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# Les auto-anticorps : marqueurs immunologiques de l'hétérogénéité de la sclérodermie systémique

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**JURY**

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**RESUME**

La sclérodermie systémique (ScS) est une connectivite associant atteinte vasculaire, auto-immunité et fibrose. Cette pathologie est associée à une morbi-mortalité importante, et les ressources thérapeutiques sont limitées. La physiopathologie de la ScS n'est que partiellement connue, mais il apparait que les liens entre le système immunitaire et la fibrose sont étroits. Ainsi, la ScS peut être considérée comme un modèle prototypique d'étude des liens immunité-fibrose. Il s'agit d'une maladie hétérogène, c'est-à-dire que les phénotypes cliniques présentés par les patients sont variables, rendant complexe l'établissement d'une classification des patients en groupes homogènes. Mieux comprendre cette hétérogénéité est un préalable indispensable à la constitution d'endotypes, permettant l'étude des mécanismes physiopathologiques propres à chacun d'entre eux.

L'objectif de cette Thèse a été de mieux appréhender cette hétérogénéité clinique et d'étudier la place des marqueurs immunologiques, en particulier les auto-anticorps, en tant que biomarqueurs de cette hétérogénéité.

Le premier travail a été une classification sans a priori des patients de la cohorte européenne EUSTAR (European Scleroderma Trials and Research Group) par une analyse en cluster sur 24 variables sélectionnées (atteintes cliniques, auto-anticorps). Deux puis 6 groupes de patients homogènes ont été obtenus, dont la survie était significativement différente. Ce travail a suggéré qu'il existait des groupes homogènes de patients au-delà de la dichotomie historique forme cutanée diffuse vs. limitée. La présence d'atteintes viscérales et le statut des auto-anticorps apparaissent comme des éléments importants dans la constitution des groupes.

Le deuxième travail s'est intéressé au dosage des chaines légères libres sériques (serum free light chain : SFLC) dans la ScS. Le taux de SFLC est plus élevé chez les patients ScS que chez les contrôles et est associé à des paramètres de gravité de la

maladie tels que le score de Rodnan, les scores d'activité, les pressions pulmonaires et la DLCO. Cette étude apporte des arguments supplémentaires pour évoquer la participation active des lymphocytes B à la physiopathologie de la ScS.

Nous avons ensuite réalisé une estimation de la prévalence des anticorps anti-ARN polymérase de type III dans notre cohorte de patients avec ScS avant d'inclure ces données dans une revue systématique avec méta-analyse. Ce travail a montré que la prévalence de ces anticorps était hétérogène entre les centres. Les facteurs potentiels pouvant expliquer une partie de cette hétérogénéité sont des facteurs géographiques, suggérant l'implication de facteurs génétiques et/ou environnementaux.

Le dernier travail a été dédié à l'hypertension artérielle pulmonaire (HTAP) des connectivites, en particulier de la ScS. A partir d'une cohorte de patients provenant du centre de référence de l'HTAP du Royaume-Uni, les anticorps anti-U1RNP ont été analysés en tant que marqueur pronostique. Ces anticorps sont associés de façon significative à une meilleure survie des patients avec connectivite et HTAP. Dans la ScS, on observe une tendance vers une meilleure survie également chez les patients porteurs de ces anticorps.

Les auto-anticorps constituent donc des biomarqueurs diagnostiques et pronostiques puissants dans la ScS. Ils permettent de mieux cerner l'hétérogénéité de cette pathologie, et devraient probablement être intégrés dans les futures classifications. Leur rôle pathogénique reste encore à démontrer. Les perspectives de notre travail sont d'identifier de nouveaux auto-anticorps et d'étudier leurs effets sur le fibroblaste, cellule effectrice centrale de la fibrose.

**TITRE EN ANGLAIS**

Auto-antibodies in systemic sclerosis: immunological markers of heterogeneity

**RESUME EN ANGLAIS**

Systemic sclerosis (SSc) is a connective tissue disease (CTD) characterized by vasculopathy, auto-immunity and fibrosis. This condition is associated with a significant morbi-mortality, and the therapeutic armamentarium is limited. The pathological mechanisms of SSc are partially known, but the links between the immune system and fibrosis appear tight. ScS can be considered as a prototypical model for the study of the links between immunity and fibrosis. There is a high heterogeneity in SSc. The clinical phenotypes of patients are highly variable, making the classification of patients into homogeneous groups complex. A better understanding of this heterogeneity could lead to the identification of endotypes, which are needed to study the pathophysiological processes of each group.

This PhD Thesis aimed to better decipher this clinical heterogeneity and to assess the immunological components, in particular auto-antibodies, as biomarkers of heterogeneity.

The first work was a without any a priori cluster analysis in the EUSTAR (European Scleroderma Trials and Research Group) cohort using 24 selected variables (encompassing clinical involvement and auto-antibodies). Two then 6 homogeneous clusters were obtained, with significantly different survival curves. We suggested that there could be homogeneous groups of patients beyond the classical dichotomy diffuse cutaneous form vs. limited. Organ involvement as well as antibody status were suggested to play a major role in defining homogeneous groups of patients with different prognosis.

Our second work assessed the value of serum free light chain (SFLC) in SSc. The

SFLC level was higher in SSc patients than in controls and was associated with severity parameters, such as Rodnan skin score, disease activity score, pulmonary pressures and DLCO. This study suggested that B cells could play an active role in the mechanisms of SSc.

Then we estimated the prevalence of anti-RNA polymerase III antibodies in SSc in our cohort of patients followed by a systematic review with meta-analysis. We showed that the prevalence was highly heterogeneous between centers. Potential factors explaining partly the observed heterogeneity were geographical factors, which underscore the probable implication that genetic background and environmental factors play a role.

Finally, we focused on CTD-associated pulmonary arterial hypertension (PAH) in a large cohort of patients from the United Kingdom national reference center for PAH. We assessed whether anti-U1 RNP antibodies could be a prognostic factor in CTD-associated PAH with a focus on SSc- associated PAH. Anti-U1RNP antibodies were significantly associated with a decreased mortality in CTD-PAH patients. There was a trend towards a decreased mortality in SSc-PAH patients with anti-U1RNP antibodies.

Auto-antibodies are strong biomarkers of diagnosis and prognosis in SSc. They allow to partly capture the clinical heterogeneity of this condition, and should be integrated in the future classifications of patients. Their pathogenic role remains to be shown. We plan to identify new auto-antibodies in SSc and to study their direct effects on the fibroblast, which is the key effector cell of fibrosis.

# Abréviations

ACR : American College of Rheumatology
BAFF : B-cell activating factor
CM : connectivite mixte
CMH : complexe majeur d'histocompatibilité
CRP : C-reactive protein
CTGF : connective tissue growth factor
CVF : capacité vitale forcée
dcSSc : sclérodermie systémique cutanée diffuse
DLCO : capacité de diffusion du transfert du monoxyde de carbone
EGF : epidermal growth factor
EUSTAR : European Scleroderma Trials and Research Group
GI : atteinte gastro-intestinale
HTAP : hypertension artérielle pulmonaire
IC95% : intervalle de confiance à 95%
Ig : immunoglobulines
IL-6 : interleukine-6
IMIDs : immune-mediated inflammatory diseases
IPBDF : immunoprotéomique bidimensionnelle en fluorescence
lcSSc : sclérodermie systémique cutanée limitée
LES : lupus érythémateux systémique
MCP : articulation métacarpo-phalengienne
OR : odds ratio
PDGF : platelet-derived growth factor
PID : pneumopathie interstielle diffuse
RT-PCR : Reverse Transcriptase – Polymerase Chain Reaction
ScS : sclérodermie systémique
SFLC : serum free light chain
SRC : scleroderma renal crisis
SSc : systemic sclerosis
TGF-β : transforming growth factor beta
Treg : lymphocytes T régulateurs
VEGF : vascular endothelial growth factor

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# PARTIE I – CONTEXTE SCIENTIFIQUE

## I.1 Fibrose et auto-immunité

La fibrose correspond au mode de cicatrisation pathologique d'un tissu après une agression tissulaire. Alors qu'une réponse fibrosante peut sembler adaptée à court terme, la prolongation et l'entretien au cours du temps des mécanismes de fibrose par une inflammation chronique aboutit à une dysfonction cellulaire et une défaillance de l'organe cible. La fibrose intervient dans la physiopathologie de diverses affections telles que le diabète, certaines infections, la maladie alcoolique du foie, le cancer, l'athérosclérose, le rejet d'organe après transplantation, l'obésité et les maladies inflammatoires à composante auto-immune (IMIDs : immune-mediated inflammatory diseases) (**Figure 1**). On considère actuellement que la fibrose est responsable d'environ un tiers de la mortalité mondiale globale, avec par ordre de décroissance des étiologies : les maladies rénales chroniques, la cirrhose et les IMIDs (Longo et al., 2015).

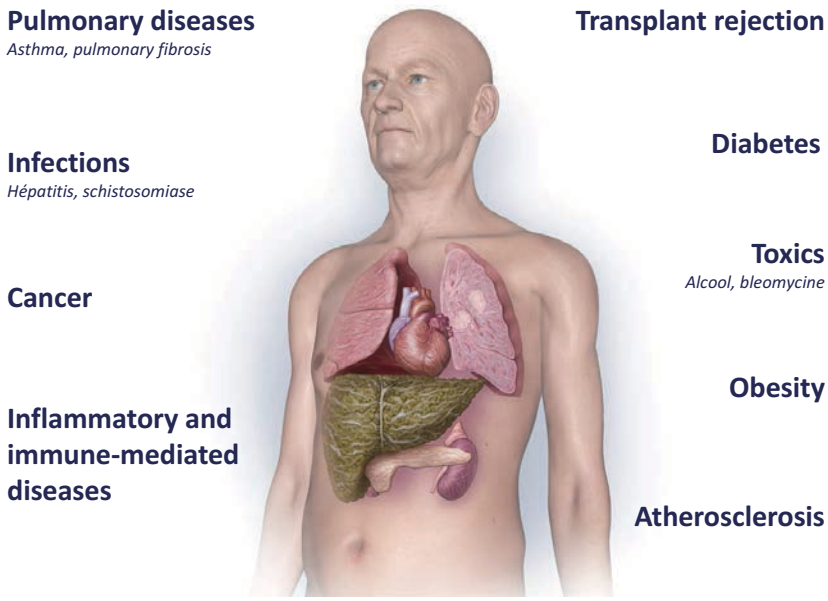



**Figure 1 :** Grandes causes d'atteintes fibrosantes des organes majeurs, d'après Longo et al., 2015

### I.1.1 Le processus fibrosant : conséquences et besoins cliniques

Quatre phases se succèdent lors de la réponse fibrosante. La première est l'initiation de la réponse, influencée par l'agression de l'organe ou du tissu. La seconde phase est l'activation des cellules effectrices et la troisième correspond à la synthèse de matrice extra-cellulaire ; le dépôt actif (et la résorption insuffisante) de matrice extra-cellulaire provoque la progression de la fibrose et finalement l'insuffisance d'organe.

Le fait que différentes maladies touchant divers organes se caractérisent par la présence d'une fibrose suggère des mécanismes pathogéniques communs. Cette réponse fibrosante est orchestrée par différents types cellulaires et des voies moléculaires spécifiques. Les composants cellulaires incluent les cellules inflammatoires (par exemple macrophages et lymphocytes T), les cellules épithéliales, les cellules endothéliales et les cellules dites « effectrices ». Ces dernières regroupent les fibroblastes, les myofibroblastes, les fibrocytes et possiblement des cellules derivées des tissus épithéliaux (après transition épithélio-mésenchymateuse). Au-delà des types cellules, certaines voies moléculaires sont centrales : par exemple la voie du transforming growth factor beta (TGF-β) est cruciale quel que soit le type de fibrose.

Bien que nous identifiions de plus en plus les mécanismes fibrosants, il existe très peu de thérapeutiques effectives, et encore moins ciblant de façon spécifique la fibrogenèse. La compréhension de la physiopathologie de la fibrose apparait donc comme essentielle pour traiter cette complication terminale grevée d'une importante morbi-mortalité (Longo et al., 2015).

### I.1.2 Interactions entre le système immunitaire et le processus fibrosant

Le système immunitaire intervient dans la régulation de la fibrose (**Figure 2**), notamment par de nombreuses interactions entre le fibroblaste/myofibroblaste et :

- Les cellules de l'immunité innée : neutrophiles, macrophages, éosinophiles, mastocytes et plaquettes
- Les cellules de l'immunité adaptative : lymphocytes Th1, Th2, Th17, Treg et lymphocytes B (Van Linthout et al., 2014).

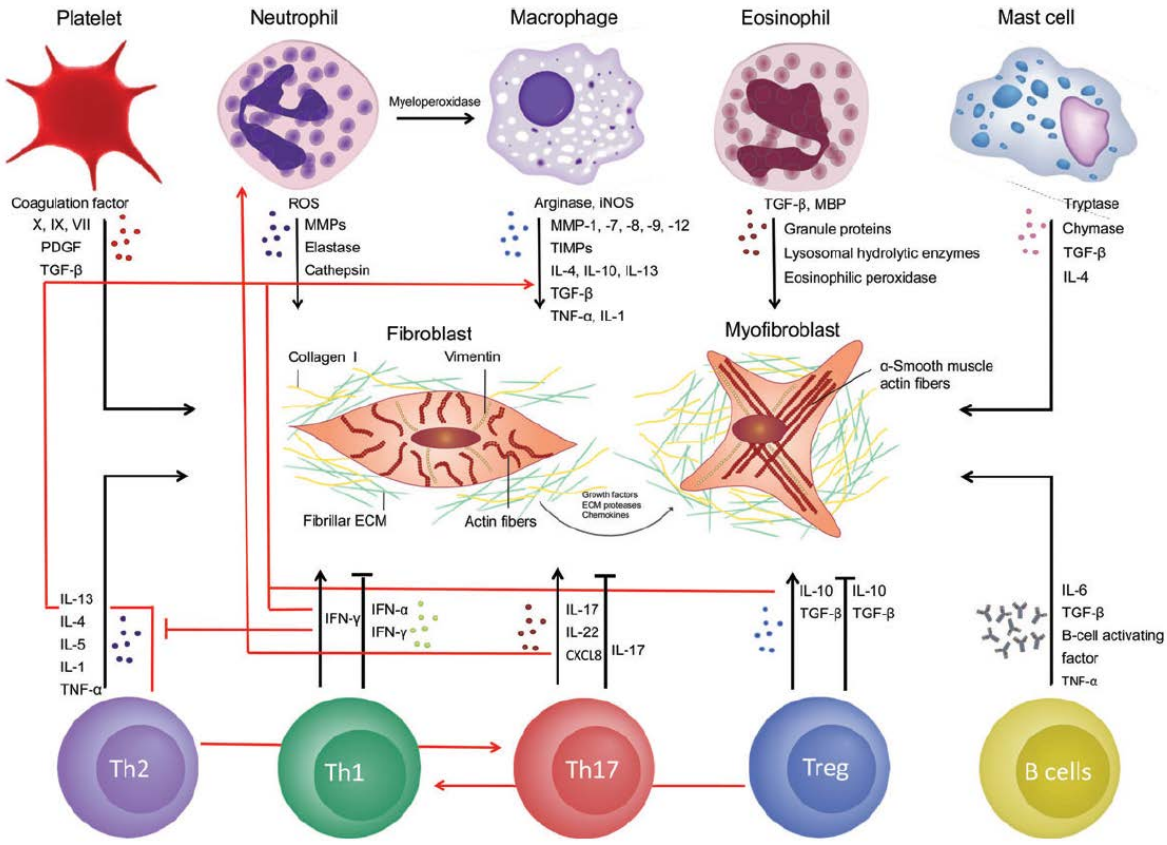



**Figure 2 :** Influence des cellules de l'immunité innée et adaptative sur le fibroblaste/myofibroblaste, d'après Van Linthout et al., 2014

### I.1.3 Place du lymphocyte B

Le rôle du système immunitaire humoral dans le processus fibrosant est encore peu connu. Néanmoins, il a été montré que le fibroblaste pouvait sécréter le facteur activant des lymphocytes B (B-cell activating factor : BAFF). En réponse, le lymphocyte B sécréterait des cytokines pro-fibrosantes telles que le TGF-β et l'interleukine-6 (IL-6) (Sanges et al., 2017). Le rôle pathogénique des anticorps est encore largement

méconnu, bien que certains travaux suggèrent un effet direct sur le fibroblaste (Van Linthout et al., 2014).

## I.2 La sclérodermie systémique : un modèle prototypique de fibrose ?

La sclérodermie systémique (ScS) est une maladie auto-immune systémique. Il s'agit d'une pathologie rare dont la prévalence est estimée entre 7 et 489 par million d'habitants et son incidence entre 0.6 et 122 par million d'habitants et par an (Chifflot et al., 2008). Elle est caractérisée par une atteinte vasculaire, une atteinte fibrosante et la présence d'une auto-immunité. Comme dans les autres connectivites les manifestations cliniques sont variées : sclérose cutanée plus ou moins étendue, reflux gastro-œsophagien, phénomène de Raynaud, ulcères digitaux, pneumopathie interstitielle diffuse, hypertension artérielle pulmonaire (HTAP), calcinose, arthrite, myosite. Ces atteintes d'organes ne sont pas présentes chez tous les patients, ou à des degrés divers. Certaines d'entre elles peuvent engager le pronostic vital.

### I.2.1 Atteintes cliniques

De nouveaux critères de classification de la ScS ont été publiés en 2013 sous l'égide de l'American College of Rheumatology (ACR) et de l'European Scleroderma Trials and Research Group (EUSTAR) (Van den Hoogen et al., 2013). Un patient est classé comme ayant une ScS si le score est ≥ 9 (**Tableau 1**). Ces critères permettent de d'associer le diagnostic de ScS avec une sensibilité de 91% et une spécificité de 92%.

| Item | Sous-item | Score |
|---|---|---|
| Epaississement de la peau au-delà des MCP | | 9 |
| Epaississement de la peau des doigts | Doigts boudinés<br>Sclérodactylie | 2<br>4 |
| Troubles trophiques | Ulcère digital<br>Cicatrice pulpaire | 2<br>3 |
| Télangiectasies | | 2 |
| Microangiopathie organique en capillaroscopie | | 2 |
| HTAP et/ou PID | HTAP<br>PID | 2<br>2 |
| Phénomène de Raynaud | | 3 |
| Anticorps spécifiques | Anti-centromère<br>Anti-Scl70<br>Anti-ARN polymérase de type III | 3 |

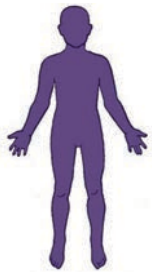
**Tableau 1** : critères ACR/EULAR 2013 de classification de la ScS, d'après (Van den Hoogen et al., 2013) ; MCP : articulation métacarpo-phalengienne, HTAP : hypertension artérielle pulmonaire, PID : pneumopathie interstielle diffuse

En 1988, LeRoy et al. ont proposé une classification des patients en deux sous-groupes (**Figure 3**) en fonction de l'atteinte cutanée (LeRoy et al., 1988) :

- **ScS cutanée limitée :** caractérisée par une atteinte cutanée distale des membres, une évolution lente avec une survenue de la maladie plusieurs années après le début du phénomène de Raynaud, peu d'atteintes sévères d'organes en dehors d'un risque tardif d'HTAP. Ces patients ont été caractérisés par une fréquence élevée d'anticorps anti-centromère.

- **ScS cutanée diffuse :** associée à une évolution plus agressive, avec atteinte rapide du tronc et des segments proximaux des membres, un risque élevé et précoce d'atteintes sévères d'organes (pulmonaire, rénale, cardiaque, intestinale…). Dans cette forme cutanée, la mise en évidence d'anticorps anti-centromère était très peu

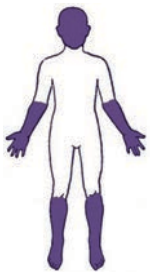
probable. Un tiers des patients présentaient des anticorps anti-Scl70 (LeRoy et al., 1988).



