or other cytokines, with a strong imprint of oxidative stress. The whole lead to the overactivity of the fibroblast with genetic dysregulation, apoptosis defect, hyperproduction of elements of extracellular matrix, and finally the phenomena of vasculopathy and fibrosis.

These advances contribute to open new therapeutic areas through the design of biologics and small molecules.

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1. Introduction

Systemic sclerosis (SSc) is a rare connective tissue disease characterized by vascular hyperactivity and remodeling, fibroblast activation and extra-cellular matrix synthesis leading to skin and organs fibrosis, and immune system dysfunction with autoimmunity hallmarks [1].

In recent years, thanks to the contribution of new experimental models and molecular biology technologies in the fields of genomic, epigenetic, transcriptomic, proteomic and metabolomic from human and animal model samples, significant progress has been made in the understanding of mechanisms contributing to the occurrence of vasculopathy and fibrosis.

In this review article, we will provide the reader with an overview of the pathogenesis of SSc, with emphasis of recent acquisitions in the field.

2. Environment, genetics, and epigenetics

2.1. Environmental factors

Many environmental factors have been assumed or identified to contribute to the development of SSc. There are 5 meta-analyses on the link between occupational exposure and SSc [2–6]. Regarding silica exposure, 15 case-control studies (overall Odds Ratio (OR) 2.81 [95%CI 1.86–4.23]; $p < 0.001$) and 4 cohort studies (overall RR 17.52 [5.98–51.37]; $p < 0.001$) reported an over risk [2] whereas for the solvents exposure, 13 case-control studies reported a positive association (overall OR 2.00 [1.32–3.02]; $p = 0.001$) [2] which concerns mostly aromatic solvents (OR 2.72 [1.21–6.09]), trichloroethylene (OR 2.07 [1.34–3.17]), halogenated solvents (OR 1.49 [1.12–1.99]), and ketones (OR 4.20 [2.19–8.06]) [6] and epoxy resins (overall OR 2.97 [2.31–3.83]; $p < 0.001$) [2]. The exposure to silica is more frequent in male gender, and they present more frequently diffuse subset, anti-topoisomerase antibodies (ATA) and interstitial lung disease

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(ILD) [7], whereas exposure to aromatic solvents and ketones is preferentially encountered in females. Elements currently identified as contributing to the pathophysiology of systemic sclerosis are illustrated in the Figure 1.

In addition, the existence of clustered cases of SSc suggested a role of infectious disease in SSc onset, particularly *Parvovirus B19* (PVB19) and *Cytomegalovirus* (CMV). Notably, Lunardi et al. have reported the presence of a “SSc peptide” recognized by serum immunoglobulin from 93% of patients with SSc. Interestingly, this peptide shows an important homology with UL94, a CMV late protein that can be recognized by purified IgG from SSc patients [8].

2.2. Genetics

Family studies in SSc patients have led to establish a genetic predisposition to the disease [9–11]. Functional gene analysis of fibroblasts of twins SSc patients showed that autologous serum stimulation led to the same genetic cellular pattern [12]. Following these first translational studies in SSc genetic, genome-wide association studies (GWAS) revealed the presence of polymorphisms associated to SSc. Candidates genes have been reported by Chairta et al. in 2017 who showed a dual division between HLA locus genes and non HLA locus genes. A functional analysis of these genes also showed the importance of chemokines, cytokines, interleukins and integrins in SSc thus reinforcing the role and the link between genetic and immune system [13]. More recently, an international GWAS study (including 4436 SSc patients and 14,751 controls) showed a significant association between SSc and the single nuclear polymorphisms (SNPs) GSDMA $p = 1.4 \times 10^{-10}$ and PRDM1 $p = 6.6 \times 10^{-10}$ which are two loci associated with the immune response [14]. Many recent studies have highlighted other gene variants implicated in innate immunity such as IRF5, IRF7 and TLR2, or in T and B cells activation including CD247, TNFAIP3, STAT4 and BLK or NF- κ B pathway [15].

2.3. Epigenetic insights

Histone post-translational modifications, DNA hypomethylation, and microRNAs or long- non coding RNAs system were reported to participate in immune activation and fibrosis processes in patients with SSc (Figure 1).

Histone post-translational modifications have been reported such as histone deacetylase genes HDAC4 and HDAC5, which represent a repressive mark of genes access, and which are upregulated in endothelial cells from SSc patients, and conversely, HDAC6 is down-regulated [16]. Expression of Krüppel-like factor 5 (*KLF5*) and Friend leukemia integration 1 (*FLI1*) transcription factors, two repressors of type I collagen gene, connective tissue growth factor (CTGF) and Transforming Growth Factor- β (TGF- β) pathways, is downregulated in SSc skin [17].

Hypomethylation of DNA in fibroblasts as well as in immune cells (CD4⁺ and CD8⁺ T-cells) from SSc patients has been reported, that contribute to drive activation of many genes implicated in SSc pathogenesis (*COL23A1*, *COL4A2* . . .) [18,19]. Several proteins are implicated in these DNA methylation alteration, such as a decrease of DNA (cytosine-5)-methyltransferase-1 (DNMT1) in fibroblasts or T-cells [20,21], or an upregulation of ten-eleven translocation methylcytosine dioxygenase (TET) enzymes in dermal fibroblasts from SSc patients [22] or bleomycin (BLM)-induced mouse model, which regulate transcriptional activation.

Non-coding RNAs were recently explored in the field of SSc pathogenesis [23]. Several of micro (mi)RNAs identified in immune cells, fibroblasts or in serum from SSc patients are upregulated like miR-618 targeting IRF8 mRNA in plasmacytoid dendritic cells (pDCs), which reduced their development and enhanced IFN- α release upon TLR9 stimulation [24]. Others, targeting matrix metalloproteinase 1 (MMP1), PPAR- γ or type I/IV collagen, induce a fibroblast-related profibrotic phenotype (miR-203-3p, miR-155, miR-130b, miR-483-5p, etc.) [25–28]. Further investigation is needed in this field.

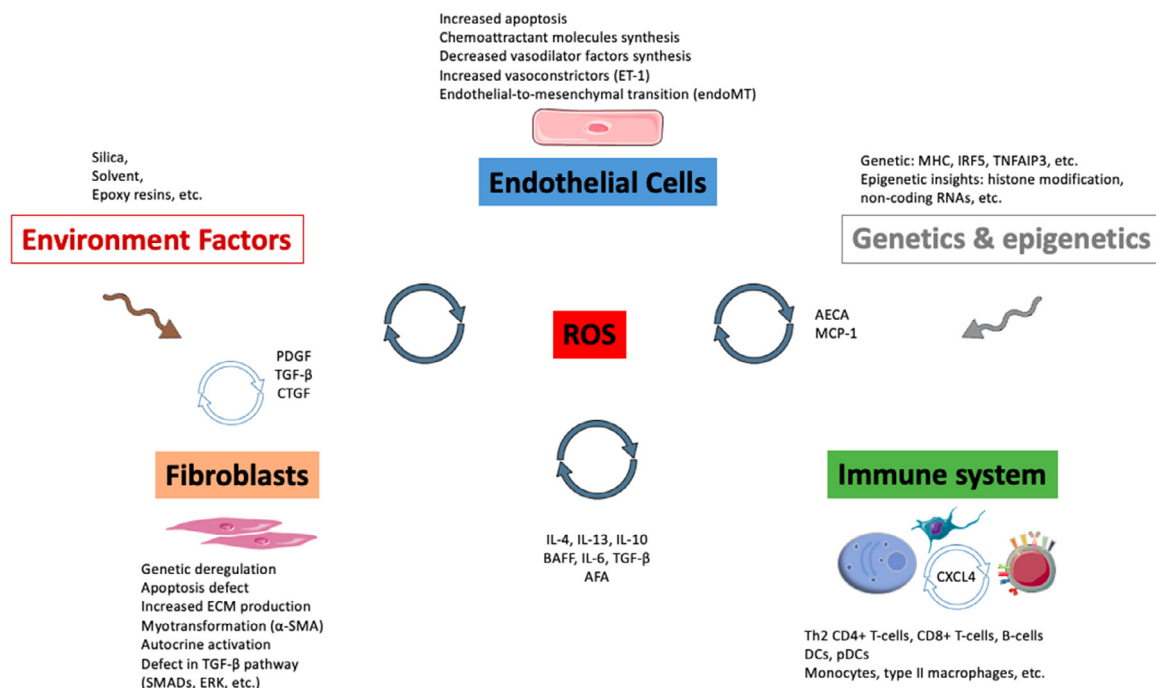


Fig. 1. Elements currently identified as contributing to the pathophysiology of systemic sclerosis. Legend: AECA: anti-endothelial cell antibodies; AFA: anti-fibroblast antibodies; BAFF: B-cell activating factor; CTGF: connective tissue growth factor; CXCL4: platelet-derived chemokine (C-X-C motif) ligand 4 / platelet factor 4; DC: dendritic cells; ECM: extracellular matrix; ET-1: endothelin-1; IL: interleukin; IRF-5: interferon response factor 5; MCP-1: monocyte chemoattractant protein 1; MHC: major histocompatibility complex; pDC: plasmacytoid dendritic cells; PDGF: platelet derived growth factor; RNA: ribonucleic acid; ROS: reactive oxygen species; SMAD: mothers against decapentaplegic family; TGF- β : transforming growth factor beta; TNFAIP3: tumor necrosis factor alpha-induced protein 3.

3. Experimental models

More than twenty animal models of SSc have been set up over the years, including 17 genetic SSc mouse models and 7 induced models [29]. The main characteristics of the major experimental models are emphasized in figure 2 (fig.2). Only the most classical/recent will be described bringing its advantages and limits.

TSK-1 mouse is a spontaneous genetic model, resulting to a partial duplication of the fibrillin-1 gene (*Fbn1*), combining skin fibrosis and markers of autoimmunity, but suffering from lack of vasculopathy and inflammation [30]. The *TSK-2 mouse model*, results from the injection of a mutagen, the ethyl-nitrosourea, inducing mutation in the alpha chain of collagen type III. Despite the presence of inflammation this model is lacking vascular involvement [31].

One of the oldest and well-described models secondary to subcutaneous bleomycin injections is the *BLM-induced mice* model [32]. This model developed dermal fibrosis associated with inflammation, a pulmonary fibrosis and autoimmune features with the positivity of antinuclear antibodies (ANA) and ATA. Although it is an easy handling model, it remains artificial and overestimates the

anti-inflammatory drug effects compared to those observed in the human SSc [33].

Friend leukemia virus integration 1 (Fli-1) deficient mice model and its variants, have several strengths. That transcription factor is a repressor of type I collagen and is a cornerstone regulator of fibrosis genesis. *Fli-1* was found to be decrease in endothelial cells, fibroblasts and keratinocytes in SSc patients' skin [34]. A homozygous *Fli-1* deletion of C-terminal transcriptional activation domain (*Fli-1^{-/-} KO mice*) generates a model with fibrosis predominantly, with upregulation of collagen mRNA and protein, associated with skin thickening and observation of collagen fibrils, without vasculopathy, inflammation or autoimmunity [35].

An interesting model of SSc has also been developed using reactive oxygen species (ROS) in BALB/c strain [36]. Subcutaneous injections of a ROS solution, containing HOCl, O₂⁻, ONOO⁻ and/or OH⁻ leads to the development of skin fibrosis but also systemic reaction with lung fibrosis and renal injury. Interestingly, exposure to these oxidized molecules triggers a systemic autoimmune response characterized by the detection of anti-topoisomerase I (ATA) and anti-centromere antibodies (ACA) in ROS-treated mice. Thus, TGF-β independent model suggests a possible new role of ROS in the

		EXPERIMENTAL MODEL	VASCULOPATHY	FIBROSIS	INFLAMMATION	AUTOANTIBODIES
		INDUCED MODELS				
		Bleomycin-induced model [32]	±	+	+	ANA, ATA
		HOCl-induced model [36]	+	+	+	ATA
		Scl-GVHD [110]	±	+	+	ANA
		Angiotensin II induced model [110]	-	+	+	-
		Topo I immunization induced SSc [110]	-	+	+	ATA
		IMU-COLV mice [111]	+	+	-	ANA
GENETIC MODELS						
	SPONTANEOUS	TSK-1 mice (TSK/+) [30]	-	+	-	ANA, ATA, anti-DNA
		TSK-2 mice (TSK2/+) [31]	-	+	+	ANA, ATA, ACA, anti-DNA
		UCD-200 & 206 chickens [112]	+	+	+	ANA
	MODIFIED	Fra-2 transgenic mice (Fra-2 ^f) [110]	+	+	-	-
		Caveolin 1 ^{-/-} mice [110]	+	+	-	-
		TGF-β transgenic mice : TBRI ^{CA} (Cre-ER) & TβRIIΔk mice [113,114]	+	+	-	-
		uPAR KO mice [115]	+	+	-	-
		Fli-1 deficient model [116]	-	+	-	-
		Endothelium cell specific Fli-1 CKO mice [117]	+	-	-	-
		Epithelial cell specific Fli-1 CKO mice [118]	-	+	+	ANA
		Fli-1 ^{+/-} KLF5 ^{+/-} mice [17]	+	+	+	ANA
		SIRT3 KO mice [119]	±	+	-	-
		VEGF ^{+tg} mice [50]	+	+	+	-

Fig. 2. Experimental models of SSc. [110-119] Legend: BLM: ACA: anti-centromere autoantibodies; ANA: anti-nuclear autoantibodies; ATA: anti-topoisomerase I autoantibodies; BLM: bleomycin (BLM)-induced mouse model; Fli-1: Friend leukemia virus integration; Fli-1 CKO mice: endothelial cell specific Fli-1 knockout mice; Fra-2: transcription factor Fos-related antigen 2; H2K1: histocompatibility 2, K1, K region (MHC class I protein binding); HOCl model: hypochlorous acid exposure; KLF5: Krüppel-like factor 5; Fli-1^{+/-} KLF5^{+/-}: double heterozygous; KO: knock-out; PAH: pulmonary arterial hypertension; ROS: reactive oxygen species; SC: subcutaneous; Scl-GVHD: Sclerodermatous Graft-Versus-Host Disease; SIRT3: mitochondria-localized deacetylase Sirtuin 3; Topo I Ab: anti topoisomerase I autoantibodies; TSK mice: Tight skin mice; UCD: University of California, David chicken model; uPAR: Urokinase-type plasminogen activator receptor.

oxidation of self-antigens that could lead to auto-immune response against modified self-antigens and particularly, topoisomerase 1 and centromere antigens.

These new and older animal models provide many opportunities to better understand the molecular mechanisms underlying the pathophysiology of SSc and the identification of potential new therapeutic targets.

4. Oxidative stress

Oxidative stress through reactive oxidizing species (ROS) represents one of the key hallmarks of SSc. ROS, including superoxide anion (O_2^*), hydrogen peroxide (H_2O_2), hydroxyl radicals (*OH), nitric oxide (*NO), peroxynitrite ($ONOO^-$) and hypochlorous acid ($HOCl$), stimulate the synthesis of proinflammatory and profibrotic cytokines such as TGF- β or Platelet-Derived Growth Factor (PDGF), increase fibroblast proliferation and activation, collagen production and vascular damage [37–39].

The inhibition of ROS-generating NADPH oxidase by diphenyleneiodonium allows to suppress the fibroblast activation (reduction of collagen, fibronectin-1 and α -Smooth Muscle Actin (α -SMA) and skin fibrosis *in vitro* and *in vivo* experiences [40].

Sildenafil, a phosphodiesterase type 5 inhibitor, currently used in the management of Raynaud phenomenon (RP) or PAH, was able to modulate oxidative stress-induced proinflammatory activation of dermal fibroblasts from SSc patients, via STAT3-, ERK-, NF- κ B-, and PKB/AKT-dependent pathways [41]. Numerous other therapies targeting oxidative stress were developed [42].

5. Vasculopathy

Vascular involvement is easily identifiable in the clinic in SSc patients who present with Raynaud's phenomenon. Early in the disease, it could be responsible for the immune response and the fibrosis it precedes [43]. Vascular damage in SSc can be understood through pathological studies which highlighted endothelial lesions but also intimal hyperplasia, inflammatory perivascular infiltrate, and rarefaction of capillaries (Figure 1).

Endothelium lesions comprised loss of cell continuity, basement membrane disruption, increase in the number of apoptotic endothelial cells, and decrease in the proliferation of their precursors [44,45]. Such endothelial architecture dysfunction is exacerbated by the decrease in the production of vasodilator factors such as nitric oxide or prostacyclin [46], and by an increase in the production of vasoconstrictor factors such as ET-1 [47] which, *in vitro*, is also able to stimulate fibrosis [48].

Intimal hyperplasia and perivascular infiltrate, responsible for thickening of the vascular wall, are explained by the excess proliferation of pericytes and VSMCs. Interestingly, these cells have at their surface PDGF receptors for which auto-antibodies have been found in SSc patients [49].

Angiogenesis defect is also marked in the SSc vasculopathy. Maurer et al. showed that mice transgenic for VEGF had a decreased number of microvessels and more marked cutaneous fibrosis in response to subcutaneous injection of bleomycin [50]. This defect in angiogenesis could also be linked to a reduced expression of desmoglein-2, reported in microvascular endothelial cells (ECs) from patients with diffuse SSc [51].

Vascular involvement is currently considered as the initial pathologic element of SSc, and the links to the immune system and fibrosis have not all been elucidated. However, ECs from patients with SSc produced a supernatant capable of activating fibroblasts which, therefore, express α -SMA, vimentin, collagen, metalloproteases (MMP) 2 and 9 and proliferate on an artificial ECM [52], whereas ECs synthesize the chemokine MCP-1 and of the cell adhesion protein VCAM-1 in excess [53], which can both recruit

inflammatory and immune cells into the vessels and engage an immune response.

6. Immune system

6.1. Innate immunity

6.1.1. Toll-Like receptors, type I interferon signature, and inflammasome

Among the 10 TLRs described in humans, a demonstration of activation has been proposed for three of them in SSc: TLRs 2, 4, and 8 showing that either their ligands and/or the receptors and their co-receptors are increased in patients with SSc [54] and linked to *in vitro* fibrosis [55].

Type I interferon signature has been deciphered in SSc. First genetic studies have highlighted transcription factors implicated in this signature such as « *Signal Transducers and Activators of Transcription 4* (STAT4) » and « *interferon regulatory factor 5* (IRF5) » [56]. Second, Plasmacytoid dendritic cells, which are a major source of IFN I have been increasingly reported in SSc [57]. Third, the signature itself has been found in SSc patients and correlate with B-cell activation factor cytokines as well as the serum levels of the N-terminal propeptide of procollagen type III (PIIINP) [58].