**dcSSc**

| ScS cutanée diffuse (dcSSc) | | ScS cutanée limitée (lcSSc) |
|---|---|---|
| Dans l'année des premiers changements cutanés | **Début du phénomène de Raynaud** | Des années avant les modifications cutanées |
| Tronc et racines des membres | **Atteinte cutanée** | Limitée aux mains, avant-bras, pieds et face ou absente |
| Frictions tendineuses | **Autres** | Névralgie trigéminée, calcinose et télangiectasies |
| Incidence précoce et significative de PID, crise rénale, atteinte gastro-intestinale et cardiaque | **Atteintes d'organes** | Incidence significative et tardive d'HTAP<br><br>PID présente ou absente |
| Absence d'anti-centromères<br>Anti-Scl70 (30% des patients) | **Auto-anticorps** | Fréquence élevée d'anti-centromères (70–80%) |
| Dilatation et destruction capillaires | **Capillaroscopie** | Anses capillaires dilatées habituellement sans effusion |

LeRoy et al. *J Rheumatol* 1988;15:202–205



**lcSSc**

**Figure 3 :** Deux formes de ScS proposées par LeRoy et al., d'après LeRoy et al., 1988

Depuis, cette classification arbitraire mais robuste sur le plan clinique a été appliquée par l'ensemble des équipes internationales. Ainsi, les grandes cohortes mondiales fournissent en général la fréquence des atteintes cliniques sur la population totale, puis classée en forme cutanée limitée/diffuse. L'exemple de la cohorte EUSTAR qui correspond au consortium européen des centres prenant en charge la ScS est présenté dans le **Tableau 2**.

| | **Total (n=7655)** | **lcSSc (n=4481)** | **dcSSc (n=2838)** |
|---|---|---|---|
| Phénomène de Raynaud, % | 96 | 97 | 96 |
| Ulcères digitaux, % | 36 | 33 | 42 |
| Atteinte articulaire, % | 32 | 22 | 49 |
| HTAP, % | 21 | 21 | 22 |
| PID au scanner, % | 52 | 44 | 64 |
| Atteinte intestinale, % | 24 | 23 | 24 |
| Crise rénale, % | 2 | 1 | 4 |

**Tableau 2** : Atteintes cliniques de la sclérodermie systémique dans la cohorte EUSTAR (European Scleroderma Trials and Research Group), d'après Meier et al., 2012 ; HTAP : hypertension artérielle pulmonaire, PID : pneumopathie interstielle diffuse

Il existe une variation des présentations cliniques en fonction des centres, probablement liée à des facteurs génétiques et/ou environnementaux. La comparaison des cohortes entre elles est également rendue complexe par les différentes définitions utilisées des atteintes d'organe. Muangchan et al. ont réalisé une méta-analyse des complications sévères d'organe à partir de 69 articles, suggérant que la plupart de ces complications surviennent chez environ 15% des patients : atteinte cardiaque 15% (intervalle de confiance (IC) à 95% : 6-24), HTAP confirmée par cathétérisme cardiaque droit 15% (IC95% : 12-17), myosite 13% (IC95% : 10-17), PID avec capacité vitale forcée (CVF) < 70% : 15% (IC95% : 12-17), arthrites 12% (IC95% : 9-16), ulcères digitaux 15% (IC95% : 10-20) (Muangchan et al., 2013).

La ScS est une pathologie associée à une morbi-mortalité significative. D'après une méta-analyse réalisée par Rubio-Rivas et al. entre 1964 et 2005, le ratio de mortalité standardisée des patients sclérodermiques comparés à la population générale a été estimé à 2.72 (IC 95% : 1.93-3.83). La survie cumulée à partir du premier phénomène hors Raynaud a été estimée à 84.1% à 5 ans et 75.5% à 10 ans (Rubio-Rivas et al., 2014). Les complications responsables de mortalité ont évolué ces dernières décennies. En comparant deux périodes de 5 ans débutant respectivement en 1972 et 1997, Steen et al. ont montré un changement notable dans les causes de mortalité des patients avec ScS (**Figure 4**). En effet, la crise rénale sclérodermique était responsable de 42% des décès en 1972-6 contre 6% en 1997-2001. A l'opposé, la fibrose pulmonaire causait le décès de 6% des patients en 1972-6 contre 33% en 1997-2001 (Steen et al., 2007).

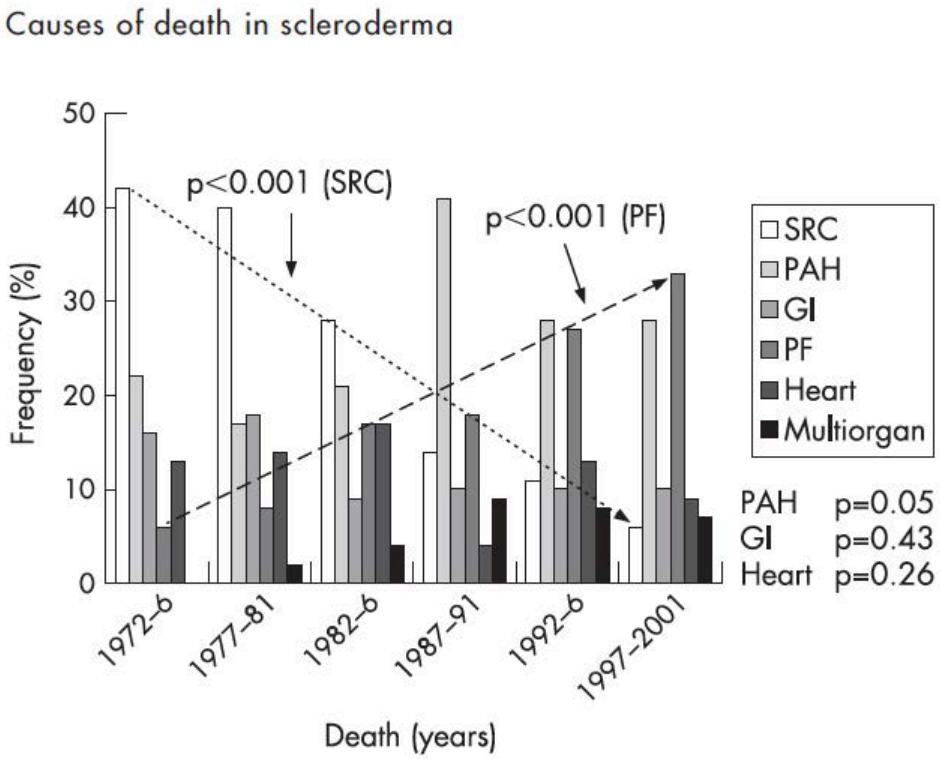



**Figure 4 :** Evolution des causes de décès liés à la ScS entre 1972 et 2001, d'après (Steen et al., 2007). SRC : crise rénale sclérodermique, PAH : hypertension artérielle pulmonaire, GI : atteinte gastro-intestinale, PF : fibrose pulmonaire, heart : atteinte cardiaque

### I.2.2 Interactions entre système vasculaire, auto-immunité et fibrose

L'origine de la ScS n'est pas connue mais elle implique probablement des facteurs environnementaux sur un fond génétique de susceptibilité. La physiopathologie est dominée par des lésions microvasculaires précoces responsables de lésions endothéliales avec la production de nombreux médiateurs favorisant une réponse inflammatoire et un remodelage vasculaire. De nombreux arguments impliquent des phénomènes d'auto-immunité dans l'entretien du processus pathologique. Les lésions de fibrose sont liées à l'accumulation de composants de la matrice extracellulaire en raison de perturbations de l'équilibre synthèse/dégradation de nombreuses substances, une activation et une différenciation de cellules mésenchymateuses liées à des facteurs autocrines et paracrines (**Figure 5**) (Allanore et al., 2016).

Il existe de nombreux modèles animaux de ScS (**Tableau 3**). Chacun présente des avantages et limites dans l'étude des grands mécanismes physiopathologiques de la ScS. Cependant aucun ne rassemble complètement les trois piliers physiopathologiques que sont l'atteinte vasculaire, l'auto-immunité et la fibrose ; il n'est donc pas possible de transposer directement les mécanismes observés chez l'animal à la complexité de la maladie humaine. Ainsi, la caractérisation précise de la pathologie humaine par l'intermédiaire d'études cliniques ou translationnelles garde toute sa place.

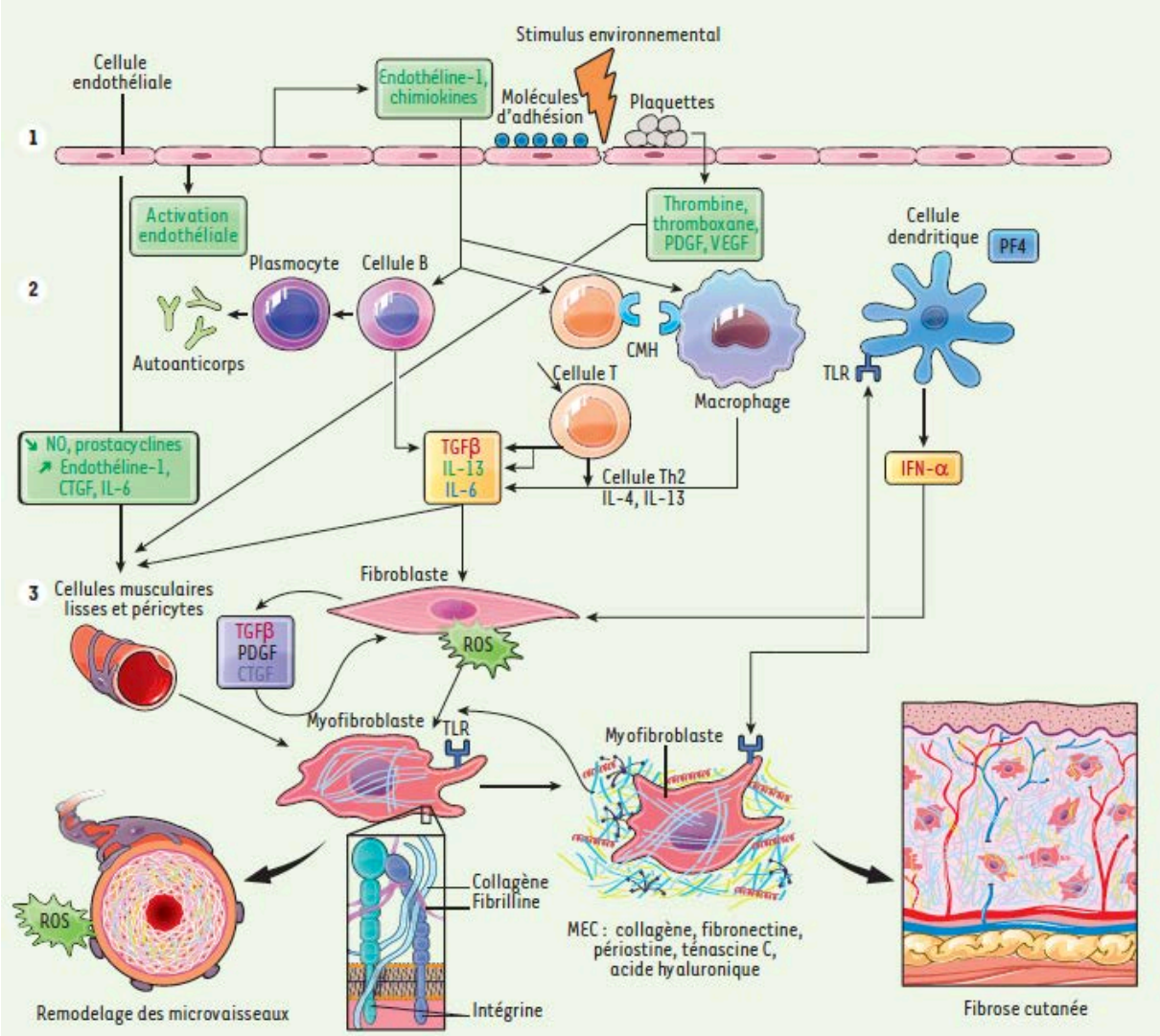



**Figure 5 :** Physiopathologie de la ScS d'après (Allanore et al., 2016). L'activation fibroblastique qui conduit à la fibrose caractéristique de la ScS résulte de lésions microvasculaires avec agression endothéliale accompagnée d'une réaction inflammatoire et immunitaire dérégulée. Étape 1. Activation endothéliale, perméabilité, expression de molécules d'adhésion et dépôt de plaquettes conduisent à la synthèse de vasomodulateurs, de cytokines, de facteurs de croissance et de chimiokines. Étape 2. Différentes cellules inflammatoires et immunitaires sont recrutées et activées. Elles produisent de l'interféron de type 1, des cytokines Th2, de l'interleukine 6, des facteurs de croissance et des autoanticorps. Étape 3. Les fibroblastes sont activés par ces stimulus, produisent de la matrice de façon dérégulée, se différencient en myofibroblastes qui entretiennent le processus avec une matrice désorganisée, des troubles métaboliques en réponse au stress mécanique et à l'hypoxie, une production d'espèces réactives de l'oxygène et de facteurs de croissance conduisant au remodelage vasculaire et à la fibrose tissulaire. PDGF : platelet-derived growth factor ; VEGF : vascular endothelial growth factor ; CMH : complexe majeur d'histocompatibilité ; CTGF : connective tissue growth factor ; TGFβ : transforming growth factor-beta ; TLR : toll-like receptor ; MEC : matrice extracellulaire ; PF4 : platelet factor 4 ; IFN-α : interféron alpha ; ROS : espèces réactives de l'oxygène ; IL : interleukine ; NO : monoxyde d'azote.

| | Site de fibrose | Atteinte vasculaire | Inflammation | Cibles de l'auto-immunité |
|---|---|---|---|---|
| **Modèles génétiques spontanés** | | | | |
| Souris Tsk1/+ | Derme | Cardiopathie et anomalies du tonus vasculaire | Absente | Fibrilline-1 |
| Souris Tsk2/+ | Derme | Absent | Modérée | Anti-nucléaires, Scl70 |
| Poulet UCD-200 | Peau et organes | Occlusions vasculaires | Infiltrats périvasculaires | ADN, histones |
| **Modèles inductibles** | | | | |
| Modèle bléomycine | Peau et poumon | Présente | Pic entre J3 et J5 puis diminue | Muqueuse gastrique |
| Modèle hypochloreux | Peau et poumon | Vasculopathies des petites artères rénales | Présente dans le derme | Scl70 ? |
| GVH sclérodermiforme | Peau | Présente | Inflammation et perturbations cytokiniques | Scl70 |
| Modèle angiotensine de type II | Présente | Présente, remodelage cardio-vasculaire | Inflammation péri-vasculaire | Non reporté |
| Scl70 et adjuvant complet de Freund | Peau et poumon | Non reporté | Perturbations cytokiniques et pic d'inflammation à 8 semaines | Scl70 |
| **Modèles transgéniques** | | | | |
| Endotheline-1 | Rein et poumon | Réduite | Présente | Non reporté |
| FRA-2 | Peau et poumon | Présente | Présente | Non reporté |
| Récepteur de type I TGF-β | Derme | Présente | Absent | Non reporté |
| Récepteur du TGF-β kinase-déficient de type II | Derme et poumon | Absente mais déclenchée par l'addition d'un inhibiteur du VEGFR | Non reporté | Non reporté |
| PDGFR-α | Peau et organes | Non reporté | Perturbations cytokiniques | Scl70 |
| **Modèles knock-out** | | | | |
| Caveolin-1 | Derme | Présente | Réponse inflammatoire augmentée | Non reporté |
| Egr-1 | Fibrose réduite | Non reporté | Inflammation réduite | Non reporté |
| Fli1 | Skin | Présente | Non reporté | Non reporté |
| MCP-1 | Fibrose réduite | Non reporté | Inflammation réduite | Non reporté |
| mPGES-1 | Fibrose réduite | Non reporté | Inflammation réduite | Non reporté |
| PPARγ | Derme | Non reporté | Inflammation augmentée | Non reporté |
| PTEN | Derme | Non reporté | Non reporté | Non reporté |
| Relaxine | Peau | Non reporté | Non reporté | Non reporté |

**Tableau 3 :** Principales caractéristiques des modèles animaux de ScS d'après Artlett et al., 2014 ; MCP-1, macrophage chemoattractant protein-1; PPARγ, peroxisome proliferator-activated receptor-gamma; PTEN, phosphatase and tensin homolog; FRA-2, Fos-related antigen-2; Egr-1, early growth response gene; Fli1, Friend leukemia integration factor-1; TGF-β, transforming growth factor-beta; PDGFR,

platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor; GvHD, graft versus host disease; Tsk1/+, tight skin 1; Tsk2/+, tight skin 2; UCD-200, University of California at Davis line 200; mPGeS-1, microsomal prostaglandin e2 synthase-1.

## I.3 Hétérogénéité des phénotypes cliniques

### I.3.1 Classification historique

La classification diffuse/limitée proposée par LeRoy et al. s'est révélée utile en pratique clinique quotidienne, et robuste. En effet, les atteintes d'organes décrites sont retrouvées dans les sous-groupes et la forme diffuse a été associée à une mortalité plus élevée (Hazard Ratio 2.40 (IC95% 1,73-3,32)) (Komocsi et al., 2012). Ainsi, une grande partie de la littérature suggère que ces deux sous-groupes diffuse/limitée diffèrent en terme d'atteintes cliniques, de profils biologiques et de pronostic (Medsger et al., 2003).

Cependant, cette distinction a été récemment remise en question par des études issues de grandes cohortes internationales, qui ont mis en évidence une hétérogénéité clinique probablement sous-estimée. Ces études suggèrent qu'il n'existe probablement pas de parallélisme clair entre la sévérité des atteintes d'organe et le degré d'extension cutanée.

- Dans une étude de 398 patients consécutifs suivis pendant 15 ans, Nihtyanova et al. ont montré que la présence d'atteintes d'organes était un indicateur puissant du pronostic, dans les deux formes cutanées de ScS. De façon intéressante, les courbes de survie étaient similaires entre les formes diffuse/limitée lorsqu'il existait une atteinte d'organe (Nihtyanova et al., 2014).
- Une étude du groupe canadien de recherche sur la sclérodermie s'est intéressée aux caractéristiques cliniques et à la survie des patients ayant une forme limitée avec anticorps anti-Scl70 et des patients ayant une forme diffuse

avec anticorps anti-centromère. Les caractéristiques démographiques et les atteintes viscérales étaient associées au statut sérologique plus qu'à la forme cutanée (Srivastava et al., 2015).

- Plus récemment, Kranenburg et al. ont aussi montré que les atteintes d'organes et la survie étaient différentes entre les patients avec une forme limitée et des anticorps anti-Scl70, et les patients avec une formée limitée sans anti-Scl70 ou avec une forme diffuse et anti-Scl70 (Kranenburg et al., 2016).

Ces travaux amènent à penser qu'une classification des patients en sous-groupes basés sur les auto-anticorps en plus de la forme cutanée, pourrait mieux prédire l'évolution clinique que la forme cutanée ou la sérologie seule.

### I.3.2 Autres classifications proposées

#### Formes de chevauchement

A partir de l'étude de 3240 patients du registre allemand de ScS, Moinzadeh et al. ont comparé l'évolution clinique de patients avec une forme de chevauchement ScS avec une autre connectivite (10% des patients) et ceux sans forme de chevauchement. Les patients avec chevauchement présentaient plus d'atteintes musculo-squelettiques que les autres, et développaient des atteintes cardiaques et pulmonaires plus précocement que les formes cutanées limitées, mais plus tardivement que les formes cutanées diffuses. Ces patients étaient également porteurs d'anticorps spécifiques (anti-U1RNP, anti-PM/Scl, anti-SS-A/SS-B) (Moinzadeh et al., 2014).

**Formes paranéoplasiques**

Les données issues des cohortes internationales ont permis de mettre en évidence un risque plus élevé de développer un cancer chez les patients avec une ScS, notamment en présence d'anticorps anti-ARN polymérase III (odds ratio OR 4,12 [2,26-7,51]), et chez les patients avec une dermatomyosite, en présence d'anticorps anti-TIF1 gamma (OR 5.11 [5.01-5.22]) (Chen et al., 2010 ; Sobanski et al., 2014 ; Moinzadeh et al., 2014). Ce risque est bien connu des cliniciens et influence aujourd'hui nos pratiques cliniques quotidiennes (recherche d'un cancer occulte chez ces patients). En 2010, l'équipe de Shah et al. (*Johns Hopkins University*) a mis en évidence un lien temporel fort entre la survenue d'une ScS et d'un cancer ; cette équipe a également montré pour la première fois un marquage immunohistochimique positif anti-ARN polymérase III sur les tumeurs de patients ayant ces mêmes anticorps présents dans leur sérum (Shah et al., 2010). Ce travail a été suivi par la publication d'un article majeur de la même équipe dans la revue *Science* en 2014 (Joseph et al., 2014). Les auteurs ont retrouvé une fréquence plus importante de mutations somatiques du gène de l'ARN polymérase III à l'intérieur des tumeurs de patients ayant des auto-anticorps anti- ARN polymérase III que chez les patients n'ayant pas ces anticorps. Ils ont également montré qu'il existait chez les patients concernés, une immunité B- et T- spécifique dirigée contre l'ARN polymérase III mutée. L'hypothèse avancée est celle d'une exposition antigénique tumorale d'une protéine mutée (l'ARN polymérase III) suivie d'une réponse immunitaire anti-tumorale. Par mimétisme moléculaire, cette immunité anti-tumorale provoquerait comme « dommage collatéral », la survenue d'une ScS. Ceci pourrait expliquer que tous les patients ayant des anticorps anti-ARN polymérase III ne souffrent pas de cancer : il s'agit potentiellement de micro-cancers au stade de dormance tumorale (non détectable actuellement) ou de cancers ayant régressé grâce à une réaction immunitaire particulièrement efficace (au prix du développement d'une

maladie auto-immune) (Shah et al., 2015).

**Classification selon les trajectoires dans le temps**

Le recueil longitudinal de variables cliniques au cours du suivi a également été utilisé pour tenter d'identifier des groupes de patients homogènes : score d'extension cutanée de Rodnan (Shand et al., 2007 ; Dobrota et al., 2007), capacité vitale forcée (Man et al., 2015 ; Schulam et al., 2015).

**Classification moléculaire : expression génique dans la peau**

L'équipe de Michael Whitfield étudie le profil d'expression de gènes impliqués dans les grandes fonctions cellulaires (telles que la synthèse de matrice extra-cellulaire, la régulation du cycle cellulaire, la sécrétion de médiateurs inflammatoires) à l'aide de RT-PCR (Reverse Transcriptase – Polymerase Chain Reaction) au sein de biopsies cutanées de patients avec ScS. A l'aide d'une analyse en cluster de l'expression intrinsèque de chacun de ces gènes, il est possible d'établir des profils homogènes de patients correspondant à un phénotype clinique particulier : « diffuse 1 », « diffuse 2 », « inflammatory », « limited » and « normal-like ». Cette approche a été proposée pour prédire la réponse thérapeutique, et pourrait s'intégrer dans une démarche de médecine personnalisée (Varga et al., 2014 ; Hinchcliff et al., 2013 ; Pendergrass et al., 2012).

Malgré les classifications proposées (**Figure 6**), la ScS demeure une pathologie hétérogène. Il semble ne pas exister une sclérodermie mais des sclérodermies. Cette importante variabilité de présentations cliniques, d'atteintes d'organes, de profils

biologiques et d'évolution complexifie la prise en charge des patients. Selon certains auteurs, l'échec de certains essais cliniques pourrait être lié à une mauvaise caractérisation des populations cibles de patients. Il apparaît nécessaire de mieux définir les phénotypes cliniques, afin de proposer au patient une prise en charge adaptée (Varga et al., 2014 ; Srivastava et al., 2015 ; Ligon et al., 2015).

Une meilleure définition des phénotypes pourrait également permettre d'avancer dans la compréhension des mécanismes physiopathologiques. Ceci rejoint le concept « d'endotype », terme pour le moment surtout utilisé dans le domaine de l'asthme et de la Broncho-Pneumopathie Chronique Obstructive (BPCO). D'après Gary Anderson, l'endotype (contraction « d'endophénotype ») est un sous-type de maladie définie sur le plan fonctionnel et pathologique par un mécanisme moléculaire ou une réponse particulière à un traitement (Anderson et al., 2008). L'endotype correspondrait donc à l'ensemble phénotype + mécanisme physiopathologique identifié.

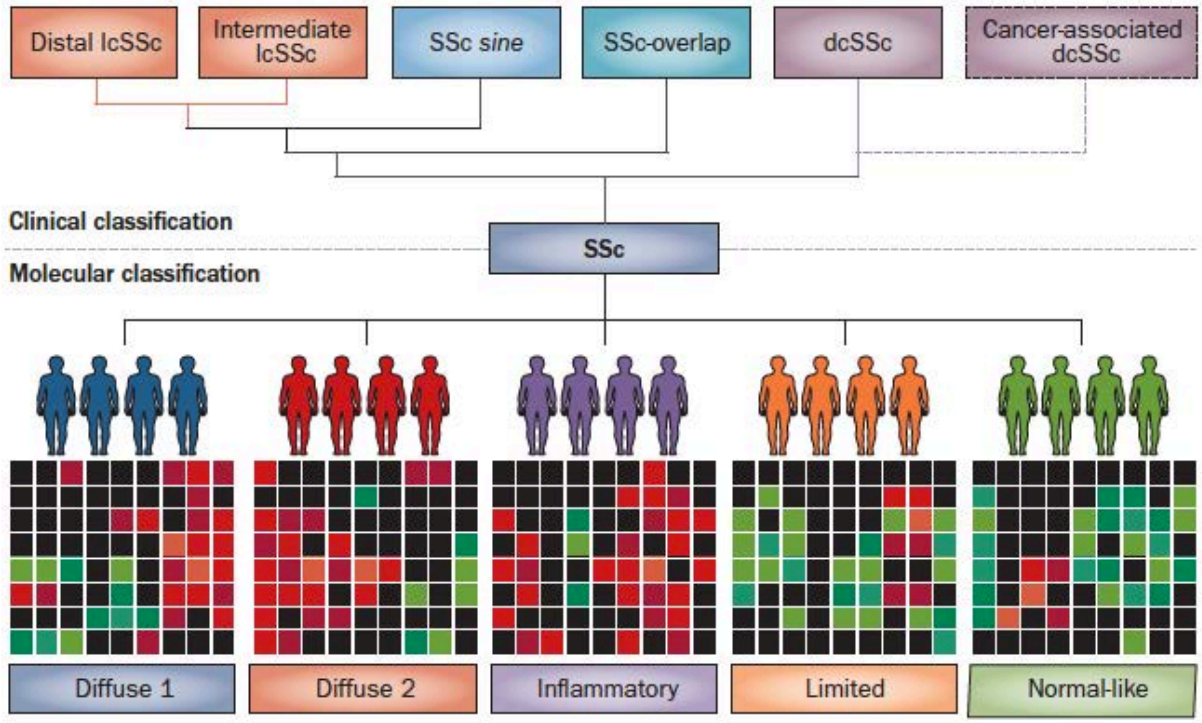



**Figure 6 :** Différentes propositions de systèmes de classification des patients avec ScS, d'après Varga et al., 2014

## I.4 Place des auto-anticorps dans la classification de la ScS

Différents auto-anticorps dirigés contre des composants cellulaires ont été mis en évidence dans le sérum des patients. Le rôle pathogène des auto-anticorps dans la ScS n'est pas encore clairement identifié. Néanmoins ils se révèlent fortement associés au phénotype clinique des patients constituant de véritables biomarqueurs diagnostiques et pronostiques de la maladie utilisés en pratique quotidienne.

### I.4.1 Biomarqueurs diagnostiques et pronostiques

Des anticorps anti-nucléaires sont retrouvés dans plus de 95 % des sérums de patients avec ScS. Habituellement dirigés contre des antigènes intra-cellulaires, leurs épitopes précis ne sont que très peu connus. Certains sont très spécifiques de la ScS, et à ce titre ont été inclus dans les nouveaux critères ACR/EULAR 2013 de la ScS (Kayser et al., 2015 ; Van den Hoogen et al., 2013). Il est frappant de constater les associations fortes entre ces anticorps et les manifestations cliniques présentes chez les patients (**Tableau 4**). Thomas Medsger avait proposé un schéma récapitulatif reproduit en **Figure 7**. A titre d'exemple, nous avons réalisé une revue systématique avec méta-analyse des associations cliniques des anticorps anti-ARN polymérase de type III dans la ScS (Sobanski et al., 2014). Ces anticorps étaient associés avec la forme diffuse (OR 4,12 [2,72-6,24]), l'atteinte articulaire (OR 1,94 [1,20-3,14]), la crise rénale (OR 8,86 [6,48-12,13]), l'atteinte cardiaque (OR 1,79 [1,10-2,91]) et la survenue d'un cancer (OR 4,12 [2,26-7,51]).

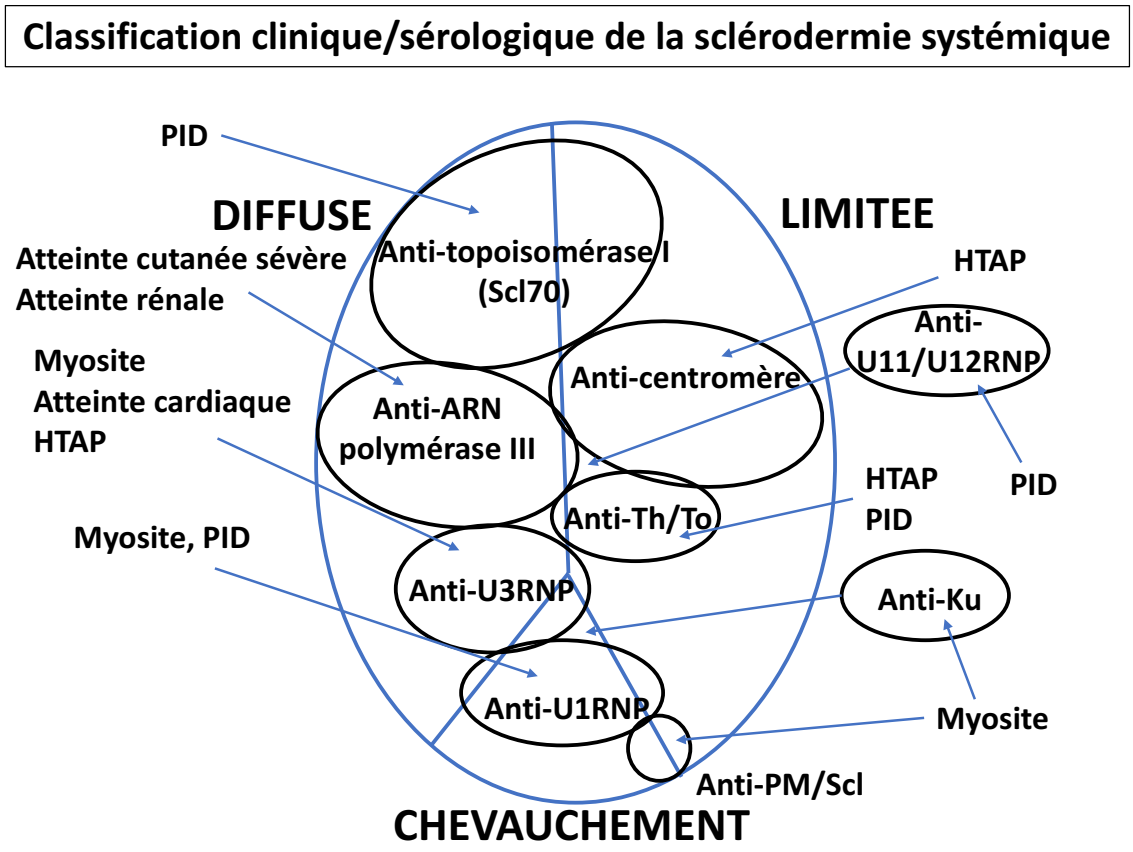



**Figure 7 :** Schéma récapitulatif des associations entre les auto-anticorps de la ScS et les phénotypes cliniques des patients. Inspiré de Thomas Medsger.

| | Fréquence (%) | Forme cutanée | Associations cliniques |
|---|---|---|---|
| Anti-centromère | 20-38 | lcSSc | HTAP |
| Anti-Scl70 (topo-isomérase de type I) | 15-42 | dcSSc | PID<br>Atteinte cardiaque |
| Anti-ARN polymérase de type III | 5-31 | dcSSc | Crise rénale<br>Friction tendineuse, synovites, myosite |
| Anti-U3RNP (fibrillarine) | 4-10 | dcSSc | Crise rénale<br>Atteinte cardiaque |
| Anti-Th/To | 1-13 | lcSSc | PID<br>Crise rénale |
| Anti-U11/U12 RNP | 3 | | PID<br>Atteinte gastro-intestinale |
| Anti-U1RNP | 2-14 | lcSSc<br>Chevauchement | Doigts boudinés, arthrites, myosite |
| Anti-Ku | 2-4 | | Myosite, arthrites |
| Anti-hUBF (NOR90) | <5 | lcSSc | |
| Anti-RuvBL1/2 | 1-2 | Chevauchement | |

**Tableau 4 :** Principaux auto-anticorps associés à la ScS, d'après Kayser et al., 2015 et Choi et al., 2016

Patterson et al. ont réalisé un immuno-dot (EUROIMMUN ®) dans une cohorte de 505 patients australiens. Ce test permet la détection simultanée des réactivités sériques les plus fréquemment rencontrées dans la ScS : CENP-A et CENP-B, ARN polymérase de type III (sous-unités 11 et 155), NOR-90, fibrillarine, Th/To, PM/Scl-75, PM/Scl-100, Ku, topoisomérase I, tripartite motif–containing protein (TRIM) 21/Ro 52, et PDGF-R. Cinq sous-groupes de patients étaient identifiés sur la base des résultats du

test, caractérisés chacun par une relative homogénéité des phénotypes cliniques (Patterson et al., 2015). Ce travail montre qu'il est possible de classer les patients en groupes homogènes à partir du statut de leurs auto-anticorps.

### I.4.2 Rôles pathogéniques potentiels

Des perturbations de l'immunité innée et acquise jouent un rôle clé dans la séquence pathogénique de la ScS. Bien que les auto-anticorps représentent des biomarqueurs diagnostiques, pronostiques d'intérêt, leur rôle pathogénique demeure relativement peu connu. En effet, la grande majorité des auto-antigènes décrits ont une localisation *a priori* intra-cellulaire, ce qui les rendrait inaccessible aux auto-anticorps circulants (Kayser et al., 2015 ; Fritzler et al., 2016). Les données de la littérature sont encore limitées (résumées dans le **Tableau 5**).

| | **Fréquence (%)** | **Rôle pathogène** | **Associations cliniques** |
|---|---|---|---|
| Anti-fibroblastes | 26-58 | Activation des fibroblastes in vitro<br>Induit un phénotype pro-adhésif et pro-inflammatoire | Association avec anti-Scl70<br>PID<br>HTAP |
| Anti-fibrilline 1 | >50 | Activation des fibroblastes in vitro<br>Stimulation du relargage de TGF- β dans la MEC | Différences ethniques de taux |
| Anti-MMP-1 et -3 | 49-52 | Inhibition de l'activité des collagénases MMP<br>Réduction du renouvellement de MEC | Fibrose cutanée, pulmonaire et des vaisseaux sanguins rénaux |
| Anti-cellules endothéliales | 44-84 | Induction de l'apoptose des cellules endothéliales in vitro<br>Stimulation du relargage de cytokines pro-inflammatoires et pro-fibrosantes dans la microvascularisation | ScS plus sévère<br>Atteintes vasculaires<br>HTAP |
| Anti-récepteur du PDGF | 33-100 | Activation du récepteur du PDGF<br>Stimulation de la production de ROS et de collagène<br>Stimulation différenciation fibroblastes en myofibroblastes<br>Induction fibrose cutanée in vivo | Cible thérapeutique potentielle du rituximab, nintedanib, imatinib et nilotinib |
| Anti-récepteur de l'angiotensine de type 1 et anti-récepteur de type A de l'endothéline 1 | 82-83 | Stimulation de la production de ROS et de collagène | ScS précoce et sévère<br>HTAP, ulcères digitaux, crise rénale, dcSSc, PID |
| Anti-IFI16 | 18 | | lcSSc, diminution DLCO, ulcères digitaux |

**Tableau 5 :** Auto-anticorps potentiellement pathogènes dans la ScS, d'après Kayser et al., 2015 et Fritzler et al., 2016

# PARTIE II – OBJECTIFS DE LA THESE ET EXPOSE DES TRAVAUX

La ScS est une pathologie associant atteinte vasculaire, auto-immunité et fibrose. Il s'agit d'un modèle prototypique d'étude des interactions entre ces trois piliers physiopathologiques, notamment entre système immunitaire et fibrose. Mieux comprendre les mécanismes responsables de la ScS, en particulier l'implication de l'auto-immunité, pourrait aider à mieux prendre en charge la fibrose, conséquence actuellement irréversible de nombreuses pathologies fréquentes.

Longtemps réduite à une présentation clinique divisée en deux sous-groupes arbitraires définis par l'extension de l'atteinte cutanée, la ScS est apparue durant cette dernière décennie, comme une pathologie hétérogène. La complexité à classer les patients en groupes homogènes ayant un phénotype clinique similaire est probablement en partie responsable de l'échec à mettre au point une thérapeutique efficace.

Différentes classifications ont été proposées, basées sur des critères cliniques, biologiques ou sur l'évolution des patients. Aucune ne permet à ce jour d'appréhender de façon satisfaisante l'hétérogénéité de la pathologie. De nouvelles classifications en sous-groupes intégrant différentes variables, en plusieurs niveaux, pourraient être proposées dans un futur proche. Les auto-anticorps, utilisés en pratique clinique quotidienne, se révèlent être des biomarqueurs diagnostiques et pronostiques puissants. Bien que leur rôle physiopathologique demeure encore en grande partie méconnu, ils permettent de dessiner des groupes de patients aux phénotypes cliniques similaires de façon relativement efficace.

1. Notre premier objectif a été de réaliser une analyse exploratoire d'une cohorte européenne de grande taille afin de mieux cerner l'hétérogénéité de la ScS. En utilisant l'analyse en « *cluster »*, l'objectif a été **d'identifier, à l'aide de variables cliniques et biologiques, et sans a priori, des groupes homogènes de patients puis de comparer leur survie**.

2. Le deuxième objectif de ce travail a été de **caractériser de potentiels marqueurs immunologiques de cette hétérogénéité**.

    a. Une première étude s'est intéressée aux **chaines légères libres sériques en tant que marqueurs des caractéristiques cliniques, de la sévérité et de l'activité de la ScS**.

    b. La seconde étude a eu pour objectif d'**estimer la prévalence mondiale globale des anticorps anti-ARN polymérase de type III dans la ScS et d'expliquer l'hétérogénéité observée dans l'estimation de cette prévalence entre les études**.

    c. La dernière étude a concerné **la place des anticorps anti-U1RNP en tant que facteur pronostique de mortalité de l'HTAP des connectivites, en particulier de la ScS**

# II.1 Analyse en cluster de la cohorte européenne EUSTAR

## II.1.1 Introduction

La ScS est une pathologie hétérogène, c'est-à-dire qu'il existe une variabilité importante entre les phénotypes cliniques présentés par les patients. La classification historique « diffuse » versus « limitée » est robuste car elle permet de diviser les patients en deux groupes distincts aux pronostics différents. Néanmoins il apparait de plus en plus clair que cette classification « arbitraire » ne permet pas de cerner l'ensemble de l'hétérogénéité : des sous-groupes de patients supplémentaires pourraient être identifiées. D'autres systèmes de classifications ont été proposés (cf. introduction générale), la plupart à l'aide de critères cliniques ou biologiques définis : statut des auto-anticorps, évolution dans le temps de paramètres cliniques… Il existe donc un « *a priori* » lors de l'établissement de telles classifications.

L'analyse en cluster est une méthode statistique qui permet de classer les patients en groupes homogènes sur la base de variables sélectionnées. Dans ce premier travail, nous avons réalisé une analyse sans *a priori* puisque toutes les variables (cliniques et biologiques) de la cohorte européenne EUSTAR étaient potentiellement incluables. Le nombre de variables réellement analysées a été réduit à 24 en raison de l'importance des données manquantes pour de nombreuses d'entre elles. En effet, la présence d'une seule donnée manquante pour une variable aurait pour conséquence l'exclusion de cette variable de l'analyse en cluster.

Cette étude a mis en évidence un premier niveau de clusterisation avec deux groupes relativement attendus. Dans le cadre d'une approche exploratoire, nous avons choisi d'augmenter le nombre de clusters jusqu'à 6 afin de déterminer s'il existait d'autres groupes homogènes de patients derrière la dichotomie « diffuse » versus « limitée ».

### II.1.2 Résumé

**Objectifs**

La ScS est une pathologie hétérogène avec des variations importantes d'atteintes de la peau, des organes viscéraux et du statut des auto-anticorps. La classification en usage est basée sur l'extension cutanée en séparant les formes cutanées limitée (lc) et diffuse (dc) mais ne permet pas d'appréhender l'ensemble de la variabilité des phénotypes cliniques. Cette étude a eu pour objectifs d'identifier et caractériser des phénotypes homogènes de patients avec ScS sans a priori en utilisant l'analyse en cluster, et comparer la survie entre les différents groupes identifiés

**Méthodes**

Etude multicentrique (137 centres) des patients de la cohorte prospective EUSTAR inclus entre Juin 2004 et Avril 2014. Les patients adultes, remplissant les critères ACR de ScS et ayant une durée de la maladie calculable ont été inclus. Au total, 11 318 patients étaient enregistrés dans la base EUSTAR. Parmi ceux-ci, 2 886 patients ont été exclus et 1 505 non analysés (en raison d'au moins une variable de clusterisation manquante). Ainsi, 6 927 patients ont été inclus dans l'analyse en cluster. L'analyse par la méthode de classification ascendante hiérarchique a été menée avec 24 variables sélectionnées. La stabilité de chacun des groupes identifiés a été étudiée. La comparaison de la survie entre les groupes a alors été réalisée avec un modèle de régression Cox à risques proportionnels.

**Résultats**

L'analyse en cluster a permis d'obtenir 2 groupes avec une stabilité moyenne. Avec un objectif exploratoire, nous avons ensuite identifié 6 groupes homogènes différents entre eux par leur présentation clinique, le statut des auto-anticorps et leur survie. Tandis que certains groupes étaient proches de la dichotomie habituelle « diffuse »

versus « limitée », d'autres présentaient des caractéristiques originales telles que des patients avec une forme limitée mais un fort taux d'atteinte viscérale et de présence d'anticorps anti-Scl70. Le pronostic était différent selon les groupes. La présence d'une atteinte d'organe avait un impact majeur sur la survie, au-delà de la forme cutanée.

**Conclusion**

Cette étude suggère que restreindre la classification des patients à l'atteinte cutanée ne permet pas de cerner l'ensemble de l'hétérogénéité de la ScS. Les atteintes viscérales et le statut des auto-anticorps pourraient avoir un rôle majeur dans la définition de groupes homogènes de patients avec des pronostics mieux définis.

**II.1.3 Article** *(soumis)*

# Phenotypes determined by cluster analysis and their survival in the prospective EUSTAR cohort of patients with systemic sclerosis

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**TOTAL WORD COUNT: 2997/3000**

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**Key Points**

**Question**: Are there homogeneous groups of patients in systemic sclerosis?

**Findings**: Using a cluster analysis, we identified homogeneous groups which differed widely by their clinical presentation, organ involvement, autoantibody status and survival.

**Meaning:** Restricting the classification of patients to the skin extension does not capture the whole heterogeneity of the disease. Organ involvement as well as antibody status are suggested to play a major role in defining homogeneous groups of patients with different prognosis.

## Abstract

### Importance

Systemic sclerosis (SSc) is a heterogeneous condition with various skin and organ involvement as well as autoantibody status. The usual subclassification is based on the skin extension by separating limited (lc) and diffuse cutaneous (dc) SSc but could not capture the whole variability of clinical phenotypes.