Inflammasomes are cytosolic receptors that regulate the activity of caspase-1 and thus the activity of the IL-1 family. Inflammasome receptors, NOD-like receptors (NLR), in particular NLRP (for NLR pyrin domain containing) have been studied in SSc. NLRP3, which has an architectural role for inflammasomes by activating caspases 1 and 5, allows the activation of IL-18, IL-33 and also of pro-IL-1 β which has the capacity to activate fibroblasts of patients with SSc [59]. The SNP rs182352 of NLRP1 was associated on the one hand with anti-topoisomerase antibodies but also with fibrosing alveolitis of SSc [60]. The expression and function of NLRP3 have been studied in fibroblasts from patients with SSc, with evidence of both overexpression of genes associated with NLRP3 in fibroblasts from SSc patients compared to healthy subjects. In this same work, the inhibition of caspase-1 decreased the secretion of collagen and the expression of the α -SMA protein by the skin fibroblasts of patients with SSc confirming the action of the inflammasome in the SSc [61].

6.1.2. Monocytes, macrophages, mastocytes, and others innate cells

Lescoat et al. recently showed that CD16-carrying monocytes were increased in patients with SSc and that this number correlates with skin and lung fibrosis severity [62]. Monocytes from SSc patients more abundantly secrete IL-6, superoxide anions, and the skin fibroblast from SSc patients has the ability to secrete monocyte chemoattractant protein-1 (MCP-1) which contributes to the tissue infiltrate and to the process of the fibrosis [63]. Finally, monocytes from SSc patients are characterized by pro-fibrosing activity in patients with PID [64], and also the ability to differentiate into cells, expressing α -SMA and producers of elements of ECM [65]. Similar to monocytes, type II macrophages are found in the blood, skin and lungs of SSc patients and implicated in SSc fibrosis [66].

Mast cells are increased in bronchoalveolar lavage and in the skin of patients with SSc [67,68], and in a mouse model cutaneous fibrosis require the presence and/or recruitment of mast cells.

Other innate immune cells such as neutrophils, eosinophils, basophils, NK cells, innate lymphocytes, and type 2 innate lymphoid cells have been reported to be increased and linked to fibrosis in SSc [69,70].

6.2. Adaptive immune response

6.2.1. Dendritic cells

Making the link between innate immunity and adaptive immunity, dendritic cells (DC) have also been studied in SSc. *Ex vivo*, DC obtained from blood monocytes of patients with SSc, stimulated

through TLR4, produce IL-10 in excess [71]. Proteomic analysis of plasmacytoid DC (pDC) supernatants from patients with SSc demonstrated an increase in the level of CXCL4, especially in patients with a diffuse form of the disease. In addition, in this work, CXCL4 was detected only in the skin of SSc patients and serum CXCL4 levels correlated with the occurrence of pulmonary fibrosis or PAH [72]. Depletion of pDCs in a mouse model of SSc secondary to the injection of bleomycin, allows the reduction of cutaneous fibrosis, the decrease of the cutaneous expression of α -SMA and decreased collagen production by fibroblasts [73].

6.2.2. T cells

Typically, the T cell response described during SSc is of the Th2 type, characterized by increased production of IL-4 and IL-13 (Figure 1). The Th2 environment in SSc has been widely reported in the blood and tissues of affected patients; it is promoted by the production of IL-4 by the CD4⁺ T cells [74] but also by CD8⁺ T cells and even by CD4⁺CD8⁺ T cells [75]. Production of IL-13 by CD8⁺ T cells has been reported in both blood and tissue of targeted SSc patients [76], it is widely accepted that stimulation of human fibroblasts by IL-4 or IL-13 causes an increase in their collagen secretion [77]. Regarding regulatory T lymphocytes (Tregs), the results obtained in the literature are contradictory [78]. Very recently, MacDonald et al. insisted on the plasticity of these cells in SSc demonstrating their ability to also produce IL-4 and IL-13 and again suggesting a pro-fibrosing role [79].

6.2.3. B cells

The role of B cells have been extensively studied and recently reviewed in SSc [80]. To sum up available data on B cells in SSc patients, one could say that B cell activation is certain, characterized by the production of SSc autoantibodies and cytokine production. Many recent studies have demonstrated that B cells play a critical role in SSc through IL-6 and TGF- β production, other immune and non-immune cells including T cells and fibroblasts, hyper-reactivity of memory B cells and fibrosis development [81].

7. Fibrosis

Fibrosis is the key feature of SSc (Figure 1); it is the consequence of the vascular and immune deregulations described above, but it is also exacerbated by specific molecular and cellular mechanisms resulting in a dysfunction or an absence of regulation of the fibroblasts. Once fibrotic mechanisms are launched, they inevitably lead to a remodeling of the extracellular matrix through collagen hyperproduction [82,83].

7.1. Soluble mediators and molecular signaling of fibrosis

7.1.1. Wnt proteins

Wnt proteins are lipid glycoproteins that bind to Frizzled receptors that have a role in the physiopathology of SSc. SSc patients downregulate Wnt antagonists DKK1 and SFRP1 [84], and stimulation of fibroblasts from healthy subjects with Wnt ligands causes their activation, and increased their migratory capacity and the expression of collagen [85,86].

7.1.2. Platelet-derived growth factor

PDGF Growth Factor stimulates fibroblast proliferation. Both PDGF itself and its receptors are increased in skin lesions, or pulmonary lesions in SSc [87–89].

7.1.3. Connective tissue growth factor

Connective tissue growth factor (CTGF), which is not expressed in skin fibroblasts in healthy individuals, it is constitutively overexpressed by fibroblasts from patients with SSc [90] and contribute to

the exaggerated synthesis of elements of ECM and the adhesion and proliferation of fibroblasts from patients with SSc. This overexpression in SSc is not dependent on TGF- β , but presumably on Sp1 [91], and correlates with the severity of lung damage even in the absence of TGF- β [92].

7.1.4. Transforming growth factor β

The growth factor TGF- β is a key cytokine in the pathophysiology of SSc [93]. TGF- β is a pleiotropic cytokine having three isoforms and whose pro-fibrosing activity depends on the SMAD signaling pathway, in particular SMAD 2 and SMAD 3. Its role in wound healing and fibrosis as a direct activator of fibroblasts is definitely acquired [94]. Stimulation of fibroblasts from healthy subjects with TGF- β induces fibrosis by causing their activation, differentiation into myofibroblasts, production of collagen, synthesis of CTGF, and expression of gene encoding ECM elements, all by increasing their capacity for adhesion, contraction, migration and survival [95]. This activation of fibroblasts is also self-maintained by an autocrine production of TGF- β which stimulates receptors whose expression is increased on their surface [96].

7.2. Cellular players in fibrosis

7.2.1. Perivascular cells and pericytes

Mesenchymal stem cells are perivascular cells involved in fibrosis. Expansion of these cells has been reported in mouse models of cardiac, renal, hepatic and pulmonary fibrosis [97]. In addition, among these cells, the pericytes grouped together markers of activation of fibroblasts such as α -SMA and Thy1 and have been described in the dermis of patients with early SSc while absent in the dermis of healthy subjects, patients idiopathic Raynaud's phenomenon or late SSc thus suggesting a pro-fibrosing role [98].

7.2.2. Fibroblasts

The role of fibroblasts in SSc, is paramount because it is the cell that produces ECM elements such as collagen, fibronectin and degrading elements. In addition, a major characteristic of fibroblasts in SSc is the acquisition of α -SMA expression and thus differentiation into myofibroblasts.

7.2.3. Genetic deregulation

Fibroblasts from disease-damaged skin of SSc patients have a different genetic profile from those of healthy subjects and from skin unaffected by the disease. Thus Meyringer et al. revealed 48 complementary DNAs differently expressed in these fibroblasts, and homologous to segments of gremlin, lysyl oxidase, c-cbl proto-oncogene and segments of genes encoding elements of the ECM [99]. In addition, some promoters functionally implemented in fibrosis mechanisms have been reported to be deregulated: thus the DKK1 and SFRP1 promoters are hypermethylated in the fibroblasts of SSc patients and the COL1A1 promoter has increased binding of its CCAAT binding transcription factor regulator [84,100]. Epigenetic changes have also been reported to explain the activation of fibroblasts. Interestingly, Wang et al. demonstrated that epigenetic modifications of the FLI1 gene lead to an increase in the production of collagen in fibroblasts [101].

7.2.4. Apoptosis defect

Among the specific abnormalities of fibroblasts from patients with SSc, an apoptosis defect has been reported with increased survival, mediated by the Fas-Fas ligand pathway, of fibroblasts from patients with SSc [102].

7.2.5. Defects in protein synthesis of ECM elements

Proteome modification has been reported for fibroblasts from patients with SSc, highlighting proteins involved in wound healing,

cytoskeletal organization, cellular and metabolic mechanisms [103]. Basal activation of fibroblasts from patients with SSc is responsible for the hyperproduction of elements of ECM.

7.2.6. Autocrine production defect

Along with this defect in the synthesis of ECM elements, fibroblasts of SSc patients promote their own activation. Thus they have an increased autocrine synthesis of CTGF, free radicals that stimulate their proliferation and collagen synthesis [104], and chemokines or cytokines. This is the case with IL-1 α , IL-6 and IL-8.

7.2.7. Failure to respond to TGF- β

Finally, it would appear that some of the fibroblast abnormalities observed in SSc are linked to an excess of TGF- β . The TGF- β pathway is also defective in fibroblasts from patients with SSc. In fact, an accumulation of expression of type I and type II receptors of TGF- β is observed on the membrane of these fibroblasts [105–107]. In addition, this increased expression is associated with abnormalities in the intracytoplasmic signaling of SMAD molecules with an accumulation of phosphorylated SMAD 3. Independently of the SMAD 2/3 molecules, the c-abl, SMAD 1 and ERK tyrosine kinase pathways are also deregulated in the fibroblasts of patients with SSc [108,109].

8. Conclusion

Many recent progresses have improved our understanding of SSc pathogenesis, which is characterized by a dysregulated fibrosis led by the activation of fibroblasts and fibrotic cells, caused by the activation of the immune system and a diffuse vasculopathy. These advances contribute to open new therapeutic areas through the design of biologics and small molecules.

Declaration of Competing Interest

The authors declare that they have no competing interest.

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Annexe 2 : Rôle du lymphocyte B dans la physiopathologie de la Sclérodermie systémique



Role of B-Cell in the Pathogenesis of Systemic Sclerosis

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Systemic sclerosis (SSc) is a rare multisystem autoimmune disease, characterized by fibrosis, vasculopathy, and autoimmunity. Recent advances have highlighted the significant implications of B-cells in SSc. B-cells are present in affected organs, their subpopulations are disrupted, and they display an activated phenotype, and the regulatory capacities of B-cells are impaired, as illustrated by the decrease in the IL-10 + producing B-cell subpopulation or the inhibitory membrane co-receptor density. Recent multi-omics evidence highlights the role of B-cells mainly in the early stage of SSc and preferentially during severe organ involvement. This dysregulated homeostasis partly explains the synthesis of anti-endothelial cell autoantibodies (AECAs) or anti-fibroblast autoantibodies (AFAs), proinflammatory or profibrotic cytokines (interleukin-6 and transforming growth factor- β) produced by B and plasma cells. That is associated with cell-to-cell interactions with endothelial cells, fibroblasts, vascular smooth muscle cells, and other immune cells, altogether leading to cell activation and proliferation, cell resistance to apoptosis, the impairment of regulatory mechanisms, and causing fibrosis of several organs encountered in the SSc. Finally, alongside these exploratory data, treatments targeting B-cells, through their depletion by cytotoxicity (anti-CD20 monoclonal antibody), or the cytokines produced by the B-cell, or their costimulation molecules, seem interesting, probably in certain profiles of early patients with severe organic damage.

Keywords: systemic sclerosis, B-cell, pathogenesis, B-cell receptor (BCR), interleukine 6 (IL-6), autoantibodies, transcriptomic, rituximab

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HIGHLIGHTS

- B-cells are key players in SSc, implicated in fibrosis and autoimmunity
- B-cell subsets are disrupted
- B-cells are activated and lose their regulation properties
- B-cells produce autoantibodies and cytokines
- B-cells crosstalk with numerous cells including fibroblasts, endothelial cells, T cells, dendritic cells, and other immune cells
- Treatments targeting B-cells, through their depletion by cytotoxicity, or the cytokines produced by the B-cell, or their costimulation molecules seem interesting, probably in certain profiles of early patients with severe organic damage

1 INTRODUCTION

Systemic sclerosis (SSc) is a rare multisystem autoimmune disease of unknown cause characterized by fibrosis, vasculopathy, and autoimmune features (1, 2). SSc is responsible for visceral manifestations such as interstitial lung disease (ILD) and pulmonary arterial hypertension (PAH), which represent the two main causes of death (3). SSc is also responsible for significant disability, handicap, and a hampered quality of life.

The pathogenesis of SSc is not fully understood. Still, significant progress has been made in the last 20 years in the understanding of the mechanisms contributing to the occurrence of vasculopathy and fibrosis. In patients with SSc, the vascular tone is dysregulated, leading to perturbed interactions between endothelial cells, vascular smooth muscle cells (VSMC), and extra-cellular matrix (ECM) components participating in vascular remodeling and occlusion. ECM accumulation is mostly the consequence of fibroblast activation (4). Since skin fibrosis is the main hallmark of this disease, research initially focused on the fibroblast. However, the demonstration in the early state of the disease of cutaneous lymphocyte infiltration before any process of fibrosis (5), the presence of auto-antibodies, imbalances in the B-cell subsets, and a hyperactivated state repositions the B-cell as an actor in the pathogenesis of SSc.

We herein provide an overview of B-cells in the pathogenesis of SSc from human data and animal models. We review B-cell distribution, B-cell dysfunction, and B-cell participation in inflammatory and fibrotic lesions, with emphasis on recently published data and their implication for patient prognosis.

Lastly, we discuss the different therapeutic options targeting B-cells or pathways involved in B-cell activation.

2 B-CELLS ARE FOUND IN AFFECTED ORGANS

Early presence of various innate or adaptative immune cells infiltrates which B and plasma cells have been demonstrated in the skin, lung, or gastrointestinal tract of SSc patients (6–10). Recent genetic and immunohistochemical data reinforce the implication of B-cells in the skin of SSc patients, from the early stages of the disease, in particular with the demonstration of the presence of CD20⁺ cells in the skin of patients and an activated B-cell genetic signature (11, 12) and illustrated in **Figure 1**. Moreover, in SSc patients, the presence of CD20⁺ B-cells in clinically affected skin seems to be greater compared to unaffected skin. Based on immunohistochemical data, the presence of inflammatory cells, including B-cells, in cutaneous samples from areas clinically involved and uninvolved by fibrosis may suggest the possibility of detecting pathological changes before the clinical onset of skin fibrosis (12).

Concerning the lung, a higher percentage of CD19⁺ B-cells in bronchoalveolar lavage fluid was found in SSc patients with ILD and could be correlated with worsening of the DLCO during pulmonary function tests (14). The occurrence of patchy lymphocytes and plasma cells or real lymphoid follicle organization in the lung examination of SSc-related ILD or PAH patients has also been described (15, 16).

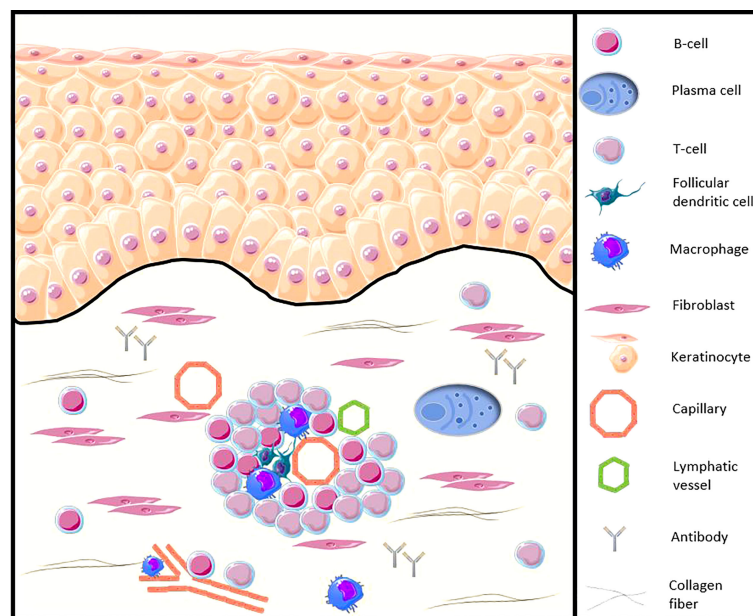


FIGURE 1 | Illustration of the B-cell presence in the SSc patients skin (adapted from Fetter et al. *Cells*, 2020 [13]). CD20⁺ B-cells and plasma cells infiltrate the skin of SSc patients, from the early stages of the disease, especially around blood vessels, accompanied by other peripheral blood mononuclear cells, such as CD3⁺ T cells or follicular dendritic cells. B-cells participate in the functions of antigen-presenting cells, production of autoantibodies and proinflammatory and profibrotic cytokines, as well as direct contact with other cells such as the fibroblast.

3 B-CELLS IN SSC EXHIBIT AN ACTIVATED PHENOTYPE IN SOME UNBALANCED SUBSETS

3.1 Distribution of B-Cell Subsets in SSc

Peripheral CD3⁻ CD19⁺ B-cells represent around 10% of total white blood cells (WBCs) and the last 30 years allowed us to isolate several peripheral B-cell subsets with specific phenotypes, including naïve, marginal zone, switched memory B-cells, and plasma cells (17). In SSc patients, the frequency of B-cells (relative count) among peripheral blood mononuclear cells is increased compared to healthy controls (18, 19). Contradictorily, data vary dramatically in the absolute count of peripheral B-cells in SSc patients according to the authors, reported to be increased (18, 20) similar (21, 22), or decreased (23) compared to healthy controls. These differences could be explained by differences in the methods used, the threshold definitions, and/or the use of glucocorticoids and/or immunosuppressant drugs. Thus, B-cell subsets in SSc patients are unbalanced, with an expansion of naïve and transitional B-cells with a decrease in memory B-cells, switched memory B-cells, and IL-10⁺-producing regulatory B-cells (Bregs), particularly in steroid-untreated patients (16, 18–20, 22, 24–27), with a higher circulating plasma cell molecular signature (28). Transitional B-cells represent a maturing subset undergoing peripheral selection, whereas switched memory B-cells are long-lived cells persisting in a quiescent state (17). These immune dysregulations lead to inadequate B-cell homeostasis and favor the emergence of autoimmune reactions.