### Objective

To identify and characterize homogeneous phenotypes in patients with SSc without any *a priori* using a cluster analysis, and compare survival among the different clusters.

### Design

Prospective EUSTAR cohort with inclusion of patients between June 2004 and April 2014 and a mean disease duration of 11.4±8.1 yrs.

### Setting

Multicenter (n=137); referral centers.

### Participants

Patients eligible for this study were aged ≥ 18, fulfilled ACR criteria for SSc, and had a calculable SSc duration. A total of 11,318 patients were recorded in the EUSTAR database. Among them, 2,886 patients were excluded and 1,505 were not analyzed (because there was at least one missing value for the clustering variables) resulting in 6,927 patients who were included in the cluster analysis

### Exposure

Non applicable

### Main outcomes and measures

The main measures were 1. 24 variables were retained for clusterisation including clinical and serological variables. 2. Death. The main outcomes were 1. Homogeneous

groups of patients by cluster analysis 2. Comparison of their clinical characteristics and survival.

**Results**

Clustering analyses resulted in an optimal definition of 2 clusters with a moderate stability. In an exploratory approach, we further identified 6 homogeneous groups which differed widely by their clinical presentation, autoantibody status and survival. While some clusters were close to the usual dichotomy between lcSSc and dcSSc, other showed original characteristics like a majority of lcSSc but a high proportion of visceral involvement and antitopoisomerase antibodies. Prognosis was different between clusters and the presence of an organ involvement had a major impact on survival beyond the skin extension.

**Conclusion and Relevance**

This study suggests that restricting the classification of patients to the skin extension does not capture the whole heterogeneity of the disease. Organ involvement and antibody status are suggested to also play a major role in defining homogeneous groups of patients with different prognosis.

## Introduction

Systemic sclerosis (SSc) is a chronic connective tissue disorder characterized by vascular involvement, fibrosis and auto-immunity. A recent work of the European League Against Rheumatism (EULAR) and American College of Rheumatology led to new and robust classification criteria for SSc [1]. Until now, the subclassification of SSc patients is only based on the skin extension as proposed by LeRoy et al. in 1988. This subclassification divide patients into two major subsets: 1. limited cutaneous SSc (lcSSc) characterized by a distal limbs skin involvement 2. diffuse cutaneous SSc (dcSSc) associated with rapid skin changes with truncal and proximal limbs skin involvement. Organ involvement is different between the two subsets with an early and significant incidence of organ complications (interstitial lung disease (ILD), renal crisis, gastrointestinal (GI) disease, myocardial involvement) [2] in dcSSc and pulmonary hypertension (PH) in lcSSc. Autoantibody status is also different in the two subsets, with a very high frequency (70-80%) of anticentromere antibodies (ACA) in lcSSc, and a higher frequency (30%) of anti-topoisomerase I antibodies (ATA) in dcSSc in the original report [2]. Moreover, mortality was higher in dcSSc patients compared to patients with lcSSc [3,4]. Altogether, previous studies suggest that these two clearly differentiated subsets (lcSSc/dcSSc) differ in terms of clinical features, biological profiles, and prognosis [5].
However, this distinction has been questioned by recent reports from large cohorts of patients that highlighted a frequently overlooked heterogeneity among clinical subsets [1,6-10], with for example patients with lcSSc but ATA and severe ILD. One way to handle heterogeneity is to perform a cluster analysis which aims to organize information so that a heterogeneous population can be grouped into relatively small number of homogeneous groups. Cluster analysis has been used in many diseases like asthma [11], chronic heart failure [12], gout [13], ANCA-associated vasculitis [14], mixed connective tissue

disease [15]. To our knowledge, cluster analyses have also been performed in two studies in SSc [16,17]. The first one has included a large number of patients from the EULAR scleroderma trials and research (EUSTAR) cohort but focused on capillaroscopic patterns [16]. A more recent one has considered a limited number of clustering variables and included a limited number of patients [17].

This study was designed to identify and characterize homogeneous phenotypes in patients with SSc included in the EUSTAR cohort using a cluster analysis and compare survival among the different clusters.

# Methods

## Patient population

This study is based on the data collected from the SSc patients participating in the multi-national, prospective and open SSc EUSTAR cohort, launched in June 2004 [18-20]. For this study, the EUSTAR database was definitively locked in April 2014. The patients eligible for this study were aged ≥ 18, fulfilled ACR criteria for SSc [21], and had a calculable SSc duration (i.e. a date of disease onset (DO, defined as the apparition of the first non-Raynaud's phenomenon) and at least one date of visit).

All patients agreed to participate in the EUSTAR database by signing informed consent forms approved by the local ethical committees. The study was conducted in accordance with the principles of the Declaration of Helsinki and local laws and guidelines for Good Clinical Practice.

## Definition and selection of variables

A total of 933 variables were found in the EUSTAR database, encompassing demographic data, disease characteristics, organ involvement, laboratory parameters, pulmonary function tests (PFTs), echocardiography, capillaroscopy results and treatment. Variables were selected for the cluster analysis taking account of missing data and relationship between variables. Twenty-four variables were retained (**Table 1** and **eFigure 2**), encompassing: (i) symptoms or organ complications observed during at least one visit (Raynaud, oesophageal, stomach and intestinal symptoms, digital ulcerations (DU), joint synovitis, joint contractures, tendon friction rubs, muscle weakness, muscle atrophy, arterial hypertension, palpitations, renal crisis), (ii) results of blood tests (positive antinuclear antibodies (ANA), ACA and ATA, CK elevation, proteinuria), (iii) other tests (conduction blocks, abnormal diastolic function, suspected PH

by cardiac echography, plain X-ray lung fibrosis, restrictive defect on PFTs), and (iv) the peak mRSS observed during the follow-up.

## Statistical analyses

### *Cluster analysis*

Cluster analysis defines the distances between subjects based on the combined values of their measured characteristics to find groups of subjects who are more similar to each other than in those in other groups. Cluster analysis was conducted from ascendant hierarchical clustering on the 24 selected variables using Ward's minimum variance method. Results were graphically presented in a dendrogram. The number of clusters was estimated using a visual distance criterion by cutting the dendrogram horizontally at the level of highest dissimilarity (i.e. where the vertical branches were the longest).

Assessment of the cluster-wise stability and reproducibility is a critical issue when conducting a cluster analysis [22]. To evaluate it, we performed 100 iterations of the clustering process (using the same number of clusters than the primary analysis) in randomly selected subsets to 50% of the original dataset, and assessed the cluster-wise stability by the mean of the Jaccard coefficients (a similarity measure between datasets) calculated between each cluster of the primary analysis and the most similar cluster obtained for each iteration [22]. A Jaccard similarity value ≤ 0.5 was considered as an indication of a weakly stable and reproducible cluster [23].

The main cluster analysis was performed on the whole population. In order to assess the influence of late complications on the clustering process, we further conducted a sensitivity analysis including only patients with a disease duration superior to 10 years i.e. with a sufficient time for the development of the expected organ involvements.

### *Survival analysis*

Survival analysis was conducted between clusters of patients, considering the time between the date of DO and the date of the latest news (i.e. the disease duration). A high proportion (52%) of patients were lost to follow-up (i.e. date of last news before January 2012), leading to a significant overestimation of the survival. Because we were unable to update data on vital status, we decided to exclude them from the survival analysis. A sensitivity analysis was conducted without excluding those patients.

Survival rates were compared in several Cox proportional hazards models: (i) unadjusted, (ii) adjusted for age at DO, (iii) adjusted in addition for sex, and (iv) adjusted in addition for immunosuppressive treatment.

All statistical analyses were performed using R software, version 2.14 [24], using “survival” and “fastcluster” packages. The threshold for statistical significance was set to $p<0.05$.

## Results

### Population

A total of 11,318 patients were recorded in the EUSTAR database on April 2014 (from 120 centers), corresponding to a registration of 34,066 visits. Among them, 2,886 patients were excluded and 1,505 were not analyzed (because there was at least one missing value for the clustering variables) resulting in 6,927 patients who were included in the cluster analysis (**eFigure 1**). Patients included in the cluster analysis were slightly older (58.7±13.2 vs. 56.3±13.9 years, p<0.001), with higher disease duration (11.4±8.1 vs. 8.7±8.1, p<0.001), proportion of dcSSc (42 vs. 38%, p=0.011) and an overall more aggressive disease as shown by organ involvements than those who were not (**Table 1**).

Forty-two percent of patients included in the cluster analysis had dcSSc and 58% had lcSSc. Patients with dcSSc were significantly younger than those with lcSSc, and had a more severe disease. ACA and ATA were present respectively in 14% and 61% in dcSSc, and 54% and 23% in lcSSc (**Table 1**).

### Cluster analysis

Clustering of patients based on the selected 24 variables resulted in an optimal number of 2 clusters: C(A) and C(B) (**Figure 1A**). The Jaccard coefficients were 0.64 for C(A) and 0.66 for C(B), indicating a moderate stability. Characteristics of these clusters are described in **Table 2**. The distribution of autoantibodies (ACA/ATA) among the subtype of SSc (lc/dcSSc) in each of the different clusters is presented in contingency tables in **eTables 1 and 2**.

***C(A) (n=3149, 45.5%).*** C(A) included mainly lcSSc patients (81%). A maximum of 1/3 of patients had complications such as intestinal symptoms, DU, joint, muscle, cardiac

and lung involvement. Fifty-four percents of patients were ACA positive and 21% were ATA positive.

***C(B) (n=3778, 55.5%).*** C(B) included 61% of patients with dcSSc. Patients in C(B) were slightly younger than in C(A), with lower age at DO. More than half of patients had intestinal involvement, joint contractures, DU, and ILD. The distribution of autoantibodies was the exact opposite of C(A) with 54% of ATA and 22% of ACA. A heatmap showing the differences between C(A) and C(B) is shown **Figure 1B**.

In an exploratory approach to better understand the heterogeneity of the disease, we attempted to increase the number of clusters. Graphical analysis of the dendrogram showed that the suboptimal number of clusters was 6 (**Figure 1A**). Consequently, there was a decrease in Jaccard indexes (between 0.32 and 0.68) (**Table 2**).

***C1 (n=1186, 17%).*** C1 was composed mainly by female patients with lcSSc (89%), older onset, a low prevalence of ILD, and a high rate of GI involvement. ACA were present in 79% of patients.

***C2 (n=720, 18%).*** C2 included mainly lcSSc (71%) patients, with high rates of suspected PH by echo (39%) and ILD (85%) with restrictive defect (61%). There was a notable proportion of ATA (35%), which was above the proportion of ACA (24%).

***C3 (n=1243, 10%).*** Patients in C3 were mainly lcSSc (79%) with a low prevalence of ILD and GI involvement. There were two times more ACA positive (48%) than ATA positive (24%) patients.

***C4 (n=1673, 18%).*** C4 contained mainly lcSSc patients (63%). Patients in this cluster had an aggressive disease as shown by high rates of DU, GI, joint, muscular, lung and cardiac involvements. ATA were found in 46% of patients and ACA in 29%.

***C5 (n=1249, 24%).*** Patients in C5 were mainly dcSSc (72%), with a significant proportion of male patients (19%). These patients had significant GI, joint and cardiac involvement and moderate lung involvement. ATA were found in 50% of patients and ACA in 20%.

***C6 (n=856, 12%).*** In C6, patients were mainly dcSSc (92%) with the highest peak mRSS (27.2), the higher proportion of males (22%), and a very active disease as demonstrated by high rates of GI, joint, muscular, cardiac, lung and renal involvements. ATA were present in 77% of patients and ACA in 12%.

A heatmap showing the differences between the 6 clusters is shown **Figure 1B**.

The sensitivity cluster analysis conducted only in patients with disease duration superior to 10 years yielded similar results (**eTable 3**).

## Survival analyses

Kaplan-Meier curves are presented in **Figure 2 and eFigure 3**. Survival rates and results of Cox regression analyses are given in **eTable 4** and **Table 3**, respectively.

Patients with dcSSc had an increased risk of death compared to patients with lcSSc (hazard ratio (HR) =2.03 [95%CI 1.61-2.56] in the fully adjusted model). A higher mortality was also observed for patients in C(B) compared to patients in C(A) (HR=2.47 [1.86-3.27]). When considering 6 clusters, we observed in the fully adjusted model a progressive increasing risk of death from C1 to C6, with a magnitude of risk superior to those observed in the two previous analyses (i.e. HR=6.14 [3.81-9.89] for C6, compared to C1).

The sensitivity analysis including patients lost to follow-up yielded similar hazard ratios when we compared survival between clusters A-B and clusters 1-6 (data not shown).

## Discussion

This study aimed to find homogenous subgroups in a large population of nearly 7,000 SSc patients using a cluster analysis. The main results are: (i) the optimal clustering resulted in 2 groups of patients which differed by their clinical and serological characteristics, severity and prognosis, and (ii) in an exploratory approach, we further found 6 homogeneous subgroups of patients which differed widely on clinical characteristics and prognosis.

The fact that we obtained 2 clusters could be seen as a confirmation of the classical dichotomy between lcSSc and dcSSc. However, in the most severe cluster C(B), up to 39% of patients had lcSSc and 22% had ACA. Conversely, in the less severe C(A), 19% of patients had dcSSc and 21% had ATA. This suggests that there was no clear parallelism between the severity of organ involvement and the skin extent of SSc. This result is in line with recent studies. For example, in a study of 398 consecutive SSc patients followed up for up to 15 years, Nihtyanova et al. showed that the presence of significant organ-based complications was a strong indicator of prognosis, in both limited and diffuse subsets. Intriguingly, survival rates were the same for lcSSc and dcSSc patients when organ involvement was present [6]. Altogether, these results suggest that, while a consensus exists on the rationale and usefulness of classifying SSc into limited and diffuse cutaneous subsets [25], this binary system could appear as a restrictive division across a continuous spectrum of various severity mainly defined by organ involvement and subsequent prognosis [10].

To more deeply clarify the heterogeneity in SSc, we decided to further describe 6 clusters beyond the two first clusters C(A) and C(B) in an exploratory approach. Some of these clusters were well awaited as they fitted the classical dichotomy between lcSSc and dcSSc. For example, C1 cluster gathered patients with the usual presentation of

lcSSc, i.e. older female patients with a low proportion of severe organ involvement, the highest proportion of ACA and an overall good prognosis. C6 corresponded to the usual presentation of dcSSc with a high proportion of male patients, the highest proportion of ATA and severe organ involvement and prognosis. Interestingly, we found other subgroups which went beyond the skin extension. Thus, C2 was characterized mainly by lcSSc but with a rather high rate of ATA and a high proportion of lung fibrosis and suspected PH. C2 had significantly worse prognosis than C1. In the same way, C4 also had a majority of lcSSc and a high rate of visceral complication. C5 was characterized mainly by dcSSc but a rather low rate of lung fibrosis and suspected PH. This suggests that subsetting based only on the extent of skin involvement may overlook the severity of organ involvement and the prognosis.

In addition, there were subgroups of patients where the classical associations lcSSc/ACA and dcSSc/ATA are not straightforward. One of the most representative cluster to illustrate this statement is C2. In this cluster, 71% of patients were lcSSc but 85% had lung fibrosis, with a low rate of ACA positivity (24%) and a significant rate of ATA positivity (35%), which is clearly unexpected in a population where lcSSc is a majority. In that group, the prognosis was worse than in C1 where patients have lcSSc but a low rate of visceral involvement, which again corroborate the data from Nihtyanova et al. [6]. In the same line, a study from the Canadian Scleroderma Research Group (CSRG) focused on the clinical characteristics and survival of ATA positive lcSSc and ACA positive dcSSc patients. Demographic and visceral organ involvement was more associated with the serologic status than with the skin subset. Survival was associated with both skin and autoantibody subsets [7]. Recently, Kranenburg et al. have also shown that ATA-positive lcSSc patients differed from ATA-negative lcSSc patients and ATA-positive dcSSc patients concerning both survival and organ involvement [26]. These studies suggest that sub-setting using antibody markers in addition to extent of

skin involvement might predict clinical outcomes better than skin or serology alone in SSc [7,26]. The importance of the autoantibody profile has also been suggested by Patterson et al. as they identified 5 groups of patients sharing specific clinical and organ associations depending on this profile [9,10]. Other attempts to categorize patients subsets based on shared clinical features, rather than a predetermined decision rule, have grouped them based on changes in mRSS over time [27], changes in the percent predicted FVC [28,29], or gene expression patterns in the skin [30,31]. Each of these approaches has resulted in a small number of subgroups that define the range of phenotypes captured by the stratification characteristics [10]. There is a growing interest for a future classification integrating patterns of underlying pathogenesis, organ involvement and prognosis, in order to personalize disease management and improve outcomes [10,25].

This study has strengths and limitations. The main strengths are the number of patients included in this large prospective and multicentric cohort, and the without any *a priori* approach employed.

The main weakness is that some clinically relevant variables were missing (e.g. other autoantibodies than ACA/ATA or degree of ILD extent on high resolution CT-scan (HRCT)) or were excluded because there was a high proportion of missing data. Moreover, some variables definitions were not fully accurate (e.g. the definitions for ILD (X-ray lung fibrosis where nowadays HRCT is widely used) and PH (suspected by cardiac echo and not confirmed by right heart catheterization)). Another limitation is that we excluded the high proportion of lost to follow-up patients from the survival analysis. However, this exclusion did not modify the comparison of survival between clusters. A potential major bias is the influence of disease duration on the clustering process, given that the rate of organ involvement tends to increase as the disease progresses.

However, we observed similar disease durations between clusters. Finally, the weak reproducibility of the explanatory approach presented here precluded the direct transition from these results to a new classification (e.g. to assign a patient to a specific group based on their characteristics). Moreover, there are well recognized differences between distinct geographical cohorts that have been seen in earlier studies [32]. However, this analysis remained very interesting to describe patterns of associations between clinical and serological characteristics, severity and prognosis in SSc patients and could help defining a new classification.

## Conclusions

In summary, this study confirmed that SSc is a highly heterogeneous disease. Our results suggest that, while a consensus exists on the rationale and usefulness of classifying SSc into limited and diffuse cutaneous subsets, this binary system could overlook the broader spectrum of clinical phenotypes defined not only by skin extension but also by organ involvement, autoantibodies and subsequent prognosis. There is a growing interest for a future SSc classification integrating these different patterns, in order to personalize disease management and improve outcomes.

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**Table 1:** Characteristics of the patients from the entire EUSTAR population and those included in the study.

| Variable | EUSTAR population | | | | | Study population | | |
|---|---|---|---|---|---|---|---|---|
| | Patients included (n=6927) | | Patients not analysed (n=1505) | | | dcSSc (42%) | lcSSc (58%) | |
| | N | % or mean±SD | N | % or mean±SD | p | % or mean±SD | % or mean±SD | p |
| Sex, female | 6924 | 86 | 1505 | 83 | <0.001 | 80 | 91 | <0.001 |
| Ethnicity, white/asian/black | 3973 | 95/3/2 | 1176 | 87/11/2 | <0.001 | 92/5/3 | 97/2/1 | <0.001 |
| Age, years | 6927 | 58.7±13.2 | 1505 | 56.3±13.9 | <0.001 | 55.6±13.0 | 60.9±13.0 | <0.001 |
| Age at SSc onset, years | 6927 | 47.3±13.3 | 1505 | 47.6±14.1 | 0.474 | 45.6±13.2 | 48.5±13.3 | <0.001 |
| Time between Raynaud's and SSc onset, years | 5868 | 3.9±8.0 | 1351 | 3.4±8.1 | <0.001 | 2.0±5.6 | 5.2±9.2 | <0.001 |
| Disease duration, years | 6927 | 11.4±8.1 | 1505 | 8.7±8.1 | <0.001 | 10.0±7.4 | 12.4±8.5 | <0.001 |
| Body mass index, $kg/m^2$ | 2483 | 23.6±4.3 | 889 | 24.4±4.8 | <0.001 | 22.9±4.0 | 24.1±4.4 | <0.001 |
| Raynaud's phenomenon, presence | 6927 | 98 | 1500 | 97 | <0.001 | 98 | 98 | 0.340 |
| Skin involvement, dcSSc | 6913 | 42 | 1437 | 38 | 0.011 | - | - | - |
| mRSS | 6927 | 12.0±9.2 | 1170 | 10.9±9.7 | <0.001 | 18.3±9.8 | 7.5±5.2 | <0.001 |
| Oesophageal symptoms, presence | 6927 | 81 | 1498 | 69 | <0.001 | 84 | 79 | <0.001 |
| Stomach symptoms, presence | 6927 | 42 | 1491 | 27 | <0.001 | 47 | 38 | <0.001 |
| Intestinal symptoms, presence | 6927 | 43 | 1497 | 33 | <0.001 | 44 | 42 | 0.027 |
| Joint contractures, presence | 6927 | 48 | 1492 | 35 | <0.001 | 64 | 36 | <0.001 |
| Joint synovitis, presence | 6927 | 26 | 1496 | 18 | <0.001 | 32 | 22 | <0.001 |
| Tendon friction rubs, presence | 6927 | 17 | 1477 | 8 | <0.001 | 28 | 9 | <0.001 |
| Digital ulceration, history or active | 6927 | 49 | 1491 | 35 | <0.001 | 58 | 42 | <0.001 |
| Muscle atrophy, presence | 6927 | 22 | 1484 | 12 | <0.001 | 30 | 16 | <0.001 |
| Muscle weakness, presence | 6927 | 39 | 1488 | 24 | <0.001 | 47 | 33 | <0.001 |
| CK elevation, presence | 6927 | 13 | 1231 | 13 | 0.711 | 18 | 9 | <0.001 |
| Systemic arterial hypertension, presence | 6927 | 34 | 1492 | 27 | <0.001 | 33 | 35 | 0.150 |
| Palpitations, presence | 6927 | 39 | 1483 | 26 | <0.001 | 41 | 38 | 0.014 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Conduction blocks, presence | 6927 | 22 | 1152 | 14 | <0.001 | 24 | 20 | <0.001 |
| Abnormal diastolic function, presence | 6927 | 33 | 1116 | 22 | <0.001 | 34 | 33 | 0.588 |
| Pericardial effusion, presence | 4442 | 11 | 920 | 8 | 0.042 | 13 | 9 | <0.001 |
| Plain X-ray lung fibrosis, presence | 6927 | 49 | 1033 | 39 | <0.001 | 63 | 39 | <0.001 |
| HRCT lung fibrosis, presence | 3424 | 57 | 816 | 53 | 0.023 | 68 | 48 | <0.001 |
| Restrictive defect on PFTs, presence | 6927 | 43 | 1083 | 33 | <0.001 | 57 | 32 | <0.001 |
| FVC, % predicted | 4349 | 89.3±21.7 | 903 | 90.0±21.8 | 0.437 | 81.4±21.1 | 94.9±20.3 | <0.001 |
| DLCO, % predicted | 6196 | 61.8±20.1 | 1026 | 66.1±21.1 | <0.001 | 57.4±19.9 | 64.9±19.7 | <0.001 |
| 6MWD, meters § | 1179 | 392±134 | 338 | 411±145 | 0.007 | 394±137 | 391±131 | 0.872 |
| Pulmonary hypertension by echo, presence | 6927 | 31 | 1173 | 22 | <0.001 | 33 | 29 | <0.001 |
| sPAP measured by echo, mmhg | 3983 | 34.5±15.3 | 727 | 34.2±15.1 | 0.041 | 34.8±16.4 | 34.2±14.5 | 0.013 |
| Renal crisis, history | 6927 | 3 | 1497 | 3 | 0.626 | 5 | 2 | <0.001 |
| Proteinuria, presence | 6927 | 12 | 1308 | 10 | 0.082 | 15 | 9 | <0.001 |
| CRP elevation, presence | 4736 | 36 | 1100 | 31 | <0.001 | 44 | 30 | <0.001 |
| Antinuclear antibodies, presence | 6927 | 96 | 1412 | 94 | <0.001 | 97 | 96 | 0.400 |
| Anti-centromere antibodies, presence | 6927 | 37 | 1264 | 36 | 0.751 | 14 | 54 | <0.001 |
| Anti-topoisomerase I antibodies, presence | 6927 | 39 | 1270 | 36 | 0.028 | 61 | 23 | <0.001 |
| Anti-U1RNP antibodies, presence | 4054 | 5 | 807 | 7 | 0.006 | 5 | 5 | 0.770 |
| Anti-PM/Scl antibodies, presence | 3335 | 3 | 648 | 4 | 0.278 | 5 | 2 | <0.001 |
| Anti-RNA polymerase III antibodies, presence | 3163 | 4 | 563 | 6 | 0.025 | 6 | 3 | <0.001 |
| Steroids, active or past use | 4647 | 43 | 1081 | 38 | 0.006 | 55 | 34 | <0.001 |
| Immunosuppressive drugs, active or past use | 4631 | 42 | 1085 | 44 | 0.162 | 60 | 28 | <0.001 |

Oesophageal symptoms: dysphagia and/or reflux; stomach symptoms: early satiety and/or vomiting; intestinal symptoms: diarrhoea, bloating and/or constipation; mRSS : modified Rodnan skin score; PFTs: pulmonary function tests; FVC: forced vital capacity; DLCO: diffusing capacity of the lung for carbon monoxide; 6MWD: 6-minute walking distance
Numbers are given as % or mean±SD. Characteristics of patients were compared using Student's test for continuous variables and Fisher's exact test for categorical variables. Clustering variables are underlined. § n=1517

**Table 2:** Characteristics of the 2 and 6 clusters found in the analysis

| | **2 clusters** | | **6 clusters** | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C(A) | C(B) | C1 | C2 | C3 | C4 | C5 | C6 |
| Jaccard index | 0.64 | 0.66 | 0.39 | 0.32 | 0.57 | 0.38 | 0.68 | 0.4 |
| N | 3149 | 3778 | 1186 | 720 | 1243 | 1673 | 1249 | 85 |
| **DEMOGRAPHICS** | | | | | | | | |
| Sex, *female* | 90 | 84 | 94 | 88 | 88 | 88 | 81 | 79 |
| Ethnicity, *white/asian/black* | 94/5/2 | 96/2/2 | 97/2/1 | 88/10/2 | 94/4/2 | 96/2/2 | 94/3/3 | 96/2 |
| Age, *years* | 59.2 | 58.2 | 61.3 | 60.1 | 56.6 | 61.2 | 55.8 | 55. |
| Age at SSc onset, *years* | 47.9 | 46.7 | 48.9 | 48.3 | 46.7 | 48.1 | 46.0 | 45. |
| Time between Raynaud's and SSc onset, *years* | 4.8 | 3.1 | 5.5 | 4.4 | 4.4 | 3.9 | 2.8 | 2.2 |
| Disease duration, *years* | 11.3 | 11.5 | 12.5 | 11.8 | 9.9 | 13.2 | 9.8 | 10. |
| Body mass index, $kg/m^2$ | 24.1 | 23.2 | 24.3 | 24.5 | 23.6 | 23.6 | 23.3 | 22. |
| | | | | | | | | |
| **SSc CHARACTERISTICS** | | | | | | | | |
| **Cutaneous involvement** | | | | | | | | |
| Skin involvement, *dcSSc* | 19 | 61 | 11 | 29 | 21 | 37 | 72 | 92 |
| mRSS, *mean peak value* | 6.6 | 16.5 | 6.6 | 7.2 | 6.3 | 9.2 | 19.0 | 27. |
| **Gastro-intestinal involvement** | | | | | | | | |
| Oesophageal symptoms, *presence* | 73 | 88 | 88 | 76 | 58 | 91 | 79 | 95 |
| Stomach symptoms, *presence* | 26 | 55 | 52 | 16 | 7 | 60 | 36 | 70 |
| Intestinal symptoms, *presence* | 33 | 50 | 64 | 21 | 11 | 57 | 34 | 63 |
| **Joint involvement** | | | | | | | | |
| Joint contractures, *presence* | 24 | 67 | 29 | 17 | 23 | 65 | 55 | 91 |
| Joint synovitis, *presence* | 14 | 37 | 15 | 13 | 15 | 37 | 25 | 53 |
| Tendon friction rubs, *presence* | 4 | 28 | 6 | 3 | 4 | 19 | 19 | 57 |
| **Vascular involvement** | | | | | | | | |
| Raynaud's phenomenon, *presence* | 98 | 99 | 99 | 98 | 97 | 99 | 98 | 99 |
| Digital ulceration, *history or active* | 32 | 63 | 35 | 24 | 33 | 62 | 50 | 85 |
| **Muscular involvement** | | | | | | | | |
| Muscle atrophy, *presence* | 16 | 59 | 27 | 8 | 10 | 69 | 33 | 77 |
| Muscle weakness, *presence* | 6 | 35 | 9 | 3 | 6 | 38 | 17 | 57 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CK elevation, *presence* | 6 | 18 | 7 | 7 | 5 | 17 | 13 | 26 |
| **Cardiac involvement** | | | | | | | | |
| Systemic arterial hypertension, *presence* | 31 | 37 | 38 | 28 | 26 | 44 | 26 | 38 |
| Palpitations, *presence* | 25 | 51 | 38 | 32 | 9 | 64 | 28 | 57 |
| Conduction blocks, *presence* | 12 | 30 | 16 | 14 | 6 | 39 | 16 | 34 |
| LVEF<50, *presence* | 3 | 7 | 3 | 3 | 2 | 6 | 5 | 10 |
| Abnormal diastolic function, *presence* | 24 | 42 | 27 | 33 | 15 | 54 | 24 | 43 |
| Pericardial effusion, *presence* | 7 | 14 | 7 | 11 | 4 | 15 | 9 | 18 |
| Pulmonary hypertension by echo, *presence* | 21 | 39 | 24 | 39 | 8 | 44 | 24 | 50 |
| sPAP measured by echo, *mmhg* | 32.5 | 36.0 | 33.0 | 36.7 | 29.4 | 37.3 | 32.4 | 38. |
| **Interstitial lung disease** | | | | | | | | |
| Plain X-ray lung fibrosis, *presence* | 29 | 65 | 8 | 85 | 17 | 72 | 46 | 80 |
| HRCT lung fibrosis, *presence* | 38 | 70 | 22 | 78 | 29 | 73 | 56 | 82 |
| Restrictive defect on PFTs, *presence* | 24 | 58 | 13 | 61 | 14 | 60 | 42 | 77 |
| FVC, *% predicted* | 97.8 | 82.7 | 101.2 | 86.7 | 99.9 | 84.4 | 87.5 | 72. |
| DLCO, *% predicted* | 68.0 | 56.6 | 69.8 | 57.7 | 72.3 | 55.2 | 62.5 | 50. |
| 6MWD, *meters* | 411 | 381 | 400 | 405 | 427 | 366 | 418 | 36 |
| **Renal involvement** | | | | | | | | |
| Renal crisis, *history* | 2 | 4 | 2 | 1 | 2 | 4 | 3 | 8 |
| Proteinuria, *presence* | 7 | 16 | 6 | 8 | 7 | 15 | 11 | 26 |
| **Blood tests** | | | | | | | | |
| CRP elevation, *presence* | 24 | 45 | 25 | 29 | 20 | 43 | 36 | 62 |
| Hypocomplementemia, *presence* | 10 | 13 | 13 | 7 | 8 | 14 | 10 | 12 |
| Antinuclear antibodies, *presence* | 96 | 97 | 98 | 94 | 95 | 97 | 95 | 98 |
| Anti-centromere antibodies, *presence* | 54 | 22 | 79 | 24 | 48 | 29 | 20 | 12 |
| Anti-topoisomerase I antibodies, *presence* | 21 | 54 | 8 | 35 | 24 | 46 | 50 | 77 |
| Anti-U1RNP antibodies, *presence* | 5 | 5 | 3 | 8 | 5 | 7 | 3 | 4 |
| Anti-PM/Scl antibodies, *presence* | 2 | 4 | 1 | 3 | 1 | 4 | 4 | 6 |
| Anti-RNA polymerase III antibodies, *presence* | 3 | 5 | 2 | 3 | 4 | 3 | 6 | 6 |
| **Treatment** | | | | | | | | |
| Steroids, *active or past use* | 27 | 55 | 22 | 45 | 24 | 57 | 44 | 65 |
| Prednisone, *mg/day* | 2.8 | 5.7 | 2.0 | 5.5 | 2.3 | 5.6 | 4.6 | 7.3 |
| Immunosuppressive drugs, *active or past use* | 27 | 54 | 17 | 44 | 27 | 48 | 54 | 66 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Mortality** | | | | | | | | |
| Number of deaths per 1000 patient-years | 10.3 | 22.6 | 7.5 | 17.3 | 9.7 | 19.1 | 20.8 | 31. |

PFTs: pulmonary function tests; FVC: forced vital capacity; DLCO: diffusing capacity of the lung for carbon monoxide; 6MWD: 6-minute walking distance; mRSS : modified Rodnan skin score
Numbers are given as % or mean±SD. Characteristics of patients were compared using Student's test for continuous variables and Fisher's exact test for categorical variables.
Clustering variables are underlined

**Table 3:** Survival analyses

| | Univariable analysis | | Multivariable analysis | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Age at disease onset | | Age at disease onset<br>Sex | | Age at disease onset<br>Sex<br>Immunosuppressive treatment | |
| | N=3352<br>HR (95%CI) | p | N=3352<br>HR (95%CI) | p | N=3352<br>HR (95%CI) | p | N=2887<br>HR (95%CI) | p |
| lcSSc | Reference | | Reference | | Reference | | Reference | |
| dcSSc | 1.90 (1.64-2.19) | <0.001 | 2.39 (2.07-2.77) | <0.001 | 2.14 (1.85-2.48) | <0.001 | 2.03 (1.61-2.56) | <0.001 |
| Cluster A | Reference | | Reference | | Reference | | Reference | |
| Cluster B | 2.23 (1.88-2.65) | <0.001 | 2.40 (2.02-2.85) | <0.001 | 2.26 (1.91-2.69) | <0.001 | 2.47 (1.86-3.27) | <0.001 |
| Cluster 1 | Reference | | Reference | | Reference | | Reference | |
| Cluster 2 | 2.32 (1.62-3.31) | <0.001 | 2.10 (1.46-3.00) | <0.001 | 1.97 (1.38-2.82) | <0.001 | 1.64 (0.88-3.03) | 0.119 |
| Cluster 3 | 1.30 (0.89-1.91) | 0.172 | 1.63 (1.11-2.38) | 0.012 | 1.62 (1.11-2.37) | 0.013 | 1.97 (1.10-3.54) | 0.023 |
| Cluster 4 | 2.47 (1.86-3.27) | <0.001 | 2.49 (1.88-3.30) | <0.001 | 2.40 (1.81-3.19) | <0.001 | 2.77 (1.74-4.39) | <0.001 |
| Cluster 5 | 3.03 (2.23-4.11) | <0.001 | 3.77 (2.77-5.12) | <0.001 | 3.37 (2.47-4.58) | <0.001 | 3.22 (1.93-5.36) | <0.001 |
| Cluster 6 | 4.40 (3.30-5.87) | <0.001 | 5.85 (4.38-7.81) | <0.001 | 5.20 (3.89-6.95) | <0.001 | 6.14 (3.81-9.89) | <0.001 |

**Figure Legends**

**Figure 1. A.** Dendrogram of the 6,927 patients included in the cluster analysis. The length of vertical lines represents the degree of similarity between patients. **B.** Heat map showing the clinical characteristics in each cluster.

**Figure 2. A.** Main characteristics of the 6 clusters. **B.** Representative proportions of the main clinical characteristics for each cluster. **C.** Kaplan-Meier survival curves of the 6 clusters. **D.** Forrest plot showing mortality hazard ratios and 95% confidence intervals in the 6 clusters.

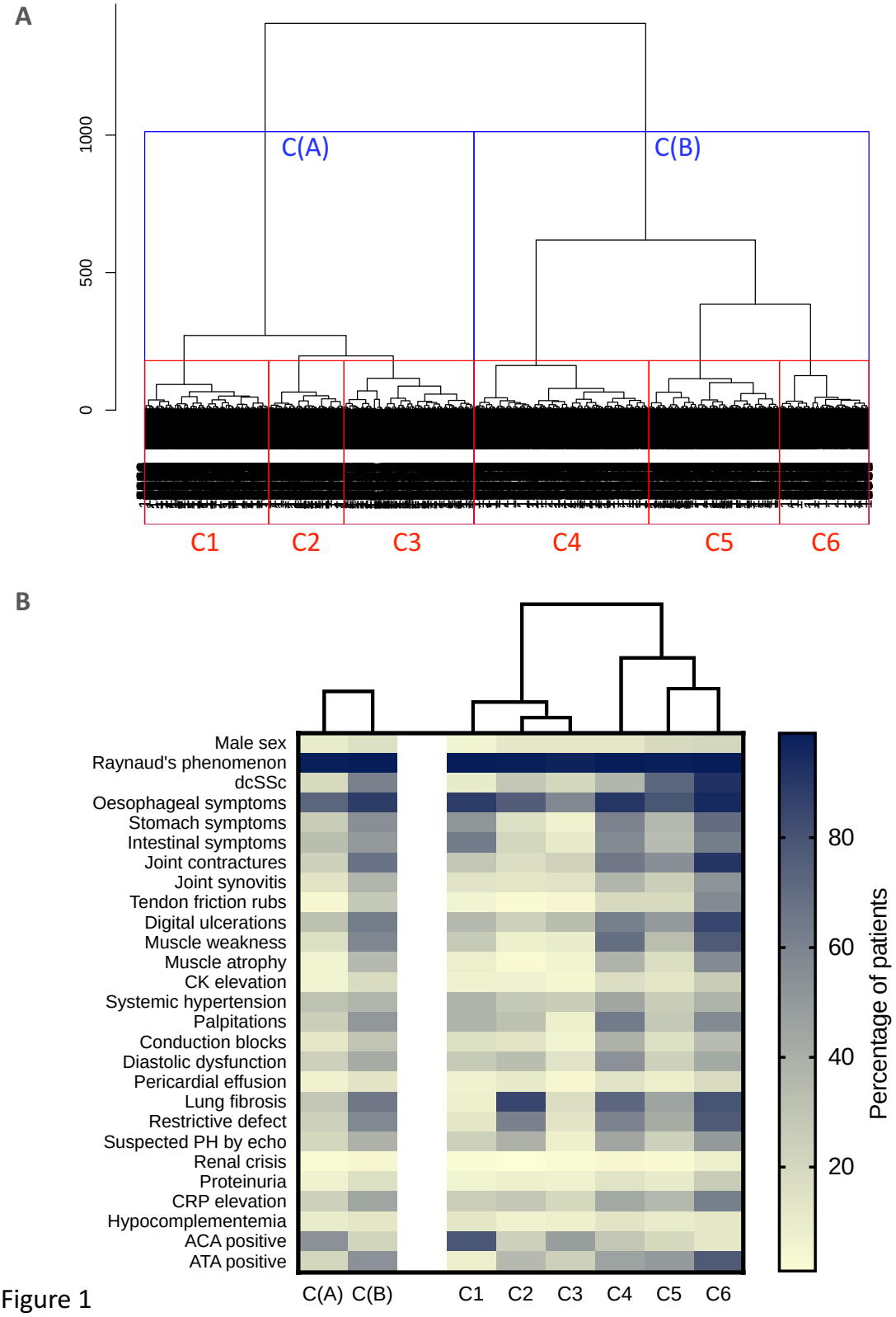
A
1000
500
0
C(A)
C(B)
C1
C2
C3
C4
C5
C6
B
Male sex
Raynaud's phenomenon
dcSSc
Oesophageal symptoms
Stomach symptoms
Intestinal symptoms
Joint contractures
Joint synovitis
Tendon friction rubs
Digital ulcerations
Muscle weakness
Muscle atrophy
CK elevation
Systemic hypertension
Palpitations
Conduction blocks
Diastolic dysfunction
Pericardial effusion
Lung fibrosis
Restrictive defect
Suspected PH by echo
Renal crisis
Proteinuria
CRP elevation
Hypocomplementemia
ACA positive
ATA positive
C(A) C(B) C1 C2 C3 C4 C5 C6
80
60
40
20
Percentage of patients

Figure 1

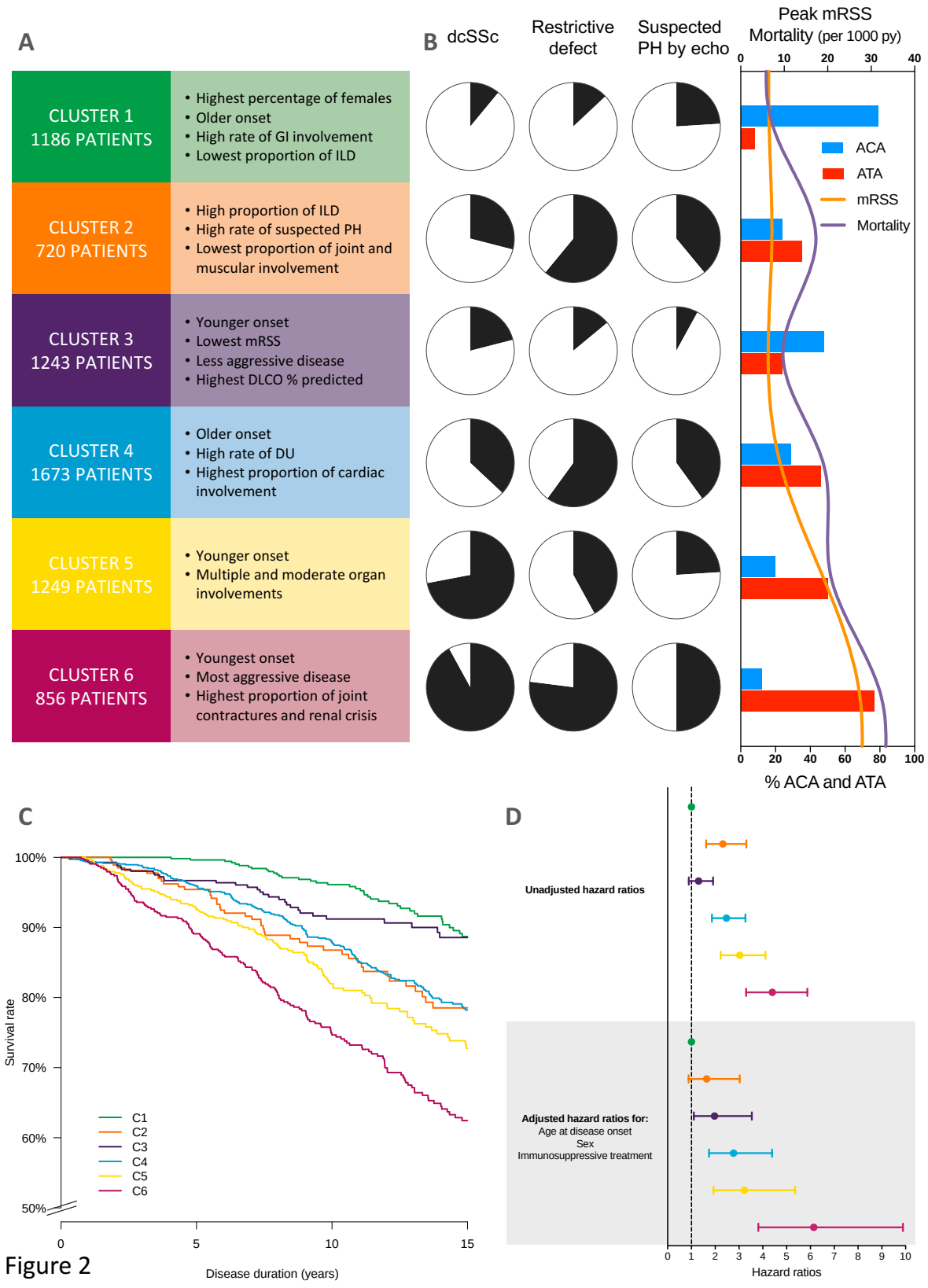
A
CLUSTER 1
1186 PATIENTS
• Highest percentage of females
• Older onset
• High rate of GI involvement
• Lowest proportion of ILD
CLUSTER 2
720 PATIENTS
• High proportion of ILD
• High rate of suspected PH
• Lowest proportion of joint and muscular involvement
CLUSTER 3
1243 PATIENTS
• Younger onset
• Lowest mRSS
• Less aggressive disease
• Highest DLCO % predicted
CLUSTER 4
1673 PATIENTS
• Older onset
• High rate of DU
• Highest proportion of cardiac involvement
CLUSTER 5
1249 PATIENTS
• Younger onset
• Multiple and moderate organ involvements
CLUSTER 6
856 PATIENTS
• Youngest onset
• Most aggressive disease
• Highest proportion of joint contractures and renal crisis
B
dcSSc
Restrictive defect
Suspected PH by echo
Peak mRSS
Mortality (per 1000 py)
0 10 20 30 40
ACA
ATA
mRSS
Mortality
0 20 40 60 80 100
% ACA and ATA
C
100%
90%
80%
70%
60%
50%
Survival rate
C1
C2
C3
C4
C5
C6
0 5 10 15
Disease duration (years)
D
Unadjusted hazard ratios
Adjusted hazard ratios for:
Age at disease onset
Sex
Immunosuppressive treatment
0 1 2 3 4 5 6 7 8 9 10
Hazard ratios

Figure 2

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## II.2 Intérêt du dosage des chaines légères libres sériques dans la ScS

### II.2.1 Introduction

Notre premier travail a donc suggéré qu'il existe des groupes homogènes de patients au-delà de la dichotomie diffuse/limitée. La présence d'atteintes d'organe et le statut des auto-anticorps semblent des éléments importants pour classer les patients. Les travaux suivants ont donc eu pour objectifs d'analyser les associations cliniques de différents marqueurs immunologiques, dont les chaînes légères libres sériques (serum free light chain : SFLC).