3.2 B-Cells Overexpress Activated Phenotype in SSc

In SSc patients, the transitional and memory B-cells are constantly activated compared with controls. The expression levels of the costimulatory molecules CD80, CD86, and CD95 are increased, with an increased proportion of IL-6⁺- and IL-6 receptor⁺ (IL6R) B-cells (4, 18–20). The overexpression of CD69, CD5, CD86, CD95 markers, and the IL6R is associated with the diffuse cutaneous SSc (dcSSc) form and the early stage of disease, suggesting the implication of the activated B-cells in the active and aggressive forms of SSc (16). The decrease in the memory B-cell compartment found in SSc compared to healthy controls is probably due to increased apoptosis (18). B-cells are not only a cellular precursor of differentiated secreting-antibody cells. The B-cells are involved in various essential regulatory immune processes like the antigen presentation, T-cell maturation and differentiation, the organization of the architecture of the lymphoid organ, and the cytokine synthesis (29). The upregulation of the CD80/86–CD28 costimulatory molecules contributes to activating autoreactive T cells and increasing the secretion of profibrotic cytokines in SSc (30). Simultaneously, the ability of B-cells to produce IL-10 is altered, participating in downregulating Breg (20, 22). Additionally, B-cells from SSc patients express increased programmed death-1 (PD-1) and PD-1 ligand 2 (PD-L2), a co-stimulatory CD28/B7 family system, which contributes to the impairment of immune tolerance (31). This disrupted homeostasis of peripheral B-cell subsets in SSc

could be secondary to an overexpression of activation markers in certain subsets, counterbalanced by increased CD95-mediated apoptosis in memory B-cells (18). This disrupted homeostasis encountered in B-cells in SSc patients probably influences many of their regulatory immune functions.

4 B-CELL RECEPTOR, INTRACELLULAR SIGNALING AND INTRINSIC PATHWAYS OF B-CELLS ARE DISRUPTED IN SSC

4.1 B-Cell Receptor and Its Co-Receptors

The activation of B-cells mediated by antigen implicates the B-cell receptor (BCR), many co-receptors, and coactivation molecules (**Figure 2**). Several components of this axis are altered during the SSc. In SSc patients, the BCR diversity is altered, which is related to the alteration of B-cell development. In particular, the VDJ rearrangement is altered, leading to an excess of membrane IgD diversity and a defective mutation load identified as a SSc-related PAH signature (33). In humans, the membrane density of the CD19 co-receptor at the membrane of peripheral B-cells in SSc patients was rated 20% higher than that in healthy controls, which seems to be specific to SSc since it is not the case in SLE (18, 21). Additionally, the expression of CD21, another positive regulator of the BCR signal, is also higher at around 20% (18, 21). Several membrane inhibitors, such as CD22 or CD35, have been found decreased on B-cells, close to a factor of 2 for CD35, especially on memory B-cells in SSc patients with peripheral vascular modified Medsger scale altered (≥ 1), with diffuse cutaneous SSc (dcSSc), and ILD (19, 34).

4.2 B-Cell Activating Factors

Independently of antigen-mediated mechanisms, BAFF and APRIL pathways are implicated in the pathogenesis of SSc. B-cell activating factor (BAFF) and APRIL pathways, both members of the tumor necrosis factor (TNF) superfamily, play a critical role in the activation, survival, and maturation of B-cells, and are involved in many disorders like transplantation/graft versus host diseases (GVHD) and autoimmune diseases (35).

First, in the type 1 tight skin mouse model (TSK/+), an increase in serum BAFF levels has been reported. Its blockage inhibits the development of skin fibrosis and autoantibody production or TGF- β and IL6 mRNA levels in the skin (36). Thus, in humans, serum BAFF and APRIL levels are higher in SSc patients compared to healthy controls by about 25 to 50% for BAFF (22, 37–39), as well as the amount of one of its specific receptors (BAFFR) at the surface of B-cells (37). Positive correlations between serum levels of BAFF and APRIL and modified Rodnan skin score (mRSS), early dcSSc, ILD and IL-6, serum IgG level, and anti-Topo I Ab titers are reported (22, 37–42).

4.3 Intracellular Signaling and Intrinsic Pathways of B-Cells Are Upregulated in SSc

Beyond alterations targeting extrinsic activators, defects in intracellular B-cell signaling, mainly tyrosine kinases, have

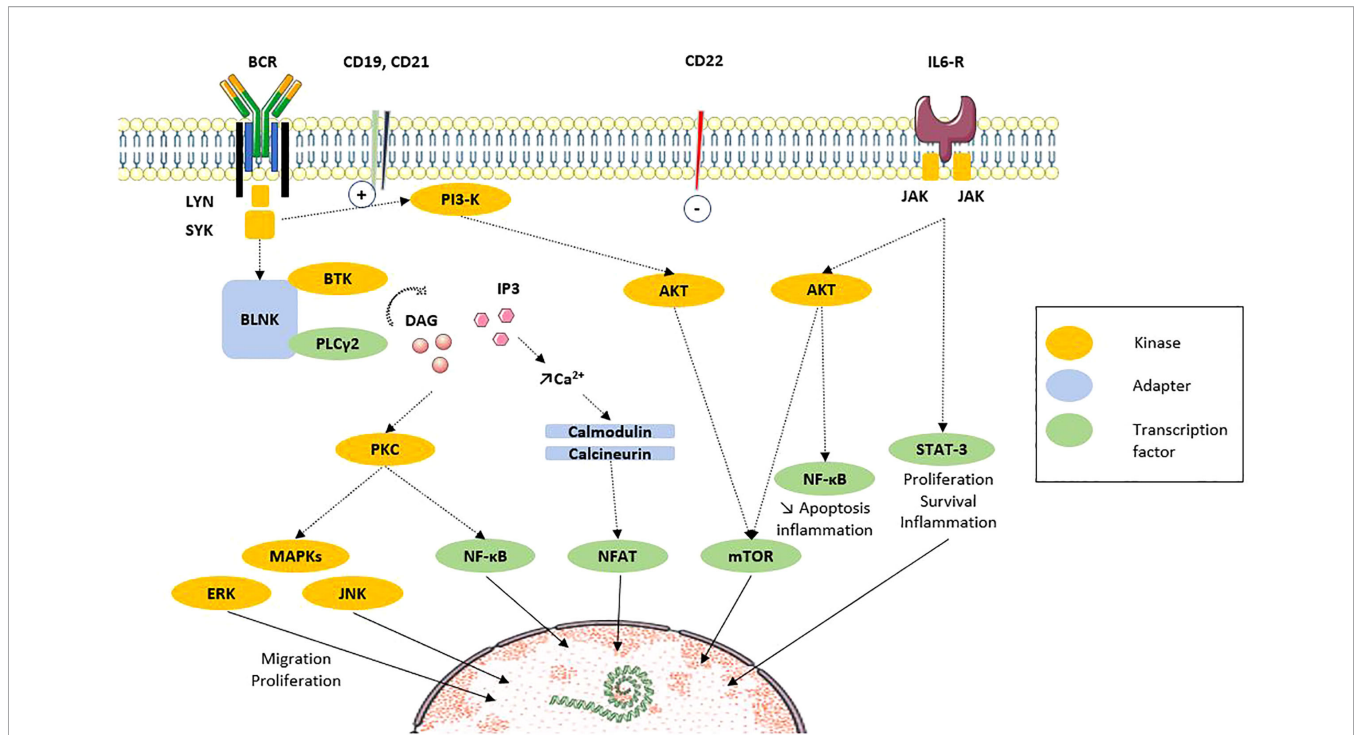


FIGURE 2 | B-cell signaling via BCR and costimulation molecules (adapted from Dal Porto et al. Mol Immunol, 2004 [32]). After ligation of antigen on the B-cell antigen receptor (BCR) at the membrane, signals are transduced and propagated by several protein phosphorylations, modifications and interactions. The end point is the regulation of transcription factors that regulate the expression of genes. Recent results highlight the participation of intracellular signaling perturbations in B-cell including defective phosphorylation of MAPK, STAT3 or mTOR molecules. Solid line: translocation. Dotted line: direct action. AKT, protein kinase B; BCR, B-cell receptor; BLNK, B-cell linker protein; BTK, Bruton's tyrosine kinase; CD, cluster of differentiation; DAG, diacylglycerol; ERK, extracellular signal-regulated kinases; IL, interleukin; IL6-R, IL6 receptor; IP3, inositol triphosphate; JAK, Janus kinases; JNK, c-Jun N-terminal kinase; Lyn, Lck/Yes novel tyrosine kinase; MAPK, mitogen associated protein kinase; mTOR, mammalian target of rapamycin; NFAT, Nuclear factor of activated T cells; PI3K, phosphoinositide 3-kinase; PLC γ , phospholipase C γ ; PKC, protein kinase C; STAT3, signal transducer and activator of transcription 3; SYK, spleen tyrosine kinase.

been documented in many autoimmune diseases, including SLE, rheumatoid arthritis (RA), and SSc. The involvement of calcium storage has also been reported.

In TSK/+ B-cells, the CD19-mediated intracellular response to stimulation appears to be exacerbated, with an increase in intracellular calcium [Ca^{2+}]_i storage (43, 44). Then, spleen tyrosine kinase (SYK) has been reported as hyperphosphorylated in B-cells of murine sclerodermatous chronic graft-versus-host disease (Scl-cGVHD) (45). Experimental use of fostamatinib, an inhibitor of SYK in Scl-cGVHD or BLM models, appears to contribute to decreasing phospho-SYK, TGF- β expression, and tissue fibrosis (45, 46).

Thus, Lyn kinase, the first signal transducing kinase involved after BCR activation, is downregulated in CD22^{low} B-cells from SSc patients (34). Moreover, after activation, defective phosphorylation of downstream MAPK, STAT3 (20, 25) or mammalian target of rapamycin (mTOR) (22) has been reported, especially in CD27⁺ transitional or memory B-cells by some authors (20, 25).

4.4 Genomic, Transcriptomic, and Proteomic Data Argue a B-Cell Signature in SSc

Large-cohort genomic studies have identified associations between genes involved in B-cell signaling and the SSc phenotype, including B-cell scaffold protein with ankyrin repeats 1 (BANK1), B lymphoid kinase protein (BLK), c-src tyrosine kinase (CSK) or intracellular protein tyrosine phosphatase (PTNP22) genes (47).

All studies on global gene expression in peripheral blood cells from SSc patients revealed the interferon IFN-inducible signature (48). Moreover, the profiles show a heterogeneity of the signatures according to certain clinical characteristics. The presence of an anti-topoisomerase or anti-U1 RNP antibody is associated with a high IFN-inducible signature, in contrast to the presence of an anti-centromere antibody or digital ulcer (49, 50).

Transcriptomic analysis of skin biopsies of SSc patients has found a B-cell signature in 69% of cases (51), with an increased expression of B-cell-related genes like genes related to light and

heavy chains of immunoglobulins, with genes that contribute to increased extracellular matrix synthesis or activation of T cells and correlated with skin thickness. These results are confirmed by immunohistochemistry staining on skin biopsy (11, 51). Whole skin transcriptomic analysis of SSc patients also showed discrimination according to the profiles of antinuclear antibodies (ANA) (52). Interestingly, adaptive immune signature, especially B-cell signature in the skin, was associated with early and diffuse SSc (51), suggesting once more time his early implication in disease and highlighting a potential therapeutic window for the use of B-cell targeting therapies.

Proteomic analyses in early dcSSc patients showed a difference in the longitudinal course of serum proteins according to the ANA status (52), confirming a B-cell implication with a continuum of differential signature from the genetic level to the protein level.

5 FUNCTIONAL CHARACTERISTICS

B-cells contribute in many ways to the pathogenesis of SSc, namely, autoantibody synthesis, proinflammatory or profibrotic

cytokine production, reduction of immunomodulatory cytokines, and/or through direct interactions with other cell types (Figure 3).

5.1 Autoantibodies Contribute to Cell Activation, Inflammation, Fibrosis, and Vasculopathy

The prevalence of anti-nuclear antibodies (Ab) in SSc patients is greater than 90% (53). Despite their important role in disease classification and prognosis staging, specific autoantibodies (mostly identified for centromeric proteins (CENP), topoisomerase I and RNA polymerase III) do not seem to play a major role in SSc pathogenesis (Table 1) (54, 69).

The anti-Topo I Ab is associated with the highest incidence of ILD and the poorest survival, followed by the anti-RNA polymerase III Ab. The anti-RNA polymerase III Ab is also associated with the highest incidence of scleroderma renal crisis (SRC). In contrast, SSc patients with an anti-U3 RNP Ab have the greatest incidence of pulmonary hypertension and SSc-related cardiac involvement (70, 71). Unbiased clustering methods incorporating autoantibody status also show their involvement in grading long-term disease prognosis (72).

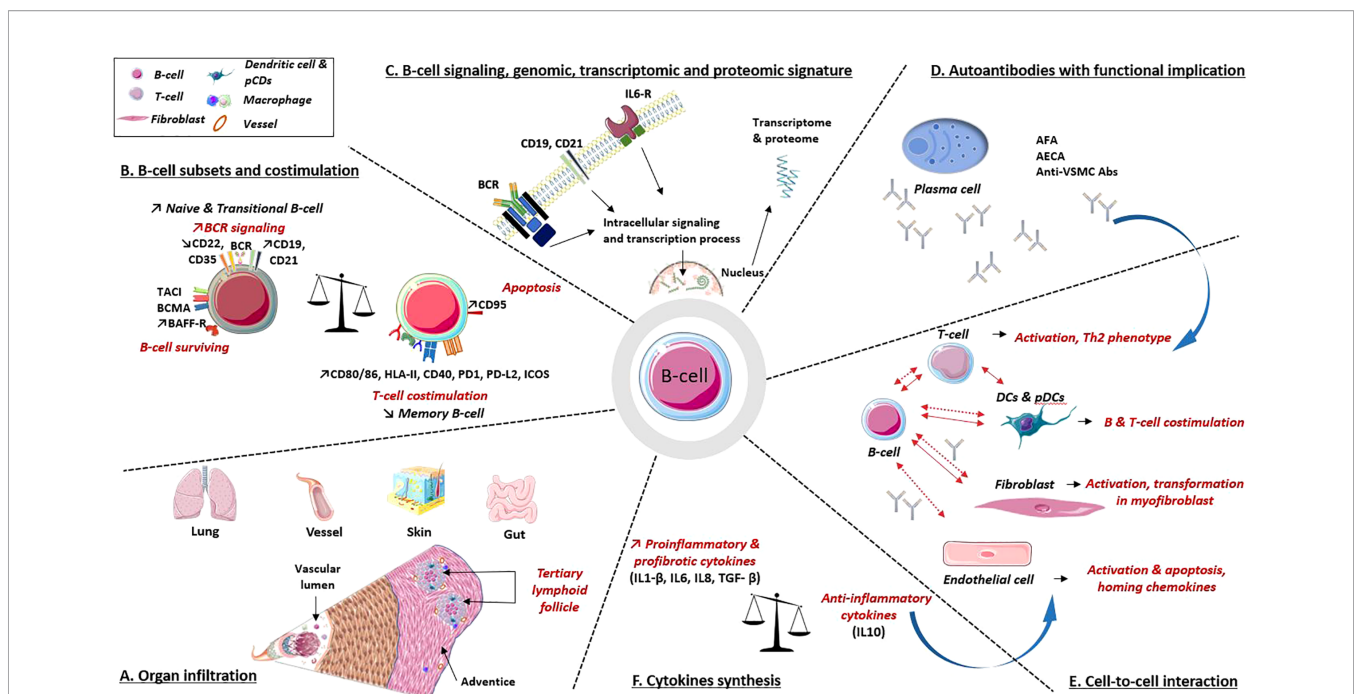


FIGURE 3 | B-cell implications in the pathogenic processes involved in systemic sclerosis. **(A)** Organ infiltration: B-cell infiltrates are present in various organs involved in SSc. These infiltrates combine B and T cells, plasma cells, dendritic cells and macrophages, underlying their interactions. **(B)** B-cell subpopulations: B-cells express at their surfaces multiple molecules involved in activation or surviving pathway, as well as decreased levels of inhibitors coreceptors. B-cells also express co-receptors required for interaction with T cell. **(C)** B-cell intracellular signaling, genomic, transcriptomic and proteomic data argue a B-cell signature in SSc. **(D)** Autoantibodies. Several autoantibodies have demonstrated their involvement in cell activation, apoptosis induction, proinflammatory and profibrotic processes. **(E, F)** Cell to cell interactions and cytokines synthesis: B-cells interact with various immune cells, fibroblast and endothelial cell through direct (solid line) and indirect contact (i.e., cytokines and antibodies; dotted line). All participate in profibrotic, proinflammatory processes, vascular remodeling and immune dysregulations. Abs, antibodies; AECA, anti-endothelial cell Abs; AFA, anti-fibroblast Abs; anti-VSMC Abs, anti-vascular smooth muscle cell Abs; BAFF-R, calcium-modulator and cyclophilin ligand-interactor and BAFF receptor; BCMA, B-cell survival membrane receptors: B-cell maturation protein; BCR, B-cell receptor; CD, cluster of differentiation; DCs, dendritic cells; HLA-II, human leukocyte antigen class II; ICOS, inducible T-cell costimulatory; IL, interleukin; IL6-R, IL6 receptor-PD-1/PD-L2: programmed death/programmed death ligand immune checkpoint; pDCs, plasmacytoid dendritic cells; TAC1, trans-membrane activator.

TABLE 1 | Autoantibodies associated with Systemic Sclerosis.

Autoantibody target	Prevalence (%)	Clinical association	Vasculopathy	Fibrosis	Other	References
Centromere	20–40	lcSSc & PAH	–	–	–	(54)
Topoisomerase I	9–42	dcSSc & ILD	–	–	–	(54)
Fibrillarin (U3 RNP)	3–20	dcSSc	+	+	–	(54, 55)
RNA polymerase III	4–11	dcSSc, SRC & malignancy	–	–	–	(54)
Th/To	2–5	SRC	–	–	–	(54)
Ku	2–5	Overlap syndrome	–	–	–	(54)
Endothelial cell [anti-VCAM, ICAM-1, NAG-2]	22–85	PAH	+	–	Leukocyte adhesion	(56–58)
Angiotensin II type 1 Receptor (AT1R)	85	PAH & Digital ulcer	+	+	–	(56, 59)
Endothelin type A receptor (ETAR)	85	PAH ulcer	+	+	–	(56, 59)
M3R	65–80	Gastrointestinal involvement	–	–	Inhibition of type 3 muscarinic receptor	(60, 61)
Vascular smooth muscle cells	80	–	+	–	–	(56–58)
Fibroblast	25–60	dcSSc with PAH, ILD	–	+	Adhesive mechanism	(62–64)
MMP-1 & MMP-3	50	dcSSc	–	+	–	(65)
PDGF-Receptor	33	–	–	+	–	(66)
MSRA	33	ILD & cardiac involvement	+	+	Reinforcement of oxidative stress	(67)
PRX I	33	ILD & cardiac involvement	+	+	–	(67)
CD22	22	–	–	–	Antagonize inhibitory CD22 signal	(68)

Anti-AT1-Rc Abs, anti-angiotensin II type 1 receptor Abs; anti-ETA-Rc Abs, anti-endothelin 1 type A receptor Abs; anti-M3R Abs, anti-muscarinic acetylcholine receptor M3; anti-MSRA Abs, anti-Methionine Sulfoxide Reductase A Abs; anti-PDGF-Receptor Abs, anti-platelet-derived growth factor receptor Abs; anti-Prx I Abs, peroxiredoxin I; anti-RNA polymerase III Abs, anti-ribonucleic acid polymerase enzyme III Abs; anti-U3-RNP Abs, anti-U3 ribonucleoprotein Abs, anti-topoisomerase 1 Ab; Anti-VCAM Abs, anti-vascular cell adhesion molecules Abs; anti-VSMC Abs, anti-vascular smooth muscle cell Abs; CCL, chemokine ligand; CD, cluster of differentiation; CENP, centromeric protein with a molecular weight of 17 (CENP-A), 80 (CENP-B) and 140 kDa (CENP-C); dcSSc, diffuse cutaneous form of systemic sclerosis; ICAM, intercellular adhesion molecules; ILD, interstitial lung disease; Ku, p70/p80 is DNA-binding protein involved in DNA repair and regulation; lcSSc, limited cutaneous form of systemic sclerosis; MMP, matrix metalloproteinases; PAH, pulmonary arterial hypertension; SRC, scleroderma renal crisis; TM4SF7 or Nag-2, cell surface tetraspanin transmembrane 4 superfamily molecules member 7; Th/To, 7-2 RNP and 8-2 RNP that are subunits of human mitochondrial RNase and RNase P ribonucleoprotein complexes.

Conversely, other autoantibodies, targeting cell surface receptors or ubiquitous molecules with potential direct pathogenic effects, have been identified in skin and pulmonary fibrosis, bowel involvement and/or vascular remodeling. The three main target cells are endothelial cells, fibroblasts, and vascular smooth muscle cells (VSMC). These autoantibodies induce activation and a proinflammatory or pro-adhesive phenotype of their cellular targets, and sometimes apoptosis (73). Thus, a direct pathogenic effect has been documented for certain anti-endothelial cells (AECA) and anti-fibroblast antibodies (AFA) (62–64, 74–76). This is the case for AECA targeting angiotensin II type I receptor or endothelin-1 type A receptor, which induce activation and apoptosis of endothelial cells with fibroblast activation, and secretion of profibrotic TGF- β cytokine (56, 77). Anti-platelet derived growth factor (PDGF)-receptor antibodies activate human VSMCs and fibroblasts and their transition into myofibroblasts, leading to fibrotic and vascular injury (66, 75). In the first publication, the presence of these anti-PDGF-receptor stimulatory antibodies was found in 100% of patients ($n = 46$). Their functional activity has been demonstrated through the involvement of the Ha-Ras-ERK1/2 activating cascade downstream of the PDGF receptor, the induction of ROS accumulation in a dose-dependent manner in *in vitro* experiments, and the promotion of type I collagen-related genes (66). Similar results have been demonstrated on human arterial smooth muscle cells ($n = 11$) with activation by anti-PDGF receptor antibodies of cell growth, migration ability, the generation of ROS, type I collagen gene expression, and the involvement of the NOX4 and mTORC1 pathways (75). These

data must be relativized slightly because the sample sizes are low and, thus far, these results have not been replicated. Proteomic studies have identified numerous antigenic targets, such as Stress-Induced-Phosphoprotein 1 (STIP 1) or alpha-enolase, two proteins involved in the contraction of vascular smooth muscle cells, with an antibody-mediated direct pathogenic effect with contraction induced by incubation with purified IgG from SSc patients (78).

5.2 Imbalance in Proinflammatory, Profibrotic and Anti-Inflammatory Cytokines Produced by B-Cells in SSc

In SSc patients, activated B-cells secrete high levels of IL-6 and TGF- β . The IL-6 levels in the supernatant of purified B-cells from SSc patients are correlated with the mRSS, suggesting a pivotal role of B-cells in the fibrosis process (16). IL-6 stimulates B and T-cell proliferation, increases the resistance to apoptosis, activates fibroblasts and reduces the regulatory functions of Tregs. The IL-6 signaling induced indirectly the collagen synthesis by the fibroblast, through the phosphorylation of STAT3, the activation of downstream targeted genes, and *via* Gremlin-1, a *Bone Morphogenetic Protein* (BMP) antagonist, which activates the non-canonical TGF- β pathway *via smad3* (79). Furthermore, the highest serum levels of IL-6 are reported in the early dcSSc compared to the other groups of patients (80). Fibroblasts are also important producers of IL-6. Fibroblasts from affected skin areas in SSc patients produce significantly more IL-6 compared with fibroblasts from unaffected skin areas, or from healthy controls (81). There is, therefore, a loop between the fibroblast

and the B-cell that is partly dependent on IL-6. Noteworthy, IL-6 and IL-10 serum levels correlated with skin and pulmonary fibrosis (82, 83). Otherwise, circulating B-cells secrete increased amounts of IL-8, IL-1 β , BAFF, and CXCL13, which are proinflammatory, profibrotic cytokines or chemo-attractant molecules, both in scleroderma mouse models and SSc patients (20, 22, 37, 84). Many of these cytokines contribute to polarizing CD4⁺ T cells into IL-4 producing Th2 cells (85), T follicular helper (Tfh) and/or Th17 cells. In SSc, a Th2 lymphocyte phenotype is observed in the early stages of disease (86), and Th2 cytokines enhance antibody production by plasma cells and contribute to stimulating collagen production by fibroblasts.

Among regulatory cytokines, a particular interest has recently been given to IL-10, produced by Breg. Bregs can decrease inflammatory and autoimmune processes (87). These cells exert suppressive effects on a large variety of cells, including autoreactive T cells, dendritic cells, or macrophages through IL-10 production/synthesis. A reduction of IL-10 synthesis has been documented, both in mouse models and in SSc patients, and is correlated with the decrease of IL-10⁺ B-cells (19, 27, 84, 88). The decrease in these cells encountered in SSc patients is associated with the presence of ILD and inversely correlated with autoantibodies levels, and with Th17 T cells (26) suggesting their high implication in the control of the profibrotic environment (26).

5.3 B and T Cells Co-Interact and Self-Activate

The identification of B- and T-cell infiltrates in SSc patient skin biopsies led to arguments for their strong interaction (12, 89) and predominantly in the early stages of disease. As expected, the production of anti-topoisomerase I Ab requires direct contact and restriction to HLA-DR and CD40/CD40-ligand costimulatory molecules in collaboration with CD4⁺ T cells (90, 91). Follicular helper T cells (Tfh) are crucial for the activation and differentiation of B-cells into plasma cells (92). Many data highlighted the interaction of Tfh and B-cells through direct contact or *via* the intermediate of cytokines (IL-21) in the fibrotic process encountered in SSc patients or murine models (93, 94). Tfh are hyperactivated in SSc patients through the higher expression of activation proteins like inducible T-cell costimulatory (ICOS) and PD-1 on their surface compared to healthy controls (93). Compared to healthy controls, circulating Tfh from SSc patients secreted higher IL-21 levels, promoting the differentiation of naïve B-cells into plasma cells and proliferation, and Ig secretion, especially in dcSSc and PAH (94).

B-cells interact with other T-cell subsets in SSc. The transmembrane glycoprotein T-cell Ig and Mucin domain protein 1 (TIM-1), a regulatory marker at the Breg surface, is implicated in the interaction between B and T cells in SSc. The frequency of peripheral blood TIM-1⁺ IL-10⁺ Breg is decreased in SSc patients compared with healthy controls, with a further defective ability to suppress CD4⁺ T-cell activation and inflammatory cytokine secretion (IFN- γ , TNF- α , IL-17) (27). However, the CD70 promoter is demethylated, favoring its overexpression on CD4⁺ T cells and seems to have contributed to the autoimmune response *via* its costimulatory activity of the B-

cell during the B-T cell interaction (95). CD8⁺ effector T cells, Th17-cells and Treg are also involved in the synthesis of many proinflammatory and profibrotic cytokines in SSc (IL-4, IL-5, IL-9, IL-13, IL-17, TGF- β , and TNF- α) (96–98).

5.4 B-Cells Interact With Dendritic Cells and Other Innate Immune Cells

Very little data are available on the B-cell/dendritic cell (DC) interaction in the SSc. Activated B-cells induce the maturation of DCs in a contact-dependent manner (CD83, CD80, CD86, CD40, and HLA-DR) through BCR signaling and the BAFF receptor. B-cell-matured DCs secondary promote polarization and activation of naive CD4⁺ T cells into Th2 cells, producing increased amounts of IL-4, IL-5, and IL-13 (99).

Otherwise, proteome-wide analysis demonstrated a high level of CXCL4 in the blood and skin of SSc patients, and primarily synthesis by the plasmacytoid DCs (pDCs). CXCL4 levels were correlated with skin and lung fibrosis and with PAH (100). Similar data were shown in the BLM model with an increased number of pDCs in the affected skin and lungs compared to wild-type mice (101). The pDCs express the BAFF ligand (35), supporting the argument for an interaction between B-cells and pDCs in the SSc.

B-cells interact with other innate immune cells such as the macrophages. Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)⁺ B effector cells stimulate macrophages through GM-CSF synthesis to induce inflammatory and fibrotic lesions. These cells belong to the memory B-cell subset and strongly produce IL-6 and TNF- α . These cells have been reported to be increased in SSc patients, especially in dcSSc and associated with ILD, suggesting their role in the pathogenesis of SSc (102).

5.5 B-Cells Activate Fibroblasts

In cellular culture, cell-to-cell contact between B-cells and dermal fibroblasts induces an increase in collagen, α -smooth muscle actin, and metalloproteinase synthesis, or cytokines and chemokines (IL-6, TGF- β 1, CCL2) (40, 41).

Antibody-mediated effects are illustrated with AFA, from the serum of SSc patients, which binds skin and lung fibroblasts, leading to proadhesive (ICAM-1), proinflammatory (reactive oxygen species (ROS)) and profibrotic (collagen) processes (66, 75, 103, 104) (Table 1).

Paracrine-mediated effects are illustrated by the ability of B-cell supernatant purified from SSc patients, rich in IL-6 and TGF- β , to induce fibroblast proliferation, collagen synthesis, and fibroblast-myofibroblast transition.

5.6 B-Cells Participate in Endothelial Cell Activation and Endothelial Damages

Interactions between B-cells, autoantibodies that they produce, and endothelial cells lead to their activation, immune cell homing, and apoptosis (56, 77). Purified IgG from AECA-positive SSc, PAH, or SLE patients is able to upregulate cell expression of various chemoattractant molecules (ICAM-1, VCAM-1, E-selectin, CCL2, or CXCL8), ROS production,

interleukins (IL-6, -8) (56–58) and promotes intima hyperplasia and contributes to vascular injury (75).

6 B-CELL AS THERAPEUTIC TARGET

Many data argue for the involvement of the B-cell in the early phase of SSc, or during some specific SSc-related involvement. Overall, the therapies directly targeting the B-cells, the cytokines produced by the B-cells during SSc, or these costimulation pathways therefore do not work in all patients, despite interesting effects (105). This suggests the potential interest of combined therapies, which have been developed in a few years. Over the past decade, interventional trials of biotherapies have been steadily increasing, representing about one-third of all therapeutic trials.

6.1 Depleting Therapies of B-Cell in SSc

6.1.1 Direct Cytotoxicity of B-cell: The Anti-CD20 Monoclonal Antibody

B-cell depletion in SSc patients is associated with a decrease of the TGF- β pathway, the collagen and myofibroblast accumulation, and the attenuation of PDGFR expression and activation in the skin (106–108). In newborn TSK mice, B-cell depletion prevents skin fibrosis and autoantibody production (88).

Impact of anti-CD20 monoclonal antibody (rituximab RTX) on the course of SSc has been reported in two small randomized and controlled trials, respectively in 16 (versus placebo, ratio 1:1) and in 60 patients (versus cyclophosphamide, ratio 1:1) with early SSc (<2 years of duration) (109, 110). Despite the small number and the need for caution in interpretation, FVC improved slightly in the RTX group compared to the decrease in the cyclophosphamide group, as well as an improvement in mRSS in the RTX group (110). The same trends have been highlighted in RA-related ILD (111, 112). A recent systematic review with meta-analysis has assessed the impact of RTX on the changes in FVC and DLCO parameters in the SSc-ILD from 20 studies (2 RCT, 6 prospective studies, 5 retrospective studies, and 7 conference abstracts) representing 575 SSc patients. In summary, RTX slightly improves FVC and DLCO at 6 and 12 months of follow-up compared to baseline. In the 2 trials comparing RTX to another immunosuppressant, RTX was similar to controls on FVC and DLCO, but exposed to a lower risk of infection (113). Data from the RECITAL RCT (NCT01862926) evaluating RTX versus cyclophosphamide (CYC) in ILD related to SSc (N = 39, 38.6%), an idiopathic inflammatory myositis (N = 45, 44.6%), or mixed connective tissue disease (N = 17, 16.8%), were recently presented at the American Thoracic Society International Conference (ATS). Patients were randomized 1:1 to receive CYC (N = 50) or RTX (N = 51). Concerning the primary endpoint at 24 weeks, both RTX and CYC improved the FVC without the superiority of the RTX but with fewer adverse events and a reduction in corticosteroid exposure compared to CYC (114). Many trials are ongoing or published testing rituximab in SSc-related ILD alone, or in association with mycophenolate mofetil, MMF

(EvER-ILD NCT02990286), or SSc-associated PAH (115). This suggests a potential place for rituximab in the therapeutic arsenal of early forms with skin or ILD manifestations. The results of larger randomized and controlled trials are awaited.

6.1.2 Direct Cytotoxicity of B-Cell: The Anti-CD19 Monoclonal Antibody

A phase I, randomized, placebo-controlled trial testing inebilizumab, an anti-CD19 monoclonal antibody in 28 SSc patients (24 in inebilizumab and 4 in the placebo group), has shown a depletion of peripheral B and plasma cells, with a possible effect on skin fibrosis (mRSS), but 2 serious adverse effects in the inebilizumab group led to the discontinuation of the trial (116).

6.1.3 Impact of Autologous Hematopoietic Stem Cell Transplantation (AHSCT) on B-Cell Homeostasis

Several data have shown an encouraging impact of AHSCT in patients with SSc (117–119). Post transplantation SSc patients have a reduced B-cell division rate, a restoration of the B-cell homeostasis, and a tolerant adaptive immune status. This is supported by post-transplant Breg levels rise, an increased Breg/memory B-cell ratio, with a long-term increase of IL10 production and recover their ability to suppress synthesis of Th1 cytokines by CD4⁺ T cells (120, 121). After transplant, the B-cell subsets show an increase of CD27/IgD⁺ naive B-cell percentage associated with a decrease of CD27⁺/IgD⁺ pre-switch memory, CD27⁺/IgD⁻ post-switched memory B-cells (122), extinction of interferon and inflammation transcriptomic molecular signatures (123), a decrease of IL-6 and TGF- β -producing B-cells (121).

6.2 Therapy Targeting Cytokines Produced by B-Cell

Serum IL-6 levels are increased in SSc patients, especially in cases of aggressive disease (16, 124). The neutralization of IL-6 by monoclonal antibodies or by immunization against a peptide from murine IL-6 in BLM mice has induced a reduction in dermal thickness (124). Additionally, the anti-IL-6 receptor monoclonal antibody (tocilizumab) induces a slightly reduction of collagen fiber bundles in the skin (125).

Based on these preliminary data, two multicenter, randomized, double-blind, placebo-controlled trials assessed the efficacy of tocilizumab on mRSS (primary endpoint) in SSc patients: the phase 2 faSScinate trial [n = 87 (126)] and the phase 3 focuSSced trial [n = 212 (127)]. Both of them failed on the primary endpoint, the difference in the mean change in mRSS. Secondary analysis showed a less consistent decline in predicted FVC in favor of the tocilizumab group. As expected, a shorter disease course and the presence of inflammatory biomarkers were associated with the best response. Its therapeutic place remains to be clarified, and further trials are necessary. The Food and Drug Administration (FDA) has just approved its use in SSc-ILD.

TGF- β appears as a central mediator in organ fibrosis and it is mostly produced by the B-cell in SSc. Its main target is the

fibroblast (4). Data from SSc patients and TGF- β transgenic mice support the rationale for targeting TGF- β (128). Fresolimumab, a pan-specific anti-TGF- β antibody, has been tested in an open-label study on early and diffuse SSc patients. The expression of TGF- β -related genes in the skin of SSc patients decreases after treatment with fresolimumab, as with myofibroblast infiltration and the skin fibrosis (129). These are very preliminary results.

6.3 Therapy Targeting Cytokine Activating B-Cell: BAFF

Belimumab is a recombinant monoclonal antibody targeting soluble BAFF that promotes the B-cell apoptosis. In a TSK mouse model, BAFF antagonist inhibited the synthesis of autoantibodies and skin fibrosis (36). One double-blind randomized and controlled trial, assessed versus placebo in association with MMF, failed on significantly improving mRSS. It showed a decrease in genetic pathways, including BCR, TLR signaling, and profibrotic genes like TGF- β which were overexpressed in improved patients (130). Combined sequential therapy after a rituximab course is currently being studied in dcSSc patients (NCT03844061).