Cette étude sur l'intérêt du dosage des SFLC s'est aussi inscrite dans une démarche de caractérisation du rôle du système humoral et en particulier du lymphocyte B dans la ScS (**Figure 8**). En effet, l'homéostasie lymphocytaire B est perturbée dans la ScS comme en témoignent une activation permanente des lymphocytes B mémoires et une susceptibilité accrue à l'apoptose. Cette perte chronique de lymphocytes B induit une production médullaire de lymphocytes B naïfs, liée à une sécrétion importante de B cell activating factor (BAFF). Sur le plan fonctionnel, les lymphocytes B sont capables de sécréter des cytokines pro-inflammatoires et pro-fibrosantes telles que l'interleukine 6 et le TGF-ß. Enfin, des données récentes suggèrent un effet potentiel des traitements induisant une déplétion lymphocytaire B tel que le rituximab (Bosello et al., 2011 ; Sanges et al., 2017 ; Sanges et al., 2017).

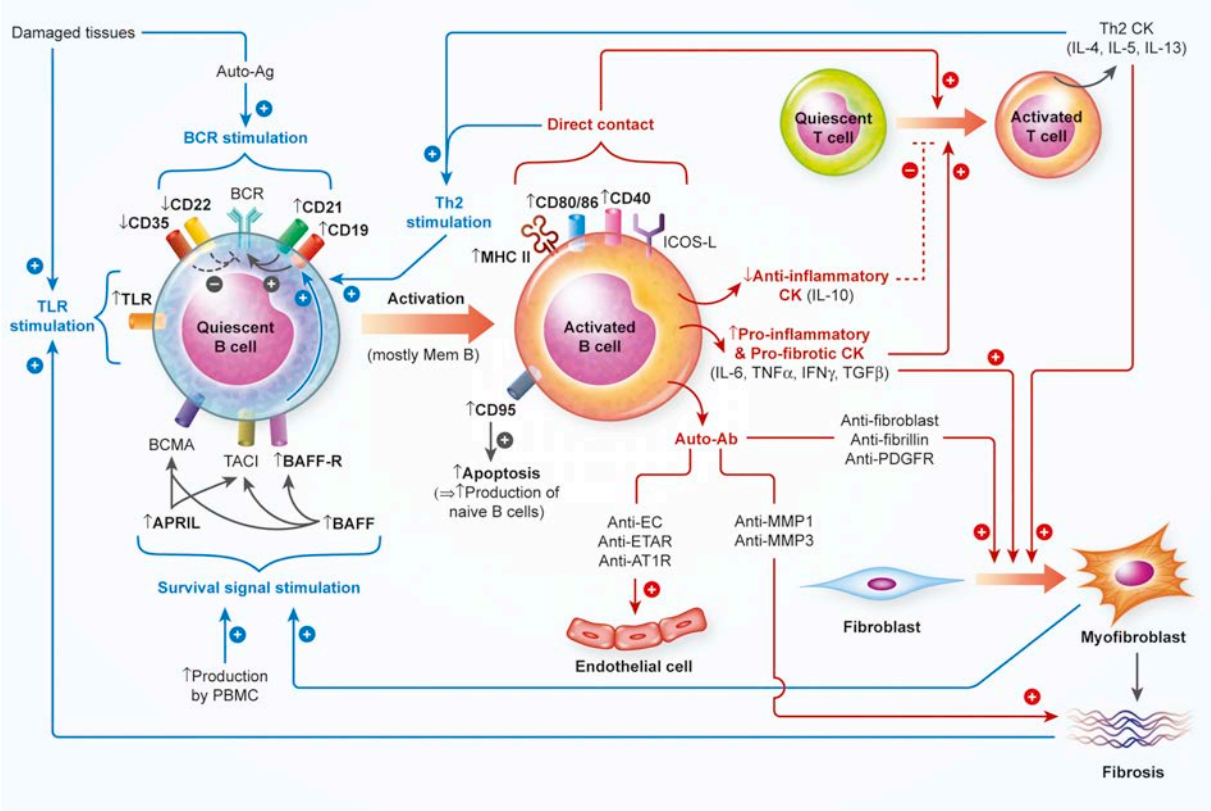


**Figure 8 :** Perturbations de l'homéostasie et des propriétés fonctionnelles du lymphocyte B durant la ScS, d'après Sanges et al., 2017

### II.2.2 Résumé

**Introduction**

Les chaînes légères libres des immunoglobulines (Ig) sont produites en excès lors de la synthèse des Ig par les lymphocytes B. Leur élévation dans le sérum (*serum free light chains* ou SFLC) est bien connue dans les gammapathies monoclonales et constitue un marqueur diagnostique et pronostique des myélomes. Leur étude dans les situations de stimulation immunitaire chronique polyclonale, et notamment dans les maladies auto-immunes, est en plein essor. Dans le lupus, la polyarthrite rhumatoïde, et le syndrome de Gougerot-Sjögren, il a été démontré que les SFLC sont plus élevées que chez les sujets sains, mais aussi que leur élévation est corrélée à l'activité de ces maladies. Aucune donnée n'existe à ce sujet dans la sclérodermie systémique (ScS).

**Patients et Méthodes**

134 patients ayant une ScS ont été inclus de façon prospective. Les données suivantes ont été recueillies : date de diagnostic, forme clinique, atteintes viscérales, manifestations biologiques, score d'activité et association à d'autres maladies auto-immunes. Le dosage des SFLC (Combylite, The Binding Site, Birmingham, RU) des facteurs rhumatoïdes et de la béta2 microglobuline a été réalisé lors de l'inclusion. Les taux de SFLC des patients ont été comparés à ceux de 401 sujets donneurs de sang (sujets contrôles) appariés par le sexe et par tranche d'âge.

**Résultats**

Les taux moyens et médians de SFLC sont significativement plus élevés chez les patients ScS (médiane 19,99 mg/l, moyenne 24,03 mg/l) comparativement aux contrôles (médiane 15,43 mg/l, moyenne 17,50 mg/l). En analyse univariée, il existe une corrélation significative entre le taux de SFLC et certaines caractéristiques cliniques de la maladie : le score de Rodnan modifié (p=0,0099), les antécédents d'ulcérations digitales (p=0,0426), l'existence d'un syndrome sec subjectif (p=0,0495),

la pression artérielle pulmonaire systolique (p=0,0369), la DLCO (p=0,0063) ainsi qu'aux scores d'activité EUSTAR (p=0,009) et de gravité MEDGSER (p=0,0001) de la maladie. Le taux de SFLC est également corrélé à la vitesse de sédimentation (p<0,0001), la CRP (p=0,0002), le taux de gammaglobulines (p<0,0001), d'Ig A, G et M, On ne retrouve par contre pas de corrélation avec l'existence d'une maladie auto-immune associée ou la présence d'auto-anticorps spécifiques de ScS.

**Conclusion**

Notre étude montre que le taux de SFLC est plus élevé chez les patients ScS que chez les contrôles et est associé à des paramètres de gravité de la maladie tels que le score de Rodnan, les scores d'activité, les pressions pulmonaires et la DLCO. Ces résultats constituent des arguments supplémentaires pour évoquer la participation active des lymphocytes B à la physiopathologie de la ScS.

**II.2.3 Article** *(Autoimmunity Reviews 13 (2014) 974–980)*

**Serum free light chains of immunoglobulins as biomarkers for systemic sclerosis characteristics, activity and severity**

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**ABSTRACT**

Systemic sclerosis (SSc) is a chronic autoimmune connective tissue disease. Humoral immunity and B cells are thought to play an important role in the pathophysiology of the disease. B cells are activated, produce specific autoantibodies and profibrotic cytokines. One way to assess B cell activation is to measure serum free light chains of immunoglobulins (sFLC) levels. We assess here sFLC levels in patients with systemic sclerosis (SSc) and their correlation with the disease characteristics, activity and severity. One hundred and thirty-four SSc patients were prospectively enrolled and compared to 401 age- and sex-matched healthy controls. sFLC levels were measured by a new quantitative immunoassay. sFLC levels were significantly higher in SSc patients than in healthy controls. sFLC levels correlated with modified Rodnan skin score and were independently associated with the presence of interstitial lung disease and its severity. In univariate analysis, sFLC levels correlated with SSc activity, as measured by the European Scleroderma Study Group activity index, and severity, as measured by the Medsger total severity score. In multivariate analysis, beta2-microglobulin levels correlated with disease activity, BAFF levels with severity and sFLC with neither of these. Other B-cell activation biomarkers (IgG, IgA, beta2-microglobulin and BAFF) were independently correlated with sFLC. sFLC levels are elevated in SSc and are independently associated with lung disease and its severity. B-cell activation biomarkers, including sFLC, beta2-microglobulin and BAFF, correlate with disease severity and activity. These results further support the role of B cells activation in the pathophysiology of SSc.

**Key words:** free light chain; BAFF; beta2-microglobulin; B cells; systemic sclerosis

## 1. INTRODUCTION

Systemic sclerosis (SSc) is a chronic connective tissue disorder characterized by vascular involvement, fibrosis and autoimmunity[1, 2]. The clinical heterogeneity of SSc is a major issue to identify and validate robust biomarkers for disease severity and activity, which are crucial to optimize the management of this disease [3, 4]. The majority of these biomarkers are collagenous or non-collagenous extracellular matrix constituents[5, 6]. Besides cellular immunity abnormalities [7], humoral immunity is increasingly thought to play a significant role in the pathophysiology of this disease[8-13]. Hypergammaglobulinaemia is associated with a more severe disease with pulmonary involvement[14, 15] SSc patients have abnormal blood B-cell compartments with an expansion of naive B cells and activated memory B cells[16]. B cells can secrete proinflammatory and/or profibrotic cytokines such as IL-6 and TGF-β leading to collagen secretion by fibroblasts[17]. B-cell activating factor (BAFF) is crucial for B-cell survival[18] and is elevated both in serum and skin of SSc patients and correlates with the skin score[19]. Finally, anti-CD20 treatment has yielded promising results in patients with severe SSc with or without interstitial lung disease (ILD)[20-23]. Taken together, these data suggest that B cells and their activation are likely to play an important role in SSc pathophysiology.

B-cell activation is associated with an exaggerated polyclonal synthesis of immunoglobulins. Immunoglobulin light chains and heavy chains are combined together during this synthesis. However, free light chains are physiologically produced in excess to heavy chains[24] and are released into the serum, from which they are rapidly excreted by the kidneys with a half-life of 2–6 hours. A new automated latex-enhanced single turbidimetric assay for simultaneous measurement of both kappa and lambda free light chains is now available to readily measure both kappa and lambda serum free light chain

of immunoglobulins (sFLC) levels[25]. sFLC are mainly used in monitoring plasma-cell dyscrasia[26]. However, a growing body of studies suggests that sFLC could be useful biomarkers in several immunopathological conditions by reflecting B-cell polyclonal activation. As an example, sFLC levels are elevated in lupus[27], rheumatoid arthritis and Sjögren syndrome[28, 29] and are associated with the disease activity.

To our knowledge, no study has yet focused on sFLC in SSc. We designed the following prospective and controlled study to address the role of sFLC as a biomarker for disease activity and severity in SSc.

## 2. METHODS

### 2.1. Patients

All consecutive patients with SSc followed in the scleroderma outpatient clinic of Lille University Hospital were prospectively included between October 2012 and October 2013 if they fulfilled the following inclusion criteria: (1) age over 18 years; (2) American College of Rheumatology (ACR) criteria for SSc[30] and/or the LeRoy's classification system (limited cutaneous or diffuse cutaneous SSc)[31]. There were no exclusion criteria. Sex- and age-matched healthy blood donors served as controls with 3 healthy controls (HC) for 1 SSc patient.

Prospective assessment at the time of blood sample collection for SSc patients gathered data on age, weight, presence of digital ulcers, gastro-oesophageal reflux disease (GERD), New York Heart Association (NYHA) functional class and distance on a non-encouraged 6-minute walk test (6MWD). Modified Rodnan skin score (mRSS) was measured in all patients. All patients underwent pulmonary functional tests. Forced vital capacity (FVC) and diffusing capacity for carbon monoxide (DLCO) were expressed as percentages of the predicted values. Muscle involvement was diagnosed if there was

muscle weakness associated with raised creatine phosphokinase (CPK) level and electromyographic, muscle MRI or muscle biopsy abnormalities[32, 33]. Joint involvement was defined by either synovitis or inflammatory arthralgia. Disease duration was measured from: onset of Raynaud's phenomenon (RP), onset of first non-RP symptom and diagnosis of SSc.

We collected from the patients' medical records: history of renal crisis, presence of ILD on high-resolution CT scan of the chest and presence of pulmonary arterial hypertension (PAH) diagnosed on right heart catheterization.

At the time of blood sample collection, we also prospectively assessed disease activity scored using the European Scleroderma Study Group activity index (EScSG-AI)[34]. The severity of organ involvement was evaluated using Medsger's severity scale for each organ as well as a total Medgser's severity score summing each scale for a maximum score of 36[35]. Physician global assessment (PGA) was measured on a visual scale ranging from 0 mm (no active disease) to 100 mm (highly active disease). Disability was assessed by the health assessment questionnaire disability index (HAQ-DI). Patients were considered to have a history of immunosuppressive drugs if they had received an immunosuppressive treatment within the 6 months before inclusion. We also collected information on the use of more than 10 mg/d of prednisone or equivalent within the 6 months before inclusion.

In accordance with French legislation, written information was provided and consent was obtained from each patient. The study was conducted in accordance with the recommendations of the Helsinki Declaration and complied with the requirements of the French Commission Nationale Informatique et Libertés (No. DC-2008-642).

### 2.2. LABORATORY ANALYSES

Serum was collected at the time of clinical assessment and stored at -80°C until assayed. sFLC levels were measured both in patients and HC using Combylite® (The Binding Site, Birmingham, UK) according to the manufacturer's recommendations. Combylite® is a single assay that measures the combined level of free kappa and free lambda immunoglobulin light chains in serum using a SPA PLUS turbidimeter[25].

The specificity of antinuclear autoantibodies was identified as part of routine clinical care using both specific immunofluorescence patterns on HEp-2 cells (Euroimmun, Lübeck, Germany) and the Luminex approach (Bio-Plex 2200, Bio-Rad® Laboratories Ltd, CA, USA) for anti-topoisomerase I antibodies (ATA) and anti-centromere antibodies (ACA) identification. Rheumatoid factor and serum beta2-microglobulin were measured in each patient. Serum BAFF level was also measured in each patient in duplicate using a microplate EIA assay (R&D Systems, Minneapolis, MN, USA). Serum electrophoresis and serum and urine immunofixation were performed in all patients to assess the presence of a monoclonal component.

### 2.3. Statistical analysis

A propensity score matching method was used to reduce recruitment bias: a group of 134 patients with SSc were matched according to propensity score with 401 HC (3 HC for 1 SSc patient), leading to an even distribution of potential confounding factors (age and sex). Qualitative variables were described by frequency and percentage. The continuous variables are reported as mean±SD. Normal distribution of quantitative variables was tested by the Shapiro–Wilk test.

Bivariate analyses were performed to study the relationships between each explanatory variable and sFLC. Pearson's or Spearman's correlation coefficient was used for

quantitative variables and Student's t-test or the Mann–Whitney test for qualitative variables. In addition, a multivariable linear regression with a stepwise selection at the level 0.2 was performed to identify a subset of the most important explanatory variables for the relationship with FLC. In order to avoid the problem of multicolinearity, which occurs when the explanatory variables are highly correlated, and to obtain a parsimonious model, we adopted the following strategy: first, variables with $p < 0.2$ were selected and included in a principal component analysis (PCA) in order to study their correlations. Then, the variables included in the multivariable regression model were selected from the PCA results (graphic correlation circle) on the basis of their clinical relevance. The stability of the model was assessed by a bootstrap method. In the final model, for each variable we computed the adjusted p-value. The same analysis was used to study the relationships between each explanatory variable and EScSG-AI, Medsger's total severity score, Medger's score and presence of ILD. Finally, receiver operating characteristic (ROC) curves were performed to find the best threshold FLC discriminating the variable Medger's score-lung (0 or 1 or 2 vs 3 or 4). All analyses were performed with SAS software, version 9.3 (SAS Institute Inc., Cary, NC, USA). All tests were performed at a significance level of 0.05.

## 3. RESULTS

### 3.1. Study population

One hundred and thirty-four consecutive SSc patients were prospectively enrolled. Baseline characteristics of patients are described in **table 1**. The control group comprised 401 HC (326 [81%] females), with a mean age of 55±11 years.

Regarding the biological characteristics of SSc patients, total gammaglobulin levels were

10.6±3.2 g/L. IgG, IgA and IgM levels were 10.8±3.2, 2.4±1.3 and 1.3±0.7 g/L, respectively. BAFF and beta2-microglobulin levels were 1133±1123 pg/mL and 2.0±0.8 mg/L, respectively. Rheumatoid factor titre was 14±26 UI/L.

### 3.2. sFLC levels in patients and controls

sFLC levels were significantly higher in SSc patients than in HC (24.0±13.8 vs 17.5±19 mg/L, p=0.0002) (**figure 1**). Eight (6%) SSc patients had a monoclonal gammopathy (IgG kappa in four patients, IgG lambda in two and IgA kappa in two). All these monoclonal gammopathies were of unknown significance with no signs of multiple myeloma. There was no significant difference in sFLC level between these eight patients and the rest of the SSc cohort.

### 3.3. Correlation between sFLC and clinical and biological characteristics of SSc

In univariate analysis, sFLC levels significantly and positively correlated with mRSS (**figure 2**) and systolic pulmonary arterial pressure (PAP) and were associated with history of digital ulcers and history of immunosuppressive drugs. sFLC levels negatively correlated with DLCO (**table 2**).

We assessed the correlation between sFLC levels and other biomarkers of B-cell activation (BAFF, rheumatoid factor, and beta2-microglobulin) as well as total gammaglobulins, IgG, IgA, IgM levels and inflammation parameters (C-reactive protein [CRP] and erythrocyte sedimentation rate [ESR]) in SSc patients (**table 2**). In univariate analysis, all these parameters were significantly correlated with sFLC levels.

In multivariate analysis, sFLC levels were independently associated with history of digital

ulcers, presence of ILD and history of immunosuppressive drugs. We also found a large correlation with IgG, IgA and beta2-microglobulin (**figure 3A**) as well as a smaller correlation with rheumatoid factor and BAFF (**figure 3B**). Conversely, there was no correlation between sFLC levels and IgM, CRP and ESR.