6.4 Proteasome Inhibitor Targeting Plasma Cell: The Bortezomib

Bortezomib, the first-in-class proteasome inhibitor, suppresses activated B-cells, plasma cells, and DC maturation and promotes immune-modulatory effects. This therapeutic class, used particularly in multiple myeloma, is very active and attractive. A human phase 2 trial is being recruited, testing a combination of bortezomib and MMF in SSc-related ILD patients (NCT02370693). In preliminary data on BLM, TSK/+, or Scl-cGVHD mouse models, bortezomib reduced skin infiltration of B-cells and the number of germinal centers, preventing dermal fibrosis with antifibrotic and anti-inflammatory efficacy as well as a T-cell polarization toward a regulatory phenotype (104, 131, 132).

6.5 Others Drugs Targeting B-Cell Intracellular Signaling and Metabolism

Several other treatments are under study in SSc and can target B-cells. For example, some immunosuppressive therapies like pomalidomide are tested in SSc-related ILD in phase 2, randomized, placebo-controlled trials (133). Currently, no studies are underway regarding the assessment of SYK inhibitors in SSc patients. Encouraging studies have been conducted in several murine models (45, 46, 134).

7 CONCLUSION

Many advances have been made in understanding the involvement of B-cells in the pathogenesis of SSc. Besides autoantibody production, B-cells produce profibrotic and proinflammatory cytokines and interact by direct contact with the fibroblast or other immune cells. This is associated with a disorder of the homeostasis of B-cell subsets, membrane or intracellular signaling, arguing for its implication in the proinflammatory and profibrotic mechanisms. Many of these B-cell abnormalities encountered in SSc patients are associated with specific prognostic profiles. Treatments targeting B-cells, through their depletion by cytotoxicity, or the cytokines produced by the B-cells, or their costimulation molecules, seem interesting, probably in certain profiles of early patients with severe organic damage. This requires additional randomized controlled trials.

AUTHOR CONTRIBUTIONS

BT, BC, and LM have made contributions to conception and design of this review, drafting and revising it. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Annexe 3 : Hypertension artérielle pulmonaire (HTAP) associée aux connectivites

**Pulmonary Arterial Hypertension associated with Connective Tissue Diseases
(CTD-PAH): recent and advanced data**

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Abstract.

Pulmonary arterial hypertension (PAH), corresponding to group 1 of pulmonary hypertension classification, is a rare disease with a major prognostic impact on morbidity and mortality. PAH can be either primary in idiopathic and heritable forms or secondary to other conditions including connective tissue diseases (CTD-PAH). Within CTD-PAH, the leading cause of PAH is systemic sclerosis (SSc) in Western countries, whereas systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) are predominantly associated with PAH in Asia. Although many advances have been made during the last two decades regarding classification, definition early screening and risk stratification and therapeutic aspects with initial combination treatment, the specificities of CTD-PAH are not yet clear. In this manuscript, we review recent literature data regarding the updated definition and classification of PAH, pathogenesis, epidemiology, detection, prognosis and treatment of CTD-PAH.

Keywords: connective tissue disease, pulmonary arterial hypertension, epidemiology, early detection, prognosis, PAH targeted therapies, immunosuppressive treatment.

Article highlights.

- Pulmonary arterial hypertension (PAH) is a rare disease and connective tissue diseases (CTD) are the second group of conditions associated with PAH after idiopathic PAH (IPAH).
- The epidemiology of CTD-related PAH (CTD-PAH) varies across the globe with systemic sclerosis (SSc) as the main cause in Western countries contrasting with systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) in Asia.
- PAH is the leading cause of morbimortality in the course of SSc and MCTD.
- The 6th PH World Symposium in 2018 proposed to redefine pulmonary hypertension (PH) from mean pulmonary artery pressure ≥ 25 mmHg to > 20 mmHg; and European Society of Cardiology (ESC) and European Respiratory Society (ERS) 2022 guidelines incorporated a revised cut-off level for pulmonary vascular resistance and a definition of exercise PH.
- Therapeutic advances in the field of PAH have improved outcomes for patients with CTD-PAH, with certain therapeutic specificities, such as the use of immunosuppressants in combination with specific PAH treatments in SLE and MCTD associated PAH.

Introduction.

Pulmonary arterial hypertension (PAH) corresponds to group 1 of pulmonary hypertension (PH) classification, which is identified by performing a right heart catheterization (RHC) (Humbert et al., 2022) and characterized by an increase of pulmonary artery pressure and pulmonary vascular resistance secondary to pulmonary vascular remodeling. PAH occurs either primarily, either idiopathic (idiopathic form, IPAH) or heritable (HPAH), due to a number of identified or unidentified genetic mutations (HPAH), or associated with other diseases, such as connective tissue diseases (CTD) (Humbert et al., 2022). CTD represent a complex and heterogeneous group of systemic autoimmune diseases characterized by dysregulation of the innate and adaptive immune system, which may manifest with various clinical manifestations (*e.g.*, skin, joint, muscle, vessels, etc.). CTD include systemic sclerosis (SSc), systemic erythematosus lupus (SLE), mixed connective tissue disease (MCTD), Sjögren's syndrome (SS), idiopathic inflammatory myopathies (IIM), and rheumatoid arthritis (RA). Some CTD can be associated with PAH with variable prevalence, defining CTD-related PAH (CTD-PAH) representing close to 25% of patients with PAH in USA (McGoon et al., 2013) and 15% in France, corresponding to the second disorders associated with PAH after IPAH (Humbert et al., 2006). Their outcomes may be globally less favorable than other forms (Chung et al., 2010; Humbert et al., 2010). The screening of these patients at risk of developing PAH is important, but differ among CTDs. The early detection may improve survival in patients with SSc-related PAH (Humbert et al., 2011).

In this current review, we will provide data on the update of PAH recommendations, specific data related to SSc-PAH and other CTD-PAH, pathogenesis, epidemiology and prognosis, as well as specificities of their treatment.

Update in definition and classification of Pulmonary Hypertension (PH).

PH is hemodynamically defined during a right heart catheterization and recently reviewed during the 6th PH World Symposium as a mean pulmonary artery pressure (mPAP) greater than 20 mmHg (Simonneau et al., 2019), rather than 25 mmHg previously (Galiè et al., 2016a). In healthy subjects, the normal mPAP at rest was evaluated to 14 ± 3.3 mmHg (Kovacs et al., 2009). After addition of 2 standard derivations, the threshold is therefore established at > 20 mmHg, and associated with poorer outcomes (Assad et al., 2017). SSc patients with previously named “borderline” mPAP (21-24 mmHg) had a higher risk to develop PH in the follow-up (20.9%, hazard ratio 3.7, $p < 0.001$) with a 3-years mortality of 18% (Coghlan et al., 2018; Valerio et al., 2013). Concerning pulmonary vascular resistance (PVR), the normal and prognostically relevant upper limit is 2 Wood units (WU) (Kovacs et al., 2012; Maron et al., 2020).

PH is characterized by an abnormal elevation of pressure in the pulmonary arteries. It can result from various mechanisms and may be associated with a wide range of cardiovascular, respiratory, and systemic diseases. Group 1 of PH corresponds to PAH, including IPAH, heritable PAH, PAH associated with toxins and drugs, CTD-PAH, PAH associated with the human immunodeficiency virus (HIV) infection, congenital heart disease (CHD), a portal hypertension, schistosomiasis, PAH with overt features of venous/capillaries and persistent PH of a newborn syndrome (**Table 1**). Pulmonary hypertension, based on pulmonary artery wedge pressure (PAWP) and PVR is subcategorized into precapillary PH (PAWP ≤ 15 mmHg and PVR > 2 WU), isolated postcapillary PH (PAWP > 15 mmHg and PVR ≤ 2 WU), combined precapillary and postcapillary disease (PAWP > 15 mmHg and PVR > 2 WU), and introduction of exercise PH (with a mPAP/cardiac output slope between rest and exercise > 3 mmHg/L/min) as detailed in **Table 2** (Humbert et al., 2022). PAH is characterized hemodynamically by pre-capillary PH in the absence

of other causes of pre-capillary PH, such as PH associated with lung diseases (group 3), chronic thrombo-embolic pulmonary hypertension (CTEPH, group 4) and PH with unclear or multifactorial mechanisms (group 5). In addition, the need to perform an exercise RHC in normal resting hemodynamics to unmask left ventricular failure is emphasized, particularly in patients with SSc (Stamm et al., 2016). Fluid challenge allows to unmask left ventricular diastolic dysfunction in patients with PAWP \leq 15 mmHg with a clinical phenotype suggestive to left heart disease. This should be considered, especially in the course of SSc, where left ventricular diastolic dysfunction is common (González García et al., 2022; Hui et al., 2021), but additional data are needed (Humbert et al., 2022).

The application of the new criteria on the prevalence of PAH has a low impact, with the reclassification of only 1.4% of SSc patients into the SSc-PAH group with a mPAP $>$ 20 mmHg and a PAWP \leq 15 mmHg (Xanthouli et al., 2020). Due to the multiple mechanisms involved and systemic damage during CTD, they can be implicated in the 5 groups of PH and not exclusively associated with PAH (*e.g.* PH due to left heart disease, due to lung disease/hypoxia, to pulmonary artery obstruction or to unclear and/or multifactorial mechanisms) (Flament et al., 2016; Haque et al., 2021; Szodoray et al., 2012).

Epidemiology of CTD-PAH.

The prevalence of the different subsets of PAH is similar between the different registries, with IPAH as the main cause, accounting for approximately 50-60% of PAH, followed by CTD-PAH (15-30%), then PAH associated with congenital heart disease (CHD-PAH) and associated with portal hypertension (Badesch et al., 2010; Escribano-Subias et al., 2012; Humbert et al., 2006;

Peacock et al., 2007; Thenappan et al., 2007). The proportion of CTD within PAH increases over time (Thenappan et al., 2007), probably related to a higher concern about screening in the CTD population. PAH can occur in several CTD, such as SSc, SLE, MCTD, Sjögren's syndrome with various prevalence. The PAH occurrence is common in the setting of SSc and MCTD, and more rare in the other CTD. The proportion of each underlying connective tissue disease within CTD-PAH varies according to geographical area (**Figure 1**).

In SSc, PAH is frequent and represents a severe complication, occurring in 8 to 12% of cases based on RHC (Avouac et al., 2010; Coghlan et al., 2014; Jais et al., 2008). Its prevalence may be overestimated when echocardiography alone is used to diagnose PAH. There are geographic variations in its prevalence. SSc is the leading cause of CTD-PAH in Europe and North America with a proportion of 60 to 75% (Badesch et al., 2010; Condliffe et al., 2009; Escribano-Subias et al., 2012; Humbert et al., 2006; Peacock et al., 2007). In contrast, in Asia, SSc accounts for only 6 to 22% of patients among CTD-PAH cases, far beyond SLE in terms of frequency (Y.-J. Hao et al., 2014; R. Zhang et al., 2011). Pulmonary disease (ILD and PAH) PAH is the leading cause of morbidity and mortality (Elhai et al., 2017; Tyndall et al., 2010), and sometimes PAH surpasses ILD as the leading cause of SSc-related mortality, depending on the registry (Y. Hao et al., 2016). The frequent coexistence of interstitial lung disease (ILD), pulmonary veno-occlusive disease (PVOD), or left ventricular diastolic dysfunction in the setting of SSc makes the subject of SSc-PAH more complex than other forms such as IPAH of hereditary forms.

In SLE, the prevalence of PAH is low, estimated between 3 and 4% and exclusively based on echocardiography data (T. L. Pan et al., 2000; Prabu et al., 2009; Yang et al., 2013). To our knowledge, there is no large cohort available that has assessed the incidence of PAH in SLE based on RHC. The proportion of PAH associated with SLE in CTD varies depending on the studied

population and geographical location. In southeast Asia, SLE represents the most common cause of CTD-PAH, with a prevalence around 35-60% (Y.-J. Hao et al., 2014; Lin et al., 2020; R. Zhang et al., 2011). In the French pulmonary hypertension registry, the identification of anti-Sm, anti-SSA, and anti-SSB autoantibodies is a risk factor associated with the development of SLE-PAH. In a Chinese cohort of 292 patients with RHC-confirmed SLE-PAH compared to 1986 SLE patients without PAH, the presence of serositis, anti-U1 RNP autoantibodies, and a diffusion capacity of carbon monoxide in the lung (DLCO)/%Predicted < 70% were independently associated with the detection of PAH (N. Zhang et al., 2019). Furthermore, the presence of U1-RNP autoantibodies is associated with better survival (Hachulla et al., 2018). Among Chinese SLE patients and using a clustering method, different risk profiles for PAH have been identified based on SLE characteristics and disease duration. A "vasculitis cluster" phenotype has been identified, characterized by high SLE activity associated with systemic organ involvement and early-onset PAH (usually < 2 years). Conversely, the "vasculopathic cluster" phenotype was characterized by low systemic activity and a purely vascular phenotype with a later-onset PAH and an improved survival (Sun et al., 2019).

In MCTD the prevalence of PAH was reported to be very high, between 17 and 24% in historical series, probably highly overestimated, with patients populations assessed mainly by echocardiography (Alpert et al., 1983; Burdt et al., 1999; Sullivan et al., 1984; Wigley et al., 2005). More recently the prevalence of PH was reported to be much lower, of 3.4% (5/147 patients), with only two patients with PAH (1.4%) (Gunnarsson et al., 2013). In Japan, MCTD is the leading CTD associated with PAH based on RHC, accounting for 43%, followed by SLE (29%). (Shirai et al., 2012). Pericarditis, ILD, thrombocytopenia, anti-Sm autoantibodies reactivity and the absence of polyarthritis were identified as independent predictive factors associated with the presence of PAH

in MCTD. The estimated survival rates at 1-, 5- and 10-years after the PAH diagnosis were 83%, 67% and 56%, respectively (Chaigne et al., 2023). Thus, PAH represents the first cause of mortality in the setting of MCTD (Hajas et al., 2013; Hassan et al., 2023).

In primary Sjögren syndrome (SS), the occurrence of PAH is exceptional in Western countries, and represents a very small proportion of CTD. In contrast, the frequency of PAH associated with SS is high in Asia, as illustrated in Japanese (10%) (Shirai et al., 2012) and Chinese cohorts (15%) (Y.-J. Hao et al., 2014; R. Zhang et al., 2011; Zhao et al., 2017). Raynaud's phenomenon, cutaneous vasculitis, hypergammaglobulinemia and U1-RNP or SSA/Ro autoantibodies reactivities have been reported more frequently in SS-PAH patients compared to SS patients without PAH (Launay et al., 2007).

Regarding the association between rheumatoid arthritis and PAH, the data are much more controversial as it has been shown that the prevalence is similar to the general population. (\approx 0.35%) (Montani et al., 2018). PAH associated with idiopathic inflammatory myopathy are extremely rare in the absence of an overlap syndrome with another CTD (Sanges et al., 2016) and there is a lack of solid epidemiological data based on RHC.

Specificity of CTD-PAH pathogenesis.

CTD can cause pulmonary hypertension (PH) through several mechanisms. SSc is the leading disease that can be accompanied by PAH, but also other forms of PH, such as those related to left ventricular dysfunction (*i.e.* group 2 of PH), frequently with a diastolic component, those related to restrictive interstitial lung disease (*i.e.* SSc-ILD, group 3), or pulmonary veno-occlusive disease (PVOD) (Haque et al., 2021; Young, Vummidi, et al., 2019). The underlying

pathophysiological mechanisms are distinct, but all of them can lead to right heart failure and poor prognosis. Mutations in PAH have been identified in heritable PAH, IPAH, PVOD/pulmonary capillary haemangiomas (PCH) and anorexigen-associated PAH but not in CTD-PAH (Morrell et al., 2019). The histological hallmarks of PAH encountered in IPAH are represented by plexiform lesions, in situ thrombosis, concentric intimal proliferation and media thickening with muscularization of small arteries (Humbert et al., 2019).

In the SSc-PAH, the histopathological phenotype is more fibrotic and less inflammatory, with predominantly fibrous occlusion and intimal thickening of medium-sized arteries and branching small vessels (Sasaki et al., 2011). Fibrinoid vasculitis and plexiform lesions are rare. Moreover, the vascular remodeling involving pulmonary veins and veinules is more common, illustrated by a high occurrence of PVOD, which is classified in the group 1 of PH and represented by severe patchy congestion of capillaries and occult alveolar hemorrhage (Overbeek et al., 2009). PVOD is illustrated on the high-resolution chest CT scan by the presence of lymph node enlargement, centrilobular ground-glass opacities and septal lines (Günther et al., 2012). In SSc- and MCTD-PAH, the association of arteriolar and venular involvement is more common than in PAH without CTD, with a prevalence of 75% compared to 17%. Venular obstructive involvement predominates in veins and preseptal veinules (Dorfmueller et al., 2007; O'Callaghan et al., 2011). Histopathological characteristics of MCTD-PAH are rather close to those of SSc, with fibrous intimal thickening and rare fibrinoid vasculitis and plexogenic pulmonary arteriopathy.

The histopathological lesions encountered in SLE-PAH are different from those encountered in SSc-PAH and substantially identical to those observed in IPAH. Plexogenic pulmonary arteriopathy and fibrinoid vasculitis with immune complex deposition of IgG, IgM, C3 and C1q have been observed in the vessel wall, particularly in intima and media. In contrast, a

rather rare to slight presence of fibrotic lesions was observed (Quismorio et al., 1984; Sasaki et al., 2011; Yeo & Sinniah, 1975). Phenomena of additional thrombosis are possible, with intraluminal occlusion by thrombi in the more proximal pulmonary arteries in the context of chronic thromboembolic PH (*i.e.* CTEPH, group 4 of PH), particularly in the setting of an underlying antiphospholipid syndrome (Lang & Madani, 2014).

Few data exist on histopathologic characteristics specifically associated with SS and no conclusions can be drawn.

Other inflammatory markers are found in the setting of PAH. They were first described in IPAH (Humbert et al., 1995). An increased inflammatory signature has been demonstrated, mainly in IPAH and HPAH. This is illustrated by the synthesis of various pro-inflammatory molecules by fibroblasts, endothelial cells and vascular smooth muscle cells, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)- α and chemokines (*eg.* chemokine ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1), RANTES chemokines or fractalkine/CX3CL1). (Cracowski et al., 2014; Dorfmueller et al., 2002; Humbert et al., 1995; Perros, Dorfmueller, Souza, Durand-Gassel, Godot, et al., 2007; Sanchez et al., 2007). Beyond PAH, this pro-inflammatory signature has also been described in the pathophysiology of SSc, with the involvement of oxidative stress and the production of numerous pro-inflammatory molecules (Thoreau et al., 2021). Moreover, an accumulation of immune cells including macrophages, dendritic cells, CD8⁺ and CD4⁺ T-cells, B-cells, has demonstrated in media and adventitia tunics of vascular remodeling (Cohen-Kaminsky et al., 2014; Dorfmueller et al., 2007; Perros, Dorfmueller, Souza, Durand-Gassel, Mussot, et al., 2007; Perros et al., 2012; Savai et al., 2012) in IPAH and CTD-PAH (Huertas et al., 2020; van Uden et al., 2019). In addition, T-cell phenotype dysregulations were observed in IPAH, HPAH and SSc-PAH, with a defect in regulatory T-cell functions and polarization towards a pro-

inflammatory Th17 phenotype (Huertas et al., 2016). Finally, the description of neo-organization into ectopic tertiary lymphoid follicles has been observed along the pulmonary vascular walls during IPAH (Perros et al., 2012), and to a lesser extent few organized lymphocytes in the lungs of some patients with SSc-PAH (Dumoitier et al., 2017).

In addition, autoantibodies have been identified in PAH, primarily documented in IPAH and SSc-PAH (Bussone et al., 2012; Dib et al., 2012; Karmochkine et al., 1996; Negi et al., 1998; Quismorio et al., 1984; Tamby et al., 2005, 2006; Terrier et al., 2008, 2010). These autoantibodies in SSc target the pulmonary endothelial cell (anti-endothelial cell antibodies, AECA), the fibroblast, and the vascular smooth muscle cell (anti-VSMC antibodies) (Thoreau et al., 2022). Anti-fibroblast antibodies (AFA) were detected in 40% of sera from patients with IPAH and in 30% of sera from patients with SSc-PAH (Tamby et al., 2006). The target antigens of these autoantibodies were screened by 2D electrophoresis, followed by immunoblot on specific protein extracts, and identified by mass spectrometry. These target antigens, including heat shock proteins (HSP)-27 and 70, calumenin, glucose-6-phosphate dehydrogenase, or phosphatidylinositol 3-kinase, and are involved in the regulation of cytoskeletal functions, in cellular contraction, in oxidative stress, and in cellular energy metabolism (Terrier et al., 2008).

Anti-endothelial cell antibodies were also found in SSc patients with and without PAH, and in IPAH patients (Dib et al., 2012). These antibodies identified various proteins and exhibited distinct reactivity patterns (Tamby et al., 2005). Some indicated potential roles in inducing a pro-inflammatory state in pulmonary endothelial cells through the synthesis of IL-6, or a pro-adhesive signal with (E-selectin, intercellular adhesion molecule-1 or MCP-1) (Arends et al., 2013). Anti-endothelin-1 receptor (ETAR) and angiotensin-1 receptor (AT1R) autoantibodies from sera of

patients with CTD-PAH increase pulmonary arteriolar vasoconstriction capacity (Becker et al., 2014).

Furthermore, anti-VSMC antibodies were identified. Patient serum IgG caused these cells to exhibit enhanced *in vitro* contractions in a collagen matrix. The implicated target antigens were identified as being stress-induced phosphoprotein-1 (STIP-1) and alpha-enolase (Bussone et al., 2012). It is thus hypothesized that these autoantibodies, through various mechanisms on the cells of the pulmonary vascular wall, contribute to vascular remodeling.

Prognosis of CTD-PAH.

The PAH, all causes confounding, displays a poor prognosis, with a 1-, 3-, 5- and 7-years survival rate of 85%, 68%, 57% and 49% respectively (Benza et al., 2012). Studies on several registers have assessed risk scores in PAH, including the REVEAL register, Swedish PAH register, COMPERA and the French Pulmonary Hypertension Network (Benza et al., 2015; Boucly et al., 2017; Hoeper et al., 2017; Humbert et al., 2006, 2010; Kylhammar et al., 2018). The WHO FC, pericardial effusion, high BNP levels, low DLCO and 6MWD appear as major predictors of mortality, whatever the underlying form of PAH (Farber et al., 2015; McGoon & Miller, 2012).

Historically within PAH, CTD-PAH displayed a worse survival compared to IPAH, with an over risk evaluated to 1.59 (McGoon & Miller, 2012). Patients with CTD-PAH displayed better hemodynamic parameters than IPAH, but a lower 6-min walk distance (6MWD) and DLCO, a higher prevalence of pericardial effusion, a higher B-natriuretic peptide (BNP) levels, and a lower 1-year survival and freedom from hospitalization compared to IPAH, evaluated to 86 vs 93% ($p<0.0001$) and 67 vs 73% ($p=0.03$) respectively (Chung et al., 2010).

Within CTD-PAH, SSc-PAH presents a unique phenotype. Many studies highlighted lower outcomes in SSc-PAH compared to IPAH or other CTD-PAH without SSc, and a poorer survival (Khanna et al., 2021). SSc-PAH patients are older compared to SLE and MCTD patients, are more frequently Caucasians, without difference according gender. Moreover, SSc-PAH showed similar hemodynamics but a higher proportion of WHO functional class (FC) IV, higher BNP levels, lower DLCO and significantly the poorest 1-year survival (82% in SSc-PAH vs 94% in SLE-PAH), and no significant compared to MCTD-PAH (88%) (Chung et al., 2010). Recent European data from COMPERA registry found a worse survival of SSc-PAH patients compared to SLE-related and MCTD- PAH (Distler et al., 2023). Regarding SLE-PAH, the data of French pulmonary hypertension registry established a 3- and 5-year survival of 89% and 84%, respectively (Hachulla et al., 2018).

Several factors have been identified as predictors of mortality during CTD-PAH. In SSc-PAH studied in the UK registry, age, male gender, lower mixed venous oxygen saturation and high WHO functional class were independent predictors of a poorer survival (Condliffe et al., 2009). The American PHAROS registry found male gender (Hazard Ratio, HR 3.11), diffuse cutaneous form (HR 2.12), systolic PAP on echocardiography (HR 1.06), mean PAP on right heart catheterization (HR 1.03), 6-min walk distance (HR 0.92), and DLCO (HR 0.65) as predictors of worse survival in SSc-PAH (Kolstad et al., 2018). The association of male gender and risk of death from SSc-PAH was identified in the French cohort (HR 4.76). (Launay et al., 2013).

In the SLE-PAH French registry, the anti-U1 RNP reactivity was predictive of a better survival (Hachulla et al., 2018, p. 201). In the Chinese CSTAR-PAH cohort, cardiac index was the only independent predictor of survival. Patients with a serositis or six-minute walk distance > 380 meters displayed a benefit to intensive immunosuppressive therapy and better outcome (Qian et

al., 2019). In a French study, 10-years survival was found to be equivalent between MCTD-PAH, SSc-PAH and SLE-PAH (Chaigne et al., 2023). In this French cohort, tobacco is the only predictive factor associated with death in MCTD-PAH.

Overall, the previously accepted idea that the response to PAH targeted therapies was lower in CTD-PAH compared to other PAHs is changing with the accumulation of recent data and the generalization of combination therapy (Coghlan et al., 2017; Gaine et al., 2017; Humbert et al., 2017). A recent meta-analysis including 11 randomized controlled trials (1,267 patients with CTD-PAH) and 19 registries (4,008 CTD-PAH patients) showed that PAH targeted therapies induced a 36% reduction of morbidity/mortality risk in CTD-PAH patients (HR = 0.64; 95% CI, 0.51-0.81) compared to controls and similar to overall PAH population. Therapeutic advances over the past 10 years are associated with a better prognosis. Patients with CTD-PAH treated after 2010 have a better survival than those treated before 2010 (73% vs 65%) (Khanna et al., 2021).

Early detection of CTD-PAH and evaluation strategies.

The symptoms of PAH are aspecific and it may be asymptomatic for a long time. During CTD, dyspnea or fatigue, both on exertion and at rest, can be secondary to several organ damage (*i.e.*, interstitial lung disease, PAH, heart or musculoskeletal involvement, anemia, etc.). PAH is a serious complication in CTD with a great impact on prognosis (Fei et al., 2014; Tyndall et al., 2010). The difference in the prevalence of PAH within various CTD explains a different screening strategy depending on the underlying CTD. In essence, there is a validated regular screening strategy for asymptomatic SSc patients, and a targeted screening approach, driven by a high-risk phenotype or the presence of suggestive signs for other CTD.

In SSc, PAH prevalence and the annual incidence of approximately 1.5% justifies a systemic screening strategy (Nihtyanova et al., 2014; Vandecasteele et al., 2017). The cumulative incidence of PH increases with the duration of follow-up, reaching 18% in the diffuse cutaneous form and 24% in the limited cutaneous form after 15 years of disease duration (Nihtyanova et al., 2014). In patients with SSc, PAH is often diagnosed at an advanced stage, with severe functional and hemodynamic impairment (Weatherald et al., 2019). PAH, accounting for approximately 30% of SSc-related deaths, thus becomes a major screening concern (Steen & Medsger, 2007). Early screening of SSc for PAH demonstrated an impact on prognosis in a detection cohort compared to symptomatic patients, diagnosed by routine practice. Patients with an early detection of PAH displayed a lower New York Heart Association (NYHA) functional class, lower mPAP and PVR, a higher cardiac index and a better survival (1-year survival rate 100% versus 75%, and 8-year survival rate 64% versus 17% respectively) (Humbert et al., 2011). More recently, the PHAROS study showed a better survival rate with a 1-, 3- and 5-years survival rate of 95%, 75% and 63% respectively, probably related to earlier and more systematic screening and follow-up (Kolstad et al., 2018), in line with data from the French registry (Weatherald et al., 2018).

In other CTD, a systematic screening is not recommended due to the lower prevalence. The PAH screening is warranted when suggestive clinical predictors are present, especially with the use of echocardiography. Confirmation through RHC remains the gold standard for the diagnosis of PAH. In the presence of overlap between SSc and another CTD, the annual screening strategy applied to SSc is recommended. (Humbert et al., 2022).

Screening methods are multiple, based on clinical evaluation (*e.g.* WHO functional class), biomarkers, pulmonary function test, echocardiography, or a combination of all (Coghlan et al.,

2014; Frost et al., 2019; Humbert et al., 2011; Meune et al., 2016; Morrisroe et al., 2017, p. 20). In patients with unexplained dyspnea or symptoms/signs that raise suspicion of PH, a comprehensive and step-by-step diagnostic approach should be taken into consideration (diagnostic algorithm). In step 1 (suspicion), the evaluation includes a patient interview with a collection of family history, a clinical examination to identify suggestive signs and record cardiopulmonary vital signs (*e.g.*, blood pressure, oximetry, etc.), a resting electrocardiogram, and blood tests with measurements of BNP/NT-pro-BNP. This step aims to determine the suspicion of potential pulmonary or cardiac disease. Step 2 (detection) includes non-invasive pulmonary tests such as pulmonary function test (PFT) and echocardiography. The peak tricuspid regurgitation velocity (TRV, at least ≥ 2.8 m/s without additional sign) and other additional suggestive signs that indicate the likelihood of PH, such as the enlargement of the right heart chambers, are used for the assessment of PH probability, as detailed in **Table 3**. This step is used to assign a risk level for PH (low, intermediate, or high). Step 3 (confirmation) involves performing a RHC in patients with an intermediate or high risk level to confirm PH and determine its subgroup (Humbert et al., 2022).

In SSc patients, screening only based on echocardiography or other tests alone lack sensitivity. Several algorithms based on clinical characteristics, echocardiography, and biochemical measurements such as BNP/NT-pro-BNP have been developed to identify patients who should undergo RHC, as the DETECT (Coghlan et al., 2014) or the Australian Scleroderma Interest Group (ASIG) (Thakkar et al., 2012) algorithms, with an equal predictive accuracy (Y. Hao et al., 2015). Recommendation for the screening of PAH-SSc is emphasized in **Table 4**. With regard to the application of the DETECT algorithm, a patient with course > 3 years from first non-Raynaud's symptom, forced vital capacity (FVC) $\geq 40\%$ and a predicted DLCO $< 60\%$, in step 1 a combination of current or past presence of telangiectasias, PFT parameters (FVC %

predicted/DLCO % predicted ratio), right axis deviation on electrocardiogram, presence of anti-centromere antibodies (ACA), serum urate, and N-terminal-pro-brain natriuretic peptide (NT-pro-BNP) levels is collected. In step 2, two echocardiographic variables were included (right atrium area and TRV) in order to determine the need to perform a RHC. The DETECT algorithm is more efficient than echocardiography alone in detecting patients at risk of PAH and requiring RHC. The DETECT algorithm missed 4% of PAH patients against 29% with the 2009 European Society of Cardiology/European Respiratory Society guidelines (Coghlan et al., 2014). The systematic screening of PAH is included in the guidelines for SSc management (Hachulla et al., 2021; Khanna et al., 2013). Therefore, it is recommended to screening patients with clinical assessment, PFT including DLCO, transthoracic echocardiogram, and NT-pro-BNP measurement in all patients with SSc and scleroderma spectrum disorders such as MCTD or CTD with features of SSc (Humbert et al., 2022; Khanna et al., 2013; Young, Nagaraja, et al., 2019) .

All detection methods suffer from limitations, with their own sensitivities and specificities. The threshold for referring to an RHC reference center must therefore be low, in order to achieve a low rate of false negatives. In the setting of increased sPAP upon echocardiography, RHC is the gold standard to confirm PH and subtypes including PAH, based on hemodynamic parameters, including the measurement of mPAP, PAWP and PVR (Humbert et al., 2022; Simonneau et al., 2019). This is an invasive but relatively safe procedure with an overall adverse event rate of 1.1% (95% CI 0.8% to 1.3%) with a majority of mild to moderate intensity of complications (Hoepfer et al., 2006).

Update in PAH targeted therapies in CTD-PAH.

Alongside specific treatments for PAH, general measures combine maintaining physical activity and supervised rehabilitation and nutritional status, vaccination against influenza or SARS-CoV-2 viruses, use of oxygen on a case-by-case basis or diuretics if necessary, correction of iron-deficiency anemia by intravenous iron supplementation, education and psychosocial support (Humbert et al., 2022). Regarding PAH targeted therapies, the recent advances do not relate to the discovery of new therapeutic targets but in the combination of specific treatments along with a therapeutic escalation based on improved risk stratification. Lung transplantation may be the final step in the most severe and advanced cases after maximum medical treatment (Galiè et al., 2016a, 2019; Humbert et al., 2022).

PAH targeted therapies include endothelin receptor antagonists (ERAs), phosphodiesterase 5 inhibitors (PDE5i) and the soluble guanylate cyclase stimulator (riociguat) targeting the nitric oxide pathway, prostacyclin analogs (PCAs) (*e.g.* iloprost, epoprostenol, etc.) and a prostacyclin receptor agonist (selexipag). The use of specific PAH targeted therapies for CTD-PAH is comparable to that recommended for IPAH. This strategy is based on assessing mortality risk using a multi-stratified model (low, intermediate, or high risk) and concurrently implementing a therapeutic escalation strategy that depends on the reevaluation of risk in terms of treatment response (Humbert et al., 2022). This gradual therapeutic strategy is summarized in **Figure 2**. Performing a vasoreactivity test during RHC, recommended for idiopathic, heritable, and drug-associated forms, is not recommended for CTD-PAH. Calcium channel blockers are therefore not a preferred treatment option in the therapeutic strategies for CTD-PAH. Briefly and in accordance with the risk stratification strategy, PAH patients without cardiopulmonary comorbidities and evaluated as low or intermediate risk in an expert center should start an initial oral combination

with an ERA and a PDE5i while a high-risk patient will begin an initial combination including intravenous prostacyclin analog. Patients with cardiopulmonary comorbidities, regardless of the risk classification, should initiate monotherapy with PDE5i or ERA. Patients require regular follow-up, including risk stratification and drug tolerance assessment.

During the follow-up, the patients who achieve a low-risk profile with the initial strategy should maintain the combination associated with a structured follow-up. If the initial strategy results to intermediate-risk profile while ERA/PDE5i combination, prostacyclin receptor agonist (selexipag) should be added or the PDE5i should be switched to soluble guanylate cyclase stimulator (riociguat). In patients who are at intermediate–high or high risk, adding intravenous epoprostenol or subcutaneous/intravenous treprostinil should be considered. These patients should be referred to a lung transplantation center (Humbert et al., 2022). Recently, a new therapeutic pathway has been explored via a fusion protein targeting the extracellular domain of the receptor for activin type IIA (ActRIIA), a growth factor of the TGF- β pathway. Two recent randomized placebo-controlled trials involving around 15% of patients with CTD-PAH demonstrated the efficacy of sotatercept in reducing pulmonary vascular resistance and improving walking distance at 24 weeks, in association with other PAH-specific treatments (Hoepfer et al., 2023; Humbert et al., 2021).

To date, 43 randomized controlled trials (RCTs) have been published about PAH targeted therapies, including 21 RCTs testing a monotherapy vs placebo, 20 RCTs testing patients already treated with sequential combination therapy vs background therapy plus placebo, 2 RCTs testing in treatment-naïve patients an initial combination vs an initial monotherapy (Galiè et al., 2019; Hoepfer et al., 2023; Humbert et al., 2021). Published RCTs including at least 10% of patients with CTD-PAH, WHO/NYHA classes III and IV, and more than 30 patients in overall are summarized

in the **Table 5**. The primary endpoint evolved over time, moving from the 6MWD difference to more robust and clinically relevant endpoints such as clinical deterioration (*e.g.* hospitalization, etc.) or composite endpoint including mortality. It should also be noted that intermediate criteria such as the 6MWD can be influenced by other components than the PAH during a CTD, such as musculoskeletal damage.

In a meta-analysis including 25 RCTs, whatever the cause of PAH, a PAH specific monotherapy initiated in treatment-naïve patients was associated with an improvement of exercise capacity, hemodynamics and a 43% reduction of mortality (RR = 0.57; 95% CI, 0.35 to 0.92) compared to placebo (Galiè, Manes, et al., 2009). Thus, combination therapy can be used sequentially or initially. In incident treatment-naïve PAH patients, an initial combination therapy compared to monotherapy improve symptoms, exercise capacity and outcome (Galiè et al., 2015; Hassoun et al., 2015). In prevalent PAH patients already treated, sequential combination therapy strategy improve exercise capacity, hemodynamics and outcome compared to PAH patients without modification of the previous treatment, and that was associated with a reduction of clinical deterioration risk of -35% (RR = 0.65; 95% CI, 0.58 to 0.72)) (Lajoie et al., 2016; Pulido et al., 2013; Sitbon et al., 2015a).