### 3.4. Correlation between sFLC and SSc activity and severity

sFLC levels correlated positively and significantly with the EScSG-AI (r=0.28, p=0.009, **figure 4A**), with PGA (r=0.21, p=0.03) and with the Medsger's total severity score (r=0.33, p=0.0001, **figure 4C**). Patients with an sFLC level <20 mg/L (median value) had a mean EScSG-AI of 1.2±1.4.vs 1.8±1.6 (p=0.04) for patients with an sFLC level >20 mg/L (**figure 4B**) and a mean Medsger's total severity score of 4.6±2.5 vs 6.2±3.1 (p=0.001) (**figure 4D**). sFLC levels positively correlated with Medsger score-general, Medsger score-skin and Medsger score-lung but not with the other components of the Medsger's score (data not shown).

We then assessed which clinical and biological parameters were associated with EScSG-AI and the Medsger's total severity score both in univariate and multivariate analysis, after exclusion of the parameters already included in the EScSG-AI and Medsger's severity score (**supplemental tables 1 and 2**). In univariate analysis, besides sFLC, diffuse SSc, ATA, PAH, NYHA functional class, systolic PAP, FVC, 6MWD, thyroiditis, HAQ-DI, PGA, IgA, beta2-microglobulin, BAFF and CRP were significantly associated with the EScSG-AI. In multivariate analysis, only FVC (negative association) and beta2-microglobulin (positive association) were independently correlated with the EScSG-AI.

For disease severity, in univariate analysis, besides sFLC levels, diffuse SSc, ATA and ACA, 6MWD, thyroiditis, HAQ-DI, PGA, corticosteroids, history of immunosuppressive drugs, IgA, beta2-microglobulin, BAFF, CRP and ESR were associated or correlated with the Medsger's total severity score. In multivariate analysis, diffuse SSc, HAQ-DI, corticosteroids and BAFF were significantly and independently associated with the Medsger's total severity score.

### 3.5. SFLC AND PRESENCE AND SEVERITY OF LUNG INVOLVEMENT

As ILD was independently associated with sFLC levels, we determined which parameters were associated with the presence of ILD. In multivariate analysis, FVC, mRSS, history of immunosuppressive drugs, sFLC levels, IgM levels and ATA were independently associated with the presence of ILD (data not shown). sFLC levels had an odds ratio of 1.08 (95% confidence interval: 1.03–1.14) for the presence of ILD.

As sFLC levels were associated with the presence of ILD and correlated with the lung component of the Medsger's severity score, we assessed which variables were associated with the severity of the lung involvement (**supplemental table 3**). In multivariate analysis, ATA, 6MWD, history of immunosuppressive drugs and sFLC levels were significantly and independently associated with the lung component of Medsger's severity score.

## 4. DISCUSSION

The main results of this study are: (1) patients with SSc had higher sFLC levels than HC; (2) higher sFLC levels were independently associated with the presence of ILD and the lung severity score in SSc; (3) sFLC levels were correlated with mRSS and with SSc activity and severity in univariate analysis but not in multivariate analysis; other B-cell activation biomarkers, i.e. beta2-microglobulin and BAFF, were independently associated with activity and severity, respectively; (4) in SSc patients, sFLC levels were independently and significantly correlated with beta2-microglobulin and BAFF but with neither CRP nor ESR.

To our knowledge, this is the first study to focus on sFLC in SSc patients. We have demonstrated that patients with SSc had higher sFLC levels than age- and sex-matched HC. sFLC have been found to be elevated in several other autoimmune

diseases, such as rheumatoid arthritis, lupus and Sjögren syndrome[27-29]. Elevated sFLC are usually thought to represent B-cell hyperactivity with increased formation of immune complexes. In line with this hypothesis, we also found that sFLC levels were significantly correlated with other B-cell activation biomarkers, such as BAFF, rheumatoid factor and beta2-microglobulin, as in Sjögren syndrome[28].

Several clinical characteristics of SSc were correlated or associated with sFLC levels in our study. sFLC levels positively correlated with mRSS and negatively correlated with DLCO in univariate analysis. We also found that sFLC levels were independently associated with the presence and the severity of ILD. As mRSS (reflecting skin involvement) and ILD are the main fibrotic manifestations of SSc, we suggest that sFLC levels are associated with the extent of fibrosis in SSc. Previous studies have shown that plasma IgG levels were associated with the presence of ILD and its severity in SSc[14, 15]. IgG levels in bronchoalveolar lavage fluid are raised in patients with SSc and active ILD[36]. In addition, plasma cell infiltration into lung is the earliest abnormality in ILD associated with SSc[37].

sFLC levels have been shown to be correlated with disease activity in several autoimmune diseases, such as lupus, Sjögren syndrome and rheumatoid arthritis[27-29]. In SSc patients, we have also demonstrated that sFLC levels are positively correlated with disease activity as measured by the EScSG-AI. Interestingly other B-cell activation markers were also associated with the EScSG-AI: IgA, BAFF and beta2-microglobulin but not IgG or total gammaglobulins. In multivariate analysis, only FVC and beta2-microglobulin, but not sFLC levels, were associated with SSc activity. The fact that sFLC levels were correlated with the EScSG-AI whereas IgG and total gammaglobulins were not remains unexplained. One explanation could be the difference in half-lives (2–4 h for sFLC and 20–25 days for IgG). As a result of a faster

turnover, sFLC might have a better sensitivity than IgG for measuring SSc activity[24]. The fact that IgA levels but not IgG or IgM levels were associated with SSc activity needs to be discussed, but here too we have no definite explanations. IgA has a shorter half-life of 6 days and could be more sensitive to disease activity. ATA is mainly of IgG isotype but can also be of IgA isotype especially when there are high titres of antibodies[38]. A few studies have suggested a link between ATA level and the severity and activity of SSc[39]. Therefore, a high IgA level could reflect higher ATA titres and greater severity; however, we did not directly measure ATA isotypes in our study. Interestingly, IgA rheumatoid factor has also been associated with more severe rheumatoid arthritis[40].

Beta2-microglobulin has also been shown to correlate with the activity of some autoimmune diseases, for example in Sjögren syndrome[29]. The fact that beta2-microglobulin is an independent marker of EScSG-AI has not to our knowledge been previously reported in SSc. Beta2-microglobulin is the light chain of class I HLA and is therefore a marker of B-cell activation and to some extent of T-cell activation.

Regarding disease severity as measured by the Medsger's total severity score, we also found that sFLC levels were correlated in univariate but not multivariate analysis. In multivariate analysis, diffuse SSc and elevated BAFF were independently associated with SSc severity. Several studies have shown that BAFF levels were elevated in SSc and correlated with skin extension[41]. BAFF is known for its role in the survival and maturation of B cells. BAFF might accelerate B-cell activation and this could explain the correlation between sFLC and disease severity. Interestingly, we found a significant and independent correlation between BAFF and sFLC in SSc patients.

Altogether, our results show that B-cell activation markers—beta2-microglobulin, BAFF or sFLC—are independently associated with SSc activity and severity as well as with the severity of lung involvement. Moreover, sFLC are independently correlated with other markers, such as BAFF and beta2-microglobulin. As previously hypothesised, sFLC is a marker of B-cell polyclonal activation. However, we also suggest that sFLC could have a direct pathogenic role. Interestingly, studies have shown that sFLC could bind antigens or activate some cells, such as mast cells, which could have a role in the pathophysiology of SSc[42, 43], especially in the case of lung complications[44].

Our study has several limitations. sFLC was measured using a new assay that assesses kappa and lambda sFLC levels simultaneously. This precludes determining whether there is an imbalance between kappa and lambda in SSc. However, in polyclonal secretion of sFLC, the kappa/lambda ratio is usually normal[25, 45]. Moreover, the values of sFLC levels in polyclonal secretion are much lower than in monoclonal disorders. In lower ranges of sFLC levels, combined measurement seems to be more sensitive than the measurement (and summing) of kappa and lambda sFLC separately[46]. Of note, there was no significant correlation between sFLC levels and GFR, age, sex and weight. Finally, we did not follow sFLC levels longitudinally. Further studies will be needed to determine whether variations in sFLC titres are associated with different outcomes in SSc.

In conclusion, sFLC levels are elevated in SSc and are associated with a more severe and active disease, especially concerning lung complications. Beta2-microglobulin and BAFF, which are other B-cell activation biomarkers, are also correlated with SSc severity and activity, further supporting the role of B cells activation in the

pathophysiology of SSc. Whether sFLC levels could help to predict and follow response to B-cell-targeted therapies merits further study.

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**COMPETING INTERESTS**

David Launay has served as a consultant for Actelion, GSK and Pfizer and as a speaker for Actelion. The other authors have no conflicts to declare.

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**Table 1.** Baseline characteristics of the 134 SSc patients

| Parameter | Value |
|---|---|
| *Demographics* | |
| Gender (F/M) | 109 (81%) / 25 (19%) |
| Age, years | 57 ± 14 |
| Weight, Kg | 68 ± 15 |
| *Disease characteristics* | |
| Limited/diffuse cutaneous SSc, n | 107 (80%) / 27 (20%) |
| Disease duration from RP, years | 16 ± 14 |
| Disease duration from non-RP, years | 9 ± 7 |
| Disease duration from SSc diagnosis, years | 8 ± 7 |
| ACA positive, n (%) | 66 (49%) |
| ATA positive, n (%) | 26 (19%) |
| mRSS | 6.5 ± 6.5 |
| RP, n (%) | 131 (98%) |
| Digital ulcers (history), n (%) | 56 (42%) |
| Digital ulcers (presence), n (%) | 7 (5%) |
| GERD, n (%) | 107 (80%) |
| NYHA functional class I/II/III/IV, n | 62/45/19/8 |
| 6MWD, m | 431 ± 117 |
| PAH, n (%) | 13 (10%) |
| Systolic PAP, mmHg | 32 ± 14 |
| LVEF, % | 63 ± 6 |
| ILD, n (%) | 54 (40%) |
| DLCO, % predicted | 66 ± 20 |
| FVC, % predicted | 100 ± 23 |
| Renal crisis (history), n (%) | 3 (2%) |
| Joint involvement, n (%) | 36 (27%) |
| Muscle involvement, n (%) | 12 (9%) |
| Sjögren syndrome, n (%) | 23 (17%) |
| Thyroiditis, n (%) | 24 (18%) |
| Lupus, n (%) | 3 (2%) |
| Rheumatoid arthritis, n (%) | 5 (4%) |
| *Disease activity* | |
| EScSG-AI | 1.5 ± 1.5 |
| EScSG-AI≥3, n (%) | 23 (17%) |
| Medsger total score | 5.4 ± 2.9 |
| Medsger score-general | 0.4 ± 0.8 |
| Medsger score-vascular | 1.0 ± 0.7 |
| Medsger score-skin | 1.4 ± 1.0 |
| Medsger score-articular | 0.1 ± 0.4 |
| Medsger score-muscle | 0.2 ± 0.5 |

| | |
|---|---|
| Medsger score-digestive | 0.6 ± 0.8 |
| Medsger score-lung | 1.5 ± 1.1 |
| Medsger score-heart | 0.3 ± 0.8 |
| Medsger score-kidney | 0 |
| PGA, mm | 34 ± 31 |
| HAQ-DI | 0.4 ± 0.5 |
| *Disease treatment* | |
| Corticosteroids≥10 mg/d of prednisone equivalent, n (%) | 28 (21%) |
| Immunosuppressive drugs, n (%) | 32 (24%) |

Data are expressed mean ± standard deviation or n (%). SSc : systemic sclerosis; RP : Raynaud's phenomenon; ACA: anti-centromere antibody; ATA: anti-topoisomerase I antibody; mRSS : modified Rodnan skin score; GERD : gastro-esophageal reflux disease; 6MWD: 6-minute walking distance; PAH : pulmonary arterial hypertension; PAP : pulmonary arterial pressure; LVEF : left ventricular ejection fraction; ILD : interstitial lung disease; FVC : forced vital capacity; EScSG-AI : European Sclerodema Study Group activity index; PGA : physician global assessment; HAQ-DI : health assessment questionnaire-disability index.

**Table 2.** Correlation between sFLC, clinical and biological characteristics of SSc patients

| Parameter | sFLC mg/L, mean±SD | P value Univariate (vs the rest of the cohort) | Correlation coefficient | P value univariate | P value multivariate |
|---|---|---|---|---|---|
| **Demographics** | | | | | |
| Female | 23.5±13.1 vs | 0.42 | | | - |
| Age, years | 26.5±16.4 | | 0.14 | 0.10 | NS |
| Weight, Kg | | | -0.13 | 0.15 | NI |
| **Disease characteristics** | | | | | |
| Diffuse cutaneous SSc | 28.7±17.5 vs | 0.11 | | | NS |
| Disease duration from RP, years | 22.8±12.4 | | 0.11 | 0.20 | NI |
| Disease duration from non-RP, years | | | 0.14 | 0.11 | NS |
| Disease duration from SSc diagnosis | | | 0.14 | 0.10 | NS |
| ACA positive | | 0.62 | | | - |
| ATA positive | 23.3±10.9 vs | 0.93 | | | - |
| mRSS | 24.7±16.1 | | 0.22 | 0.01 | NS |

| | | | | | |
|---|---|---|---|---|---|
| ***Digital ulcers (history)*** | 22.6±12.4 vs | 0.04 | | | 0.03 |
| Digital ulcers (presence) | 24.3±14.1 | 0.25 | | | - |
| GERD | | 0.73 | | | - |
| NYHA III/IV | 26.3±15.3 vs | 0.64 | | | - |
| 6MWD, m | 22.4±12.4 | | -0.15 | 0.10 | NS |
| PAH | 26.3±11.0 vs 23.9±13.9 | 0.18 | | | NS |
| Systolic PAP, mmHg | 24.1±14.1 vs | | 0.18 | 0.04 | NS |
| ***ILD*** | 23.9±12.5 | 0.11 | | | 0.009 |
| DLCO, % predicted | 29.0±20.9 vs 22.8±11.0 | | -0.23 | 0.006 | NS |
| FVC, % predicted | | | -0.03 | 0.68 | - |
| Joint involvement | 24.5±6.9 vs 24.0±4.3 | 0.58 | | | - |
| Muscle involvement | | 0.53 | | | - |
| Sjögren syndrome | 26.5±16.1 vs 22.3±11.8 | 0.24 | | | - |
| Thyroiditis | | 0.62 | | | - |
| | 24.2±15.3 vs | | | | |
| | 24.0±13.2 | | | | |
| | 29.7±21.0 vs | | | | |
| | 23.4±12.8 | | | | |
| | 26.9±14.8 vs | | | | |
| | 23.4±13.5 | | | | |

| | | | | | |
|---|---|---|---|---|---|
| | 21.8±9.3 vs 24.5±14.5 | | | | |
| **Disease treatment** | | | | | |
| Corticosteroids ≥10 mg/d of prednisone equivalent | 21.6±14.1 vs 24.7±13.7 | 0.14 | | | NS |
| Immunosuppressive drugs | 18.9±10.9 vs 25.6±14.2 | 0.002 | | | 0.01 |
| **Biological parameters** | | | | | |
| Gammaglobulin levels, g/L | | | 0.59 | <0.001 | NS |
| IgG, g/L | | | 0.57 | <0.001 | 0.0003 |
| IgA, g/L | | | 0.58 | <0.001 | 0.0001 |
| IgM, g/L | | | 0.20 | 0.02 | NS |
| Beta2-microglobulin, mg/L | | | 0.54 | <0.0001 | <0.0001 |
| BAFF, pg/mL | | | 0.20 | <0.0001 | 0.01 |
| Rheumatoid factor, UI/L | | | 0.18 | 0.03 | 0.03 |
| CRP, mg/L | | | 0.32 | 0.0002 | NS |
| ESR, mm | | | 0.35 | <0.001 | NS |

Data are expressed mean ± standard deviation. SSc : systemic sclerosis; RP : Raynaud's phenomenon; ACA: anti-centromere antibody; ATA: anti-topoisomerase I antibody; mRSS : modified Rodnan skin score; GERD : gastro-esophageal reflux disease; 6MWD: 6-minute walking distance; PAH : pulmonary arterial hypertension; PAP : pulmonary arterial pressure; ILD : interstitial lung disease; FVC : forced vital capacity. NI : not included in the multivariate analysis