To date, 6 RCTs have specifically analyzed data in patients with CTD-PAH (SUPER, PHIRST, ARIES, PATENT, GRIPHON, AMBITION) (Badesch et al., 2007; Coghlan et al., 2017; Fischer et al., 2016; Gaine et al., 2017; Galiè et al., 2017; Humbert et al., 2017). Taken together, sildenafil or tadalafil in monotherapy or add-on of background therapy (Badesch et al., 2007; Galiè et al., 2017), ambrisentan in monotherapy (Fischer et al., 2016), riociguat or selexipag in monotherapy or add-on (Gaine et al., 2017; Humbert et al., 2017), or combination of ambrisentan and tadalafil (Coghlan et al., 2017) improve exercise capacity (6MWD and WHO FC),

hemodynamic parameters, quality of life in patients with CTD-PAH. Based on five RCTs, the response to the combination therapy is actually evaluated as similar between CTD-PAH and IPAH for time to a morbidity or mortality event (McLaughlin et al., 2020). Initial combination therapy reduced the risk of clinical failure as well for CTD-PAH (HR = 0.43; 95% CI 0.24 to 0.77) as for SSc-PAH (HR = 0.44; 95% CI 0.22 to 0.89) (Coghlan et al., 2017). Thus, combination therapy was also benefit for CTD-PAH compared to monotherapy, with a 51.7% risk reduction of clinical failure in CTD-PAH except SSc and 53.7% for SSc-PAH (Kuwana et al., 2020). This is consistent with other meta-analysis data taking into account the 6 RCTs that included CTDs (J. Pan et al., 2018). Recent data from COMPERA regarding SSc-PAH, survival was also better with initial combination of ERA and PED5i than a monotherapy with ERA or PDE5i (Distler et al., 2023).

Immunosuppressive treatment.

Few studies have investigated the impact of the use of glucocorticoids (GCs) and/or immunosuppressants during CTD-PAH (Ding et al., 2022) with 6 cohorts (Jais et al., 2008; Miyamichi-Yamamoto et al., 2011; Qian et al., 2019; Sanchez et al., 2006; Tanaka et al., 2002; Yasuoka et al., 2018) 1 RCT for the rituximab (Zamanian et al., 2021), one open-label for the tocilizumab (Toshner et al., 2022), and 1 cases series published (Kato et al., 2011).

Cyclophosphamide with or without glucocorticoids. One retrospective study on 28 patients with CTD-PAH (6 SSc, 13 SLE, 8 MCTD and 1 RA) reviewed the response of monthly i.v.bolus of cyclophosphamide (600 mg/m²) for at least 3 months (79% also received GCs). Cyclophosphamide was prescribed as the first-line monotherapy without PAH targeted therapy, and assessed on the maintenance of NYHA I-II functional class after at least 1 year with sustained

hemodynamic improvement and without additional specific therapy of PAH. The global response was 29%, and in subgroups, with none in SSc-PAH, 38.4% in SLE-PAH and 37.5% in MCTD-PAH (Sanchez et al., 2006). Another study assessed the response to first-line monthly i.v. pulse of 600 mg/m² cyclophosphamide for 6 months associated with oral GCs in 16 patients with SLE/MCTD-PAH and or in combination with vasodilators in seven patients. The responders represented 50% of patients treated with a first-line immunosuppressant, and 57% of patients in combination with a vasodilator (Jais et al., 2008). Miyamichi-Yamamoto et al. assessed the combination of cyclophosphamide and GCs for 12-18 months in 13 patients with CTD-PAH (7 SLE, 1 MCTD, 1 SSc and 4 SS) compared to 8 CTD-PAH patients without immunosuppressant (1 SLE, 3 MCTD, 2 SSc, 1 rheumatoid arthritis and 1 dermatomyositis), all under conventional vasodilator treatment. The response was defined by mPAP reduction <25 mmHg or a reduction from baseline >10% evaluated every 6-12 months (Miyamichi-Yamamoto et al., 2011). Patients without immunosuppressant displayed unchanged mPAP and PVR. In contrast, immunosuppressants reduced mPAP (40±9 to 29±11 mmHg, P<0.01), and trended to reduce PVR. Then, immunosuppressive therapy associated with GCs seems to be more efficient than GCs alone. The simultaneous diagnosis of PAH and CTD, and the use of immunosuppressants in addition to GCs might be independent predictors of WHO functional class improvement at 3 months (Yasuoka et al., 2018). The combined use of cyclophosphamide and corticosteroids could provide a hemodynamic and clinical benefit in patients with SLE- or CTD-PAH (Jais et al., 2008), whereas their use is not recommended in PAH associated with SSc (Humbert et al., 2022; Sanchez et al., 2006). Among responders to immunosuppressants, patients with SLE-PAH are the most frequent (evaluated around 48%), followed by patients with MCTD-PAH (19%), then SS-PAH and no response for SSc-PAH (Ding et al., 2022). Other variables were identified as associated with a better benefit to immunosuppressants, such as a WHO/NYHA I-II functional class, a higher cardiac

index and a lower PVR (Jais et al., 2008; Sanchez et al., 2006). The presence of serositis at baseline (N. Zhang et al., 2019) and early diagnosis or early initiation of the immunosuppressant were also associated with a better response (Yasuoka et al., 2018). The design of mainly retrospective studies, with few RCTs and small numbers, does not allow us to reach a high level of proof and draw conclusions on the expected benefit. Their use therefore relies on personalized, individual analysis.

Other conventional immunosuppressants. A little phase IIa placebo-controlled RCT (TransformPAH) was conducted in 23 patients with PAH, whose 22% with CTD, and tested at 16 weeks the safety and tolerability of tacrolimus as primary outcome (Spiekerkoetter et al., 2017). Tacrolimus was well-tolerated and seemed to increase the BMPR2 signal. There is no more complete data. No strong data exist regarding the use of methotrexate, mycophenolate mofetil or cyclosporine. A phase I clinical trial is ongoing testing an mTOR inhibitor (ABI-009) in 25 patients with severe PAH (NCT02587325).

The monoclonal antibody rituximab. A multicenter, double-blinded, randomized, placebo-controlled, proof-of-concept trial, in 57 patients with SSc-PAH on stable-dose standard medical therapy, comparing two infusions of 1,000 mg rituximab spaced 15 days apart against same regimen with placebo. A change in 6MWD as primary outcome at 24 weeks was observed in favor of rituximab (23.6 ± 11.1 vs 0.5 ± 9.7 meters), but without significant statistically difference (Zamanian et al., 2021). Rituximab might be an effective therapeutic alternative to improve functional capacity in SSc-PAH patients. Additional data is required.

Anti-IL-6 receptor antibody tocilizumab. One phase II open-label trial has been recently published (Toshner et al., 2022). Intravenous tocilizumab (8 mg/kg every 4 weeks) over 6 months, was administered in 19 patients with idiopathic/heritable PAH and 10 CTD-PAH patients. The primary outcome measure was the incidence of combined adverse events along with the change in

PVR at 6 months. No change in PVR was demonstrated, and four patients had to discontinue the treatment due to a serious adverse event.

Other biological treatments. Experimental data on monocrotaline rat model suggested a potential benefit of anakinra (recombinant IL-1 receptor antagonist) in the prevention of PAH development (Voelkel et al., 1994). A single-group, open-label phase IB/II pilot study evaluated the feasibility and safety of anakinra (100 mg subcutaneously daily for 14 days) in 7 enrolled patients with IPAH, and with proved right ventricle failure (Trankle et al., 2019, p. 1). Anakinra in patients with PAH and right ventricle failure is safe, and further studies are needed to assess its therapeutic potential. Perhaps it would be necessary to target patients with markers of inflammation as those who may benefit most from treatment with anti-IL-1. Remember that the anakinra often has a purely suspensive effect. Regarding other targeted therapies such as belimumab (anti-BAFF antibody) or JAK inhibitors, no controlled trials have been published. Ruxolitinib, an anti-Jak1 and Jak2 inhibitor attenuated the elevation of pulmonary arterial pressure, partially reduced the right ventricle hypertrophy and partially restored the cardiac index in two experimental models of PAH and may represent a novel therapeutic option (Yerabolu et al., 2021).

Anticoagulation.

Concerning the use of anticoagulant treatment during PAH, it is not a standard of care, and the data are contradictory but the international guidelines do not support the use of anticoagulants in the therapeutic strategy of PAH (Humbert et al., 2022).

The presence of in situ thrombosis in the small caliber pulmonary vessels of transplant or autopsy specimens from patients with SSc-PAH is the basic assumption for a potential benefit of

anticoagulant treatment. Some works seem to suggest that anticoagulants have a beneficial effect on survival in CTD-PAH (Australian multicenter cohort) (Ngian et al., 2012). A meta-analysis from 2014, analyzing 9 studies (2 prospective and 7 retrospective) with none RCT, found a benefit on survival, with a reduction mortality risk of 31% (HR = 0.69; 95% CI, 0.57-0.82) (Caldeira et al., 2014).

In contrast, some data on 275 patients with SSc-PAH (28% treated by warfarin) and 155 patients with IPAH (59% under warfarin) give anticoagulation an unfavorable prognostic impact on mortality (Canadian cohort) (Johnson et al., 2012). The COMPARE study, covering 1283 incident cases including 208 with SSc, did not find any benefit on survival from anticoagulation (Olsson et al., 2014). This is also supported by the REVEAL registry on 187 PAH, including 43 SSc-PAH, with the absence of benefit of the use of warfarin on survival ($p=0.15$). Increased mortality was even found in patients who received warfarin in the previous year or during follow-up (Preston et al., 2015). A meta-analysis of 12 non-randomized trials (2512 patients with PAH), including 4 on CTD-PAH (509 CTD-PAH with 97% of SSc), concluded in the CTD-PAH subgroup an absence of reduction in mortality (HR = 1.16; 95% CI, 0.58, 2.32), in contrast to IPAH subgroup (HR = 0.73; 95% CI, 0.56, 0.95) (Khan et al., 2018). In 2020, a systematic review with meta-analysis specifically on IPAH and including 8 studies without RCT, did not show a significant decrease of mortality risk (HR = 0.80; 95% CI, 0.60, 1.06) (Wang et al., 2020).

The only phase III, placebo-controlled RCT (SPHInX study), comparing 2.5 mg apixaban with placebo twice daily for 3 years (ratio 1:1) in SSc-PAH patients, with a composite primary outcome (time to death or clinical worsening of PAH) is ongoing (Calderone et al., 2016). There is an urgent need for randomized controlled trials to answer this question.

Conclusion.

PAH is a major cause of morbidity and mortality during the CTD. Large prospective cohorts and clinical trials provided many knowledges of the specificities related to CTD-PAH. Their epidemiology is different depending on the area of the world, with a predominance of SSc in the West, and SLE and MCTD in Asia. PAH remains a dramatic condition despite the advances over the past 20 years in the field of early detection based on composite tools, the risk stratification including multiple clinical, exercise, right ventricular function and hemodynamic components. This risk stratification allows for a graduated initiation a step by step therapeutic strategy based actually on combination drug therapies with PAH targeted therapies and potentially the use of immunosuppressants in SLE- and MCTD-PAH that may benefit from them.. Although major progresses have been made in the setting of CTD-PAH, many questions remain. One of the persistent challenges in patients with CTDPH is the management in the event the coexistence of several groups of PH. Knowledges of the specific features of CTD-PAH could pave the way for new therapeutic strategies. A multidisciplinary and integrative approach in expert center is necessary to overcome these complex diseases.

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Legend to Figures.

Figure 1: Spectrum of pulmonary arterial hypertension associated with connective tissue diseases (CTD-PAH)

Pulmonary arterial hypertension (PAH) associated with connective tissue diseases (CTD) is a heterogeneous group of diseases. Several CTD can be associated with the occurrence of PAH. The respective contributions of each CTD within the group of PAH-CTD are indicated. MCTD: mixed connective tissue disease; SLE: systemic lupus erythematosus; SS: Sjogren's syndrome; SSc: systemic sclerosis; UCTD: undifferentiated connective tissue disease.

Figure 2: Evidence-based pulmonary arterial hypertension treatment algorithm for patients with connective tissue disease-associated pulmonary arterial hypertension

CTD-PAH, PAH associated with connective tissue disease; DLCO, lung diffusion capacity for carbon monoxide; ERA, endothelin receptor antagonist; i.v., intravenous; PCA, prostacyclin analogue; PDE5i, phosphodiesterase 5 inhibitor; PH, pulmonary hypertension; PRA, prostacyclin receptor agonist; s.c., subcutaneous; sGCs, soluble guanylate cyclase stimulator.

^aCardiopulmonary comorbidities are conditions associated with an increased risk of left ventricular diastolic dysfunction, and include obesity, hypertension, diabetes mellitus, and coronary heart disease; pulmonary comorbidities may include signs of mild parenchymal lung disease and are often associated with a low DLCO (< 45% of the predicted value).

^bIntravenous epoprostenol or i.v./s.c. treprostinil.

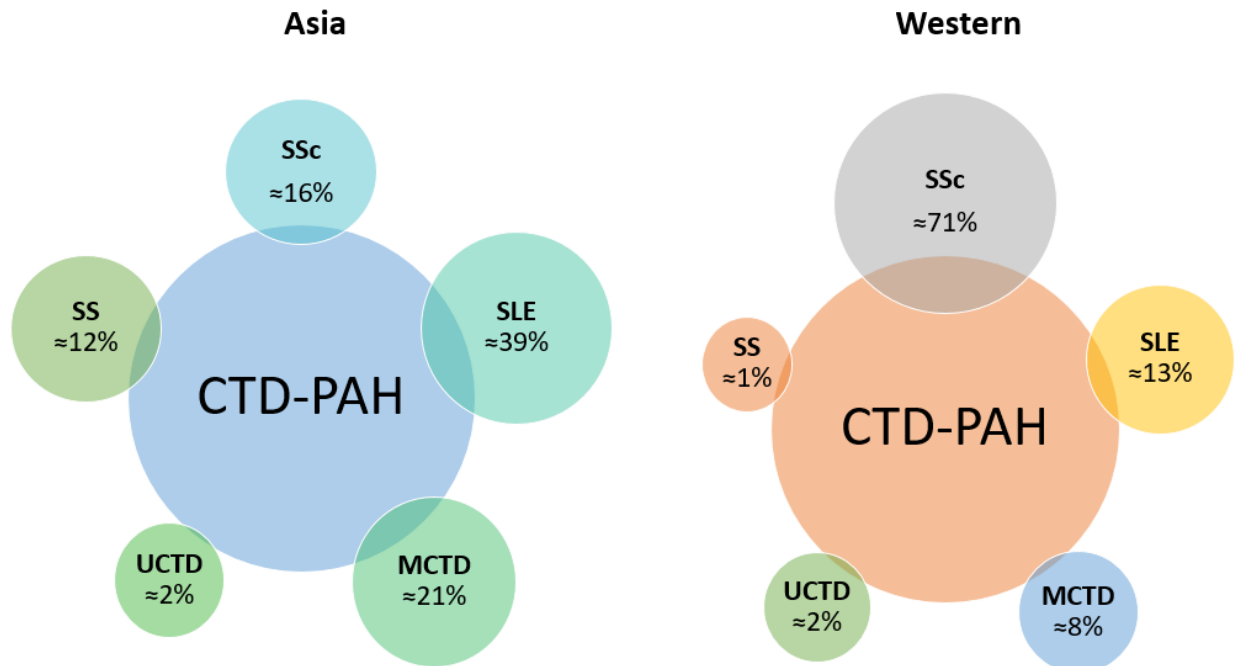


Figure 1: Spectrum and proportion of pulmonary arterial hypertension associated with connective tissue diseases (CTD-PAH) around the world.

Legend of Figure 1.

Pulmonary arterial hypertension (PAH) associated with connective tissue diseases (CTD) is a heterogeneous group of diseases. Several CTD can be associated with the occurrence of PAH. The respective contributions of each CTD within the CTD-PAH group are shown from the averages of three Asian (Y.-J. Hao et al., 2014; Shirai et al., 2012; R. Zhang et al., 2011) and three Western cohorts (Chung et al., 2010; Condliffe et al., 2009; Humbert et al., 2006). The diameter of the circles illustrates the proportion of each CTD.

MCTD: mixed connective tissue disease; SLE: systemic lupus erythematosus; SS: Sjogren's syndrome; SSc: systemic sclerosis; UCTD: undifferentiated connective tissue disease.

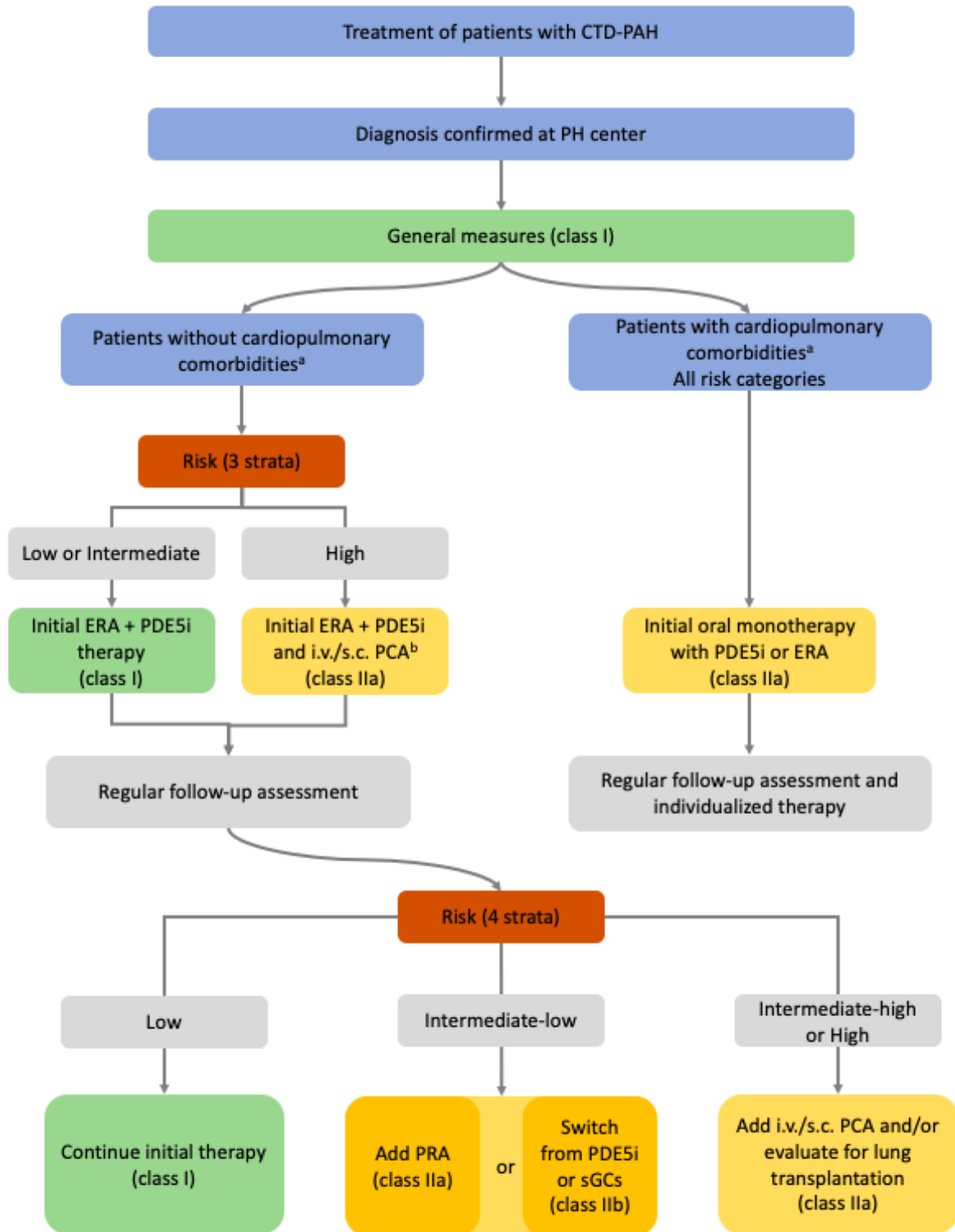


Figure 2: Evidence-based pulmonary arterial hypertension treatment algorithm for patients with connective tissue disease-associated pulmonary arterial hypertension (adapted from Humbert et al., 2022)

Legend of Figure 2.

CTD-PAH, PAH associated with connective tissue disease; DLCO, lung diffusion capacity for carbon monoxide; ERA, endothelin receptor antagonist; i.v., intravenous; PCA, prostacyclin analogue; PDE5i, phosphodiesterase 5 inhibitor; PH, pulmonary hypertension; PRA, prostacyclin receptor agonist; s.c., subcutaneous; sGCs, soluble guanylate cyclase stimulator.

^aCardiopulmonary comorbidities are conditions associated with an increased risk of left ventricular diastolic dysfunction, and include obesity, hypertension, diabetes mellitus, and coronary heart disease; pulmonary comorbidities may include signs of mild parenchymal lung disease and are often associated with a low DLCO (< 45% of the predicted value).

^bIntravenous epoprostenol or i.v./s.c. treprostinil.

Table 1: Updated classification of pulmonary hypertension (PH).

Based on 2022 ESC/ERS guidelines (from Humbert et al., 2022)

1. Group 1: Pulmonary arterial hypertension (PAH)
1.1 Idiopathic PAH:
1.1.1 Non-responders at vasoreactivity testing
1.1.2 Acute responders at vasoreactivity testing
1.2 Heritable PAH
1.3 PAH associated with drugs and toxins
1.4 PAH associated with:
1.4.1 Connective tissue disease
1.4.2 HIV infection
1.4.3 Portal hypertension
1.4.4 Congenital heart disease
1.4.5 Schistosomiasis
1.5 PAH with overt features of venous/capillaries (PVOD/PCH) involvement
1.6 Persistent PH of the newborn syndrome (PPHN)

2. Group 2: PH due to left heart disease
2.1 Heart failure:
2.1.1 PH due to heart failure with preserved LVEF
2.1.2 PH due to heart failure with reduced LVEF
2.2 Valvular heart disease
2.3 Congenital/acquired cardiovascular conditions leading to post-capillary PH

3. Group 3: PH due to lung diseases and/or hypoxia
3.1 Obstructive lung disease or emphysema
3.2 Restrictive lung disease
3.3 Other lung diseases with mixed restrictive/obstructive pattern
3.4 Hypoventilation disorders
3.5 Hypoxia without lung disease (e.g. high altitude))
3.6 Developmental lung disorders

4. Group 4: PH due to pulmonary artery obstruction
4.1 Chronic thromboembolic PH (CTEPH)
4.2 Other pulmonary artery obstructions

5. Group 5: PH with unclear and/or multifactorial mechanisms
5.1 Hematological disorders (e.g. chronic hemolytic anemia, myeloproliferative disease)
5.2 Systemic disorders(e.g. sarcoidosis, pulmonary Langerhans’s cell histiocytosis, and type 1 neurofibromatosis)
5.3 Metabolic disorders (e.g. glycogen storage disease and Gaucher disease)
5.4 Chronic renal failure with or without hemodialysis
5.5 Pulmonary tumor thrombotic microangiopathy
5.6 Fibrosing mediastinitis

CTEPH : Chronic thromboembolic pulmonary hypertension; ERS: European Respiratory Society; ESC: European Society of Cardiology; HIV: Human immunodeficiency virus; LVEF: left ventricular ejection fraction; PAH: pulmonary arterial hypertension; PCH: pulmonary capillary haemangiomatosis; PH: pulmonary hypertension; PPHN: persistent pulmonary hypertension of the newborn; PVOD: pulmonary veno-occlusive disease.

Table 2: Hemodynamic definition of pulmonary hypertension (PH) with revised criteria (adapted from Galiè et al. Eur Heart J 2016 & Humbert et al. Eur Respir J 2022).

Definition	2015 ESC/ERS characteristics (Galiè et al., 2016b)	2022 ESC/ERS revised characteristics (Simonneau et al., 2019)	Clinical group(s)
PH	mPAP \geq 25 mmHg	PAPm $>$ 20 mmHg	All groups
Pre-capillary PH	mPAP \geq 25 mmHg PAWP \leq 15 mmHg	mPAP $>$ 20 mmHg PAWP \leq 15 mmHg PVR $>$ 2 WU	Group I: PAH Group III: PH due to lung disease Group IV: CTEPH Group V: PH with unclear or multifactorial mechanisms
Post-capillary PH	mPAP \geq 25 mmHg PAWP $>$ 15 mmHg		Group II: PH due to left heart disease Group V: PH with unclear or multifactorial mechanisms
<i>Isolated post-capillary PH</i>	DPG $<$ 7 mmHg and/or PVR \leq 3 WU	mPAP $>$ 20 mmHg PAWP $>$ 15 mmHg PVR \leq 2 WU	
<i>Combined post- and pre-capillary PH</i>	DPG \geq 7 mmHg and/or PVR $>$ 3 WU	mPAP $>$ 20 mmHg PAWP $>$ 15 mmHg PVR $>$ 2 WU	
<i>Exercise PH</i>	-	mPAP/CO slope between rest and exercise $>$ 3 mmHg/L/min	

CO: Cardiac output; CTEPH: Chronic thrombo-embolic pulmonary hypertension; DPG: diastolic pressure gradient (diastolic PAP – mean PAWP); ERS: European Respiratory Society; ESC: European Society of Cardiology; PAP: pulmonary arterial hypertension; mPAP: mean pulmonary artery pressure; PAP: pulmonary artery pressure; PAWP: pulmonary artery wedge pressure; PH: pulmonary hypertension; PVR: pulmonary vascular resistance; WU: Wood unit.

Table 3: Echocardiographic signs and probability of pulmonary hypertension (adapted from Humbert et al. Eur Respir J 2022)

Peak tricuspid regurgitation velocity	Additional echocardiographic signs suggestive of PH ^a	Echocardiographic probability of PH
≤ 2.8 m/s or not measurable	No	PH probability Low
≤ 2.8 m/s or unmeasurable	Yes	PH probability Intermediate
2.9-3.4 m/s	No	
2.9-3.4 m/s	Yes	PH probability High
> 3.4 m/s	Not required	

^aOther signs:

- **Category A: Ventricles:**
 - Right ventricle/left ventricle (RV/LV) basal diameter ratio >1.0
 - Flattening of the interventricular septum (LV eccentricity index >1.1 in systole and/or diastole)
 - TAPSE/sPAP ratio < 0.55 mm/mmHg
- **Category B: Pulmonary artery:**
 - RV outflow doppler acceleration time < 105 m/s and/or mid-systolic notching
 - Early diastolic pulmonary regurgitation velocity > 2.2 m/s
 - PA diameter > aorta root diameter or PA diameter > 25 mm
- **Category C: Inferior vena cava and right atrium:**
 - Inferior cava diameter > 21 mm with decreased inspiratory collapse (< 50 % with a sniff or < 20 % with quiet inspiration)
 - Right atrial area (end-systole) > 18 cm²

LV: left ventricle; m/s, meter/second; PH, pulmonary hypertension; RV, right ventricle; sPAP, systolic pulmonary arterial pressure; TAPSE, tricuspid annular plane systolic excursion;

Signs from at least two categories (A/B/C) must be present to alter the level of echocardiographic probability of PH.

Table 4: Guidelines for screening and enhanced screening of systemic sclerosis-associated pulmonary arterial hypertension (adapted from Humbert et al., 2022)

<u>Recommendation</u>	<u>Class</u>^a	<u>Level</u>^b
In patients with SSc, an annual evaluation of the risk of having PAH is recommended	I	B
In adult patients with SSc with > 3 years' disease duration, an FVC \geq 40%, and a DLCO < 60%, the DETECT algorithm is recommended to identify asymptomatic patients with PAH	I	B
In patients with SSc, where breathlessness remains unexplained following non-invasive assessment, RHC is recommended to exclude PAH	I	C
Assessing the risk of having PAH, based on an evaluation of breathlessness, in combination with echocardiogram or PFT and BNP/NT-proBNP, should be considered in patients with SSc	IIa	B
Policies to evaluate the risk of having PAH should be considered in hospitals managing patients with SSc	IIa	C
In symptomatic patients with SSc, exercise echocardiography or CPET, or CMR may be considered to aid decisions to perform RHC	IIb	C
In patients with CTD with overlap features of SSc, an annual evaluation of the risk of PAH may be considered	IIb	C
<p>BNP, brain natriuretic peptide; CMR, cardiac magnetic resonance; CPET, cardiopulmonary exercise testing; CTD, connective tissue disease; DLCO, diffusion capacity of the lungs for carbon monoxide; FVC, forced vital capacity; NT-proBNP, N-terminal pro-brain natriuretic peptide; PAH, pulmonary arterial hypertension; PFT, pulmonary function tests; RHC, right heart catheterization; SSc, systemic sclerosis.</p> <p>^aClass of recommendation. ^bLevel of evidence.</p>		

Table 5: Characteristics of patients in RCTs including CTD-PAH patients.

Study	Year	Drug	No. of patients N	CTD-PAH N/%	SSc N/%*	SLE N/%*	MCTD N/%*	Age (years), mean±SD	Female, %	WHO FC III-IV, %	6MWD (meters), mean±SD‡	mPAP (mmHg), mean±SD‡	PAWP or PCWP (mmHg), mean±SD‡	PVR dyne/sec/cm ⁵ #, mean±SD‡	Background treatment for PAH† %
Combination															
AMBITION (Coghlan et al., 2017; Galiè et al., 2015)	2015	Combination	500	187/37%	118/63%	17/9%	23/12%	55±15	78%	69%	353±90	90±13	9±3	#825±435	Yes/5%
COMPASS-2 (McLaughlin et al., 2015)	2015	Combination	334	88/26%	NA	NA	NA	54±16	76%	58%	360±76	NA	NA	NA	!No
FREEDOM-EV (White et al., 2020)	2020	Combination	690	178/26%	NA	NA	NA	45±16	79%	34%	396±96	NA	NA	NA	Yes/100%
ERAs															
BREATHE-1 (Rubin et al., 2002)	2002	Bosentan	213	63/30%	57/75%	16/25%	NA	48±16	79%	100%	337±75	54±17	9±4	#947±609	No
ARIES-1/2 (Fischer et al., 2016; Galiè et al., 2008)	2008	Ambrisentan	393	124/32%	76/61%	18/15%	25/20%	61±15	82%	60%	345±80	49±14	NA	#886±509	No
SERAPHIN (Pulido et al., 2013)	2013	Macitentan	742	224/30%	NA	NA	NA	46±16	77%	47%	360±100	54±18	10±3	#1026±697	Yes/64%

Study	Year	Drug	No. of patients N	CTD-PAH N/%	SSc N/%*	SLE N/%*	MCTD N/%*	Age (years), mean±SD	Female, %	WHO FC III-IV, %	6MWD (meters), mean±SD‡	mPAP (mmHg), mean±SD‡	PAWP or PCWP (mmHg), mean±SD‡	PVR dyne/sec/cm ⁵ #, mean±SD‡	Background treatment for PAH† %
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PDE5i

SUPER-1 (Badesch et al., 2007; Galiè et al., 2005)	2005	Sildenafil	278	84/30%	38/45%	19/23%	8/10%	49±15	75%	61%	344±81	53±15	NA	#981±504	No
PHIRST (Galiè, Brundage, et al., 2009; Galiè et al., 2017)	2009	Tadalafil	405	95/23%	NA	NA	NA	54±15	78%	67%	344±76	53±13	NA	#879±434	Yes/53%

sGCS

PATENT-1/2 (Ghofrani et al., 2013; Humbert et al., 2017)	2013	Riociguat	443	111/25%	66/59%	18/16%	10/9%	51±17	61%	54%	365±72	91±13	9±4	#813±465	Yes/50%
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Prostacyclin Rc agonist

GRIPHON (Gaine et al., 2017; Sitbon et al., 2015b)	2015	Selexipag	1156	334/29%	170/51%	82/25%	37/11%	48±15	80%	53%	353±80	NA	NA	NA	Yes/80%
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Prostacyclin analogs

Study	Year	Drug	No. of patients N	CTD-PAH N/%	SSc N/%*	SLE N/%*	MCTD N/%*	Age (years), mean±SD	Female, %	WHO FC III-IV, %	6MWD (meters), mean±SD [‡]	mPAP (mmHg), mean±SD [‡]	PAWP or PCWP (mmHg), mean±SD [‡]	PVR dyne/sec/cm ⁵ #, mean±SD [‡]	Background treatment for PAH [†] %
Simonneau et al. (Simonneau et al., 2002)	2002	SC Treprostinil	469	50/19%	NA	NA	NA	45±1	81%	89%	326±6	61±1	10±0.3	26±1 U m ²	No
Olschewski et al. (Olschewski et al., 2002)	2002	Inhaled iloprost	206	33/16%	NA	NA	NA	52±13	67%	100%	324±95	53±13	8±4	1035±441	No
Barst et al. (Barst et al., 2003)	2003	Beraprost	116	12/10%	NA	NA	NA	42±2	86%	48%	439±11	56±2	9±1	21±2 U m ²	No
FREEDOM-C (Tapson et al., 2012)	2012	Oral Treprostinil	350	92/26%	NA	NA	NA	50±NA	81%	79%	345±74	NA	NA	NA	Yes/50%
<u>ActRIIA targeting</u>															
PULSAR (Humbert et al., 2021)	2021	Sotatercept	106	18/17%	NA	NA	NA	48±14	87%	47%	398±83	52±12	13±3	#7787±349	Yes/100%
STELLAR (Hoepfer et al., 2023)	2023	Sotatercept	323	48/15%	NA	NA	NA	48±15	79%	51%	401±82	53±14	10±3	#764±359	Yes/100%

ActRIIA: activin receptor type IIA; CTD: connective tissue diseases; 6MWD: 6-minute walk distance; MCTD: Mixed connective tissue diseases; mPAP: mean pulmonary artery pressure; N: number; NA: not available; PAH: pulmonary arterial hypertension; PAWP: pulmonary artery wedge pressure; PCWP: pulmonary capillary wedge pressure; Prostacyclin Rc agonist: Prostacyclin receptor agonist; PVR: pulmonary vascular resistance; RCTs: Randomized controlled trials; SC: subcutaneous; SD: standard deviation; sGCQ: Guanylate cyclase stimulators; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; WHO FC: World Health Organization functional class;

AMBITION: Ambrisentan plus Tadalafil in PAH; GRIPHON: Prostacyclin Receptor Agonist (Prostaglandin I2) in PAH; SERAPHIN: Study with an Endothelin Receptor Antagonist in PAH to Improve Clinical Outcome; PHIRST: PAH and Response to Tadalafil; ARIES: Randomized, Double-blind, Placebo-controlled, Multicenter, Efficacy Study of Ambrisentan for PAH (1 and 2); PATENT: PAH Soluble Guanylate Cyclase–Stimulator Trial 1; SUPER-1: Sildenafil Use in PAH; BREATHE-1: Bosentan Randomized Trial of Endothelin Antagonist Therapy; COMPASS-2: Combination of Bosentan and Sildenafil

Study	Year	Drug	No. of patients N	CTD-PAH N/%	SSc N/%*	SLE N/%*	MCTD N/%*	Age (years), mean±SD	Female, %	WHO FC III-IV, %	6MWD (meters), mean±SD‡	mPAP (mmHg), mean±SD‡	PAWP or PCWP (mmHg), mean±SD‡	PVR dyne/sec/cm ⁵ #, mean±SD‡	Background treatment for PAH† %
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Versus Sildenafil Monotherapy on PAH; FREEDOM-EV: International, Multicenter, Randomized, Double-blind, Placebo-controlled Event-driven Trial of Oral Treprostinil in Subjects with PAH; PULSAR: multicenter, randomized, doubleblind, phase 2 trial testing sotatercept versus placebo, in patients receiving receiving stable background therapy; STELLAR: multicenter, randomized, double-blind, phase 3 trial testing sotatercept versus placebo, in patients receiving receiving stable background therapy.

‡mean±SD of compared arms;

*Percent were calculated among the total number of CTDs;

†No PAH medication other than sildenafil within 3 months prior to randomization;

‡Background treatment included endothelin receptor antagonists (ERAs), phosphodiesterase 5 inhibitors (PDE5i), prostacyclin analogs (PCAs) (e.g. iloprost, epoprostenol, etc.), a prostacyclin receptor agonist (selexipag), and the soluble guanylate cyclase stimulator (riociguat);