**FIGURES**

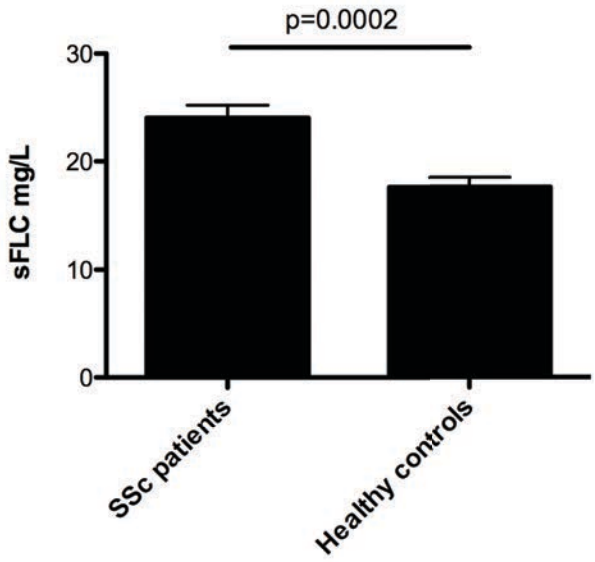


**Figure 1.** Comparison of sFLC levels between systemic sclerosis (SSc) patients and healthy controls.

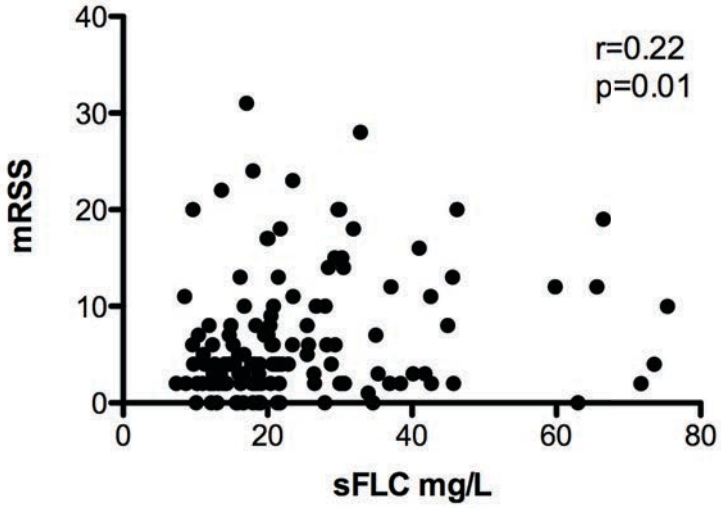


**Figure 2.** Correlation between sFLC levels and the modified Rodnan skin score.

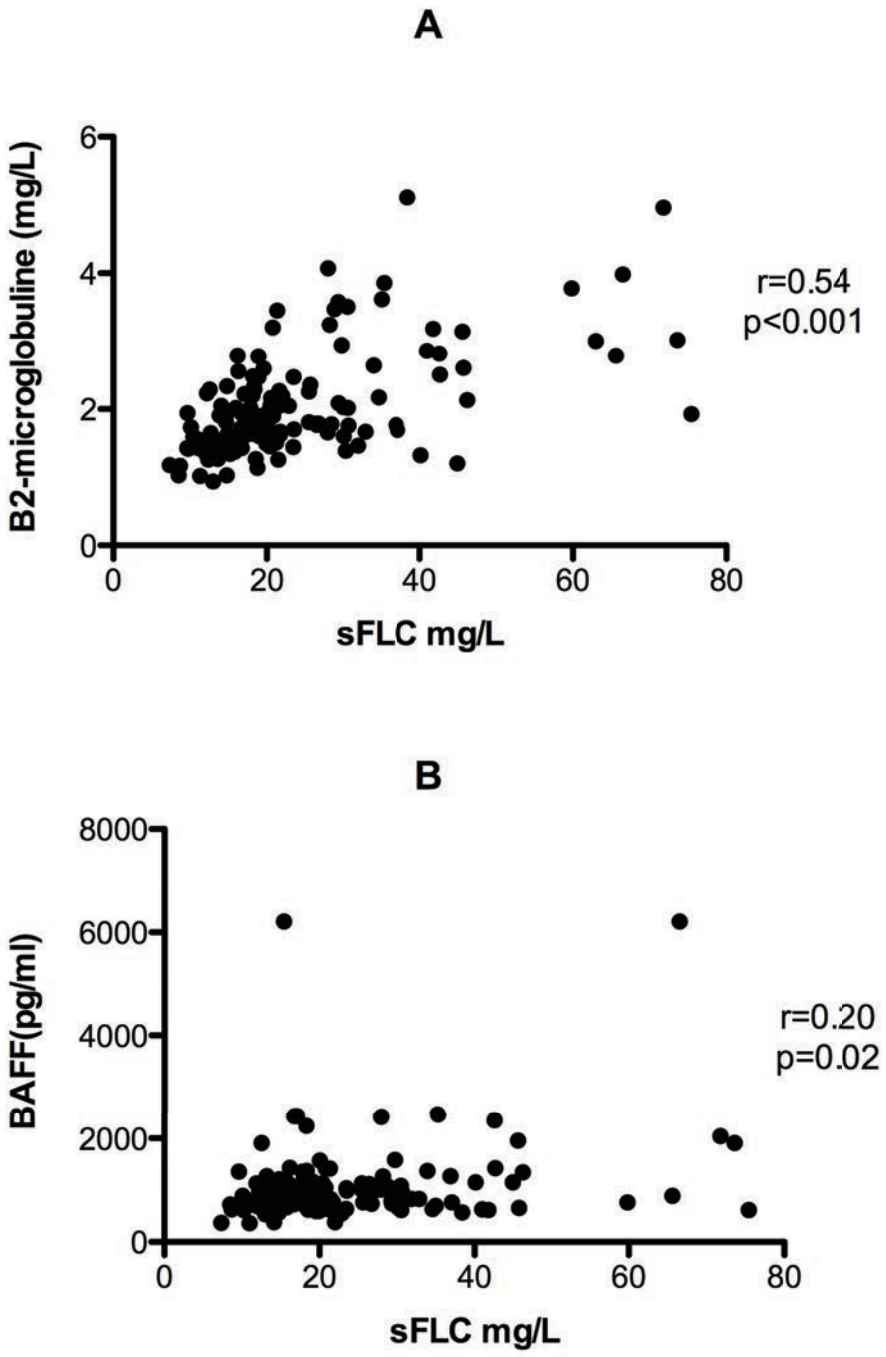


**Figure 3.** (A) Correlation between sFLC and beta2-microglobulin. (B) Correlation between sFLC and BAFF levels.

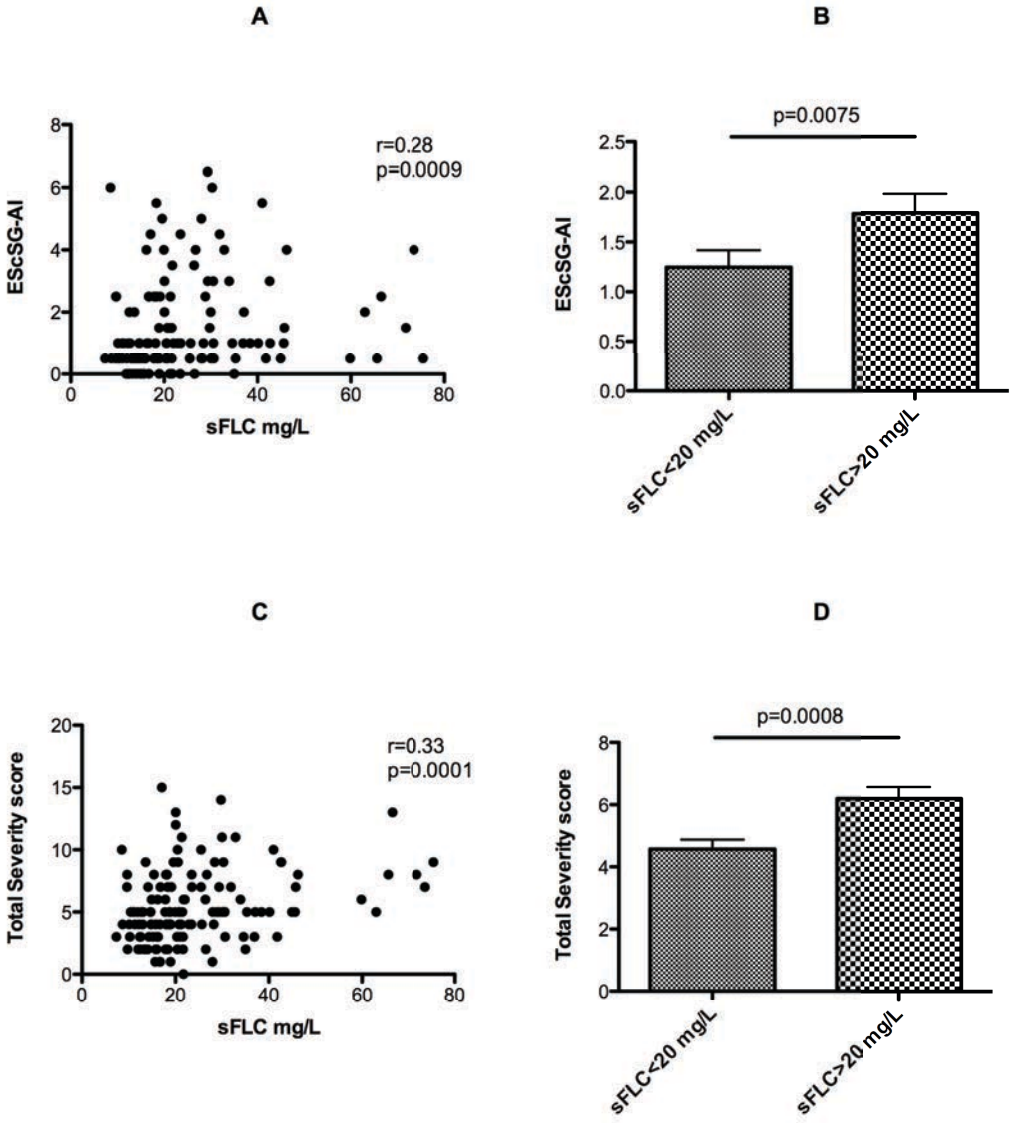


**Figure 4.** (A) Correlation between sFLC levels and systemic sclerosis activity as measured by the EScSG-AI (European Scleroderma Study Group activity index). (B) Comparison of EScSG-AI between patients with sFLC<median value (20 mg/L) and patients with sFLC>median value. (C) Correlation between sFLC and systemic sclerosis severity as measured by the total severity score. (D) Comparison of the total severity score between patients with sFLC<median value (20 mg/L) and patients with sFLC>median value.

**Supplemental Table 1.** Correlation between sFLC and other parameters with SSc activity measured by EScSG-AI

| Parameter | EScSG-AI score | P value univariate (vs the rest of the cohort) | Correlation coefficient | P value univariate | P value multivariate |
|---|---|---|---|---|---|
| **Demographics** | | | | | |
| Female gender | 1.5±1.5 vs | 0.91 | | | - |
| Age, years | 1.5±1.6 | | 0.09 | 0.29 | - |
| Weight, Kg | | | -0.15 | 0.09 | NS |
| **Disease characteristics** | | | | | |
| Diffuse cutaneous SSc | 2.2±1.7 vs | 0.01 | | | NS |
| Disease duration from RP, years | 1.3±1.4 | | -0.06 | 0.48 | - |
| Disease duration from non-RP, years | | | 0.05 | 0.52 | - |
| Disease duration from SSc diagnosis | | | 0.02 | 0.76 | - |
| ACA positive | | 0.34 | | | - |

| | | | | | |
|---|---|---|---|---|---|
| ATA positive | 1.3±1.3 vs | 0.04 | | | NS |
| GERD | 1.7±1.7 | 0.12 | | | NI |
| NYHA III/IV | 2.0±1.8 vs | 0.005 | | | NS |
| 6MWD | 1.4±1.4 | | -0.24 | 0.008 | NS |
| PAH | 1.4±1.5 vs | 0.04 | | | NS |
| Systolic PAP, mmHg | 1.9±1.6 | | 0.22 | 0.01 | NS |
| ILD | 2.2±1.5vs 1.3±1.4 | 0.06 | | | NS |
| ***FVC, % predicted*** | | | -0.30 | 0.0004 | 0.0006 |
| Thyroiditis | 2.0±1.3 vs | 0.04 | | | NS |
| HAQ-DI | 1.5±1.5 | | 0.23 | 0.007 | NS |
| PGA | | | 0.46 | <0.0001 | NI |
| | 1.8±1.8 vs | | | | |
| | 1.3±1.3 | | | | |
| | 1.1±1.3 vs | | | | |
| | 1.6±1.6 | | | | |
| **Disease treatment** | | | | | |
| Corticosteroids≥10 mg/d | 1.7±1.6 vs | 0.45 | | | - |
| of prednisone equivalent | 1.5±1.5 | | | | |

| | | | | | |
|---|---|---|---|---|---|
| Immunosuppressive drugs | 1.7±1.8 vs 1.4±1.4 | 0.55 | | | - |
| **Biological parameters** | | | | | |
| Gammaglobulin levels, | | | 0.13 | 0.12 | NI |
| g/L | | | 0.12 | 0.17 | NS |
| IgG, g/L | | | 0.25 | 0.003 | NS |
| IgA, g/L | | | 0.06 | 0.49 | - |
| IgM, g/L | | | 0.28 | 0.0009 | NS |
| sFLC, mg/L | | | 0.35 | <0.0001 | 0.001 |
| Beta2-microglobulin, | | | 0.22 | 0.009 | NS |
| mg/L | | | 0.08 | 0.32 | - |
| BAFF, pg/mL | | | 0.29 | 0.0007 | NS |
| Rheumatoid factor, UI/L | | | | | |
| CRP, mg/L | | | | | |

Data are expressed mean ± standard deviation. SSc : systemic sclerosis; RP : Raynaud's phenomenon; ACA: anti-centromere antibody; ATA: anti-topoisomerase I antibody; mRSS : modified Rodnan skin score; GERD : gastro-esophageal reflux disease; 6MWD: 6-minute walking distance; PAH : pulmonary arterial hypertension; PAP : pulmonary arterial pressure; ILD : interstitial lung disease; FVC : forced vital capacity. NI : not included in the multivariate analysis

**Supplemental Table 2.** Correlation between sFLC and other parameters with SSc severity measured by the Medsger's total severity score

| Parameter | Medsger's total severity score | P value univariate (vs the rest of the cohort) | Correlation coefficient | P value univariate | P value multivariate |
|---|---|---|---|---|---|
| **Demographics** | | | | | |
| Female gender | 5.2±2.8 vs 6.5±3.3 | 0.05 | | | NS |
| Age, years | | | 0.06 | 0.49 | - |
| **Disease characteristics** | | | | | |
| Diffuse cutaneous SSc | 7.8±3.3 vs 4.8±2.5 | <0.0001 | | | |
| Disease duration from RP, years | -0.09 | 0.28 | | | <0.0001 - |
| Disease duration from non-RP, years | 0.11 | 0.19 | | | NS |
| Disease duration from SSc diagnosis | 0.07 | 0.40 | | | - |
| ACA positive | 4.5±2.3 vs 6.2±3.2 | 0.002 | | | NI |
| ATA positive | 7.2±3.5 vs 4.9±2.6 | 0.002 | | | NS |
| 6MWD, m | | | -0.19 | 0.03 | NS |

| | | | | | |
|---|---|---|---|---|---|
| Thyroiditis | 4.5±3.4 vs 5.6±2.8 | 0.02 | | | NS |
| HAQ-DI | | | 0.42 | <0.0001 | 0.002 |
| PGA | | | 0.67 | <0.0001 | NI |
| Disease treatment | | | | | |
| corticosteroids≥10 mg/d of prednisone equivalent | 7.0±3.3 vs 5.0±2.7 | 0.004 | | | 0.02 |
| Immunosuppressive drugs | 6.5±3.4 vs 5.0±2.7 | 0.03 | | | NS |
| **Biological parameters** | | | | | |
| Gammaglobulin levels, g/L | | | 0.06 | 0.44 | - |
| IgG, g/L | | | 0.01 | 0.90 | - |
| IgA, g/L | | | 0.28 | 0.001 | NS |
| IgM, g/L | | | 0.03 | 0.68 | - |
| sFLC, mg/L | | | 0.32 | 0.0001 | NS |
| Beta2-microglobulin, mg/L | | | 0.28 | 0.0009 | NS |
| BAFF, pg/mL | | | 0.23 | 0.005 | 0.03 |
| Rheumatoid factor, UI/L | | | 0.01 | 0.90 | - |
| CRP, mg/L | | | 0.19 | 0.02 | NS |
| ESR, mm | | | 0.18 | 0.04 | NS |

Data are expressed mean ± standard deviation. SSc : systemic sclerosis; RP : Raynaud's phenomenon; ACA: anti-centromere antibody; ATA: anti-topoisomerase I antibody; mRSS : modified Rodnan skin score; GERD : gastro-esophageal reflux disease; 6MWD: 6-minute walking distance; PAH : pulmonary arterial hypertension; PAP : pulmonary arterial pressure; ILD : interstitial lung

disease; FVC : forced vital capacity; HAQ-DI : health assessment questionnaire-disability index; PGA : physician global assessment; ESR : erythrocytes sedimentation rate; Health NI : not included in the multivariate analysis

**Supplemental Table 3.** Correlation between sFLC and other parameters with severity of the lung involvement as measured by the Medsger's score

| Parameter | Medsger's lung score | P value univariate (vs the rest of the cohort) | Correlation coefficient | P value univariate | P value multivariate |
|---|---|---|---|---|---|
| **Demographics** | | | | | |
| Female | 1.5±1.1 vs 1.6±1.2 | 0.98 | | | - |
| Age, years | | | 0.09 | 0.29 | - |
| Weight, Kg | | | 0.004 | 0.96 | - |
| **Disease characteristics** | | | | | |
| Diffuse cutaneous SSc | 1.8±1.0 vs 1.5±1.2 | 0.17 | | | NS |
| Disease duration from RP, | | | -0.02 | 0.80 | - |
| years | | | 0.15 | 0.06 | NS |
| Disease duration from non-RP, | | | | | |
| years | | | 0.12 | 0.16 | NS |
| Disease duration from SSc | | | | | |
| diagnosis | 1.2±1.1 vs 1.8±1.1 | 0.002 | | | NI |
| ACA positive | 2.3±1.1 vs 1.4±1.1 | 0.0003 | | | 0.02 |
| ATA positive | | | 0.12 | 0.15 | NS |

| | | | | | |
|---|---|---|---|---|---|
| mRSS | 1.7±1.1 vs 1.4±1.1 | 0.14 | | | NS |
| Digital ulcers (history) | 1.6±1.4 vs 1.5±1.1 | 0.98 | | | - |
| Digital ulcers (presence) | 1.5±1.1 vs 1.6±1.2 | 0.89 | | | - |
| GERD | 2.3±1.3 vs 1.3±1.0 | 0.0003 | | | NS |
| NYHA III/IV | | | -0.16 | 0.09 | 0.02 |
| 6MWD | 1.6±1.1 vs 1.5±1.2 | 0.59 | | | - |
| Joint involvement | 1.7±1.0 vs 1.5±1.2 | 0.58 | | | - |
| Muscle involvement | 1.4±1.2 vs 1.6±1.1 | 0.54 | | | - |
| Thyroiditis | | | 0.29 | 0.0008 | NS |
| HAQ-DI | | | 0.45 | <0.0001 | NI |
| PGA | | | | | |
| **Disease treatment** | | | | | |
| Corticosteroids≥10 mg/d of prednisone equivalent | 2.0±1.2 vs 1.4±1.1 | 0.01 | | | NS |
| Immunosuppressive drugs | 2.2±1.2 vs 1.3±1.0 | 0.0002 | | | 0.0003 |
| **Biological parameters** | | | | | |
| Gammaglobulin levels | | | -0.03 | 0.71 | - |
| IgG | | | -0.03 | 0.69 | - |
| IgA | | | 0.13 | 0.13 | NS |
| IgM | | | -0.07 | 0.42 | - |
| sFLC | | | 0.19 | 0.03 | 0.01 |

| | | | | | |
|---|---|---|---|---|---|
| Beta2-microglobulin | | | 0.20 | 0.01 | NS |
| BAFF | | | 0.14 | 0.10 | NS |
| Rheumatoid factor | | | 0.02 | 0.80 | - |
| CRP | | | 0.15 | 0.08 | NS |
| ESR | | | 0.10 | 0.24 | - |
| GFR | | | 0.05 | 0.59 | - |

Data are expressed mean ± standard deviation. SSc : systemic sclerosis; RP : Raynaud's phenomenon; ACA: anti-centromere antibody; ATA: anti-topoisomerase I antibody; mRSS : modified Rodnan skin score; GERD : gastro-esophageal reflux disease; 6MWD: 6-minute walking distance; PAH : pulmonary arterial hypertension; PAP : pulmonary arterial pressure; ILD : interstitial lung disease; FVC : forced vital capacity; HAQ-DI : health assessment questionnaire-disability index; PGA : physician global assessment; sFLC : serum free light chains of immunoglobulins; ESR : erythrocytes sedimentation rate; GFR : glomerular filtration rate; NI : not included in the multivariate analysis

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Review

# Serum free light chains of immunoglobulins as biomarkers for systemic sclerosis characteristics, activity and severity

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ABSTRACT

Systemic sclerosis (SSc) is a chronic autoimmune connective tissue disease. Humoral immunity and B cells are thought to play an important role in the pathophysiology of the disease. B cells are activated, produce specific autoantibodies and profibrotic cytokines. One way to assess B cell activation is to measure serum free light chains of immunoglobulins (sFLC) levels. We assess here sFLC levels in patients with systemic sclerosis (SSc) and their correlation with the disease characteristics, activity and severity. One hundred and thirty-four SSc patients were prospectively enrolled and compared to 401 age- and sex-matched healthy controls. sFLC levels were measured by a new quantitative immunoassay. sFLC levels were significantly higher in SSc patients than in healthy controls. sFLC levels correlated with modified Rodnan skin score and were independently associated with the presence of interstitial lung disease and its severity. In univariate analysis, sFLC levels correlated with SSc activity, as measured by the European Scleroderma Study Group activity index, and severity, as measured by the Medsger's total severity score. In multivariate analysis, beta2-microglobulin levels correlated with disease activity, BAFF levels with severity and sFLC with neither of these. Other B-cell activation biomarkers (IgG, IgA, beta2-microglobulin and BAFF) were independently correlated with sFLC. sFLC levels are elevated in SSc and are independently associated with lung disease and its severity. B-cell activation biomarkers, including sFLC, beta2-microglobulin and BAFF, correlate with disease severity and activity. These results further support the role of B cell activation in the pathophysiology of SSc.



**Contents**



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# II.3 Prévalence des anticorps anti-ARN polymérase de type III dans la ScS

## II.3.1 Introduction

Les auto-anticorps sont des marqueurs biologiques de la ScS utilisés en pratique quotidienne. Les anticorps anti-ARN polymérase de type III appartiennent aux anticorps les plus souvent retrouvés dans le sérum des patients avec ScS. Dans la littérature, on les retrouve souvent en troisième position par ordre de fréquence, après les anticorps anti-centromère et les anticorps anti-Scl70.

Il existe cependant des différences importantes entre les études. Par exemple, les anticorps anti-ARN polymérase III sont décrits fréquemment dans les pays anglo-saxons (15% au Royaume-Uni, 17% aux Etats-Unis) mais semblent plus rares en France (entre 0 et 9%). A notre connaissance, il n'existait pas d'étude reprenant de façon systématique les prévalences de ces anticorps dans tous les articles de la littérature. Notre objectif a été d'en estimer la prévalence mondiale globale et de comprendre l'hétérogénéité de prévalence entre les centres.

### II.3.2 Résumé

**Introduction.** La prévalence des anticorps anti-ARN polymérase de type III dans la sclérodermie systémique (ScS) demeure débattue en raison d'importantes variations suivant les études. Nous avons estimé la prévalence de ces anticorps dans une nouvelle cohorte de patients sclérodermiques puis réalisé une revue systématique de la littérature avec méta-analyse afin de déterminer la prévalence mondiale globale et les facteurs de variabilités potentiels.

**Méthodes.** Les anticorps anti-ARN polymérase III ont été recherchés par deux kits ELISA commerciaux différents dans notre cohorte de patients avec ScS. Notre revue systématique a inclus tous les articles disponibles dans les bases de données PubMed et EmBase. La méta-analyse a été réalisée avec les données disponibles sur la prévalence, les caractéristiques cliniques des patients et les méthodes utilisées par les auteurs pour tester la présence d'anticorps anti-ARN polymérase III. La qualité méthodologique (risque de biais) a été évaluée par l'outil QUADAS-2 modifié.

**Résultats.** Cent trente-trois patients avec ScS (84% de femmes, 27% forme cutanée diffuse) ont été inclus. Huit (6%) et 12 (9%) patients possédaient des anticorps anti-ARN polymérase III, selon les tests utilisés. Il existait une bonne concordance entre les deux kits utilisés (kappa de Cohen $\kappa = 0.68$ ; coefficient de Spearman $R=0.729$ ; $p<0.0001$). La revue systématique de la littérature retrouvait 1961 références. Quatre-vingt dix-neuf articles ont été lus en entier, et 69 articles ont été déclarés éligibles. Trente études ont été finalement inclues dans la méta-analyse, représentant une population totale de 8437 patients. La prévalence des anticorps anti-ARN polymérase III variait entre 0 et 41%. La prévalence globale était 11% (intervalle de confiance à 95% (IC95%) : 8-14), avec cependant une hétérogénéité importante entre les études ($I^2$ 93%, $p<0.0001$). Les facteurs géographiques tels que le continent et le pays expliquaient partiellement l'hétérogénéité retrouvée. Ces facteurs étaient significativement

associés avec la prévalence (P modéré = 0.0077 pour le continent ; < 0.0001 pour le pays). Après inclusion du continent ou du pays dans notre modèle statistique, l'hétérogénéité demeurait fortement significative (p<0.0001). Il n'a pas été retrouvé d'association entre les caractéristiques cliniques des patients inclus dans les différentes cohortes et la prévalence. Lorsque seules les études avec le score méthodologique QUADAS le plus élevé étaient inclues dans l'analyse, la prévalence globale était 9% (IC95% : 7-12) avec une hétérogénéité plus faible (I2 73%). Le continent demeurait associé avec la prévalence (p=0.0013). L'hétérogénéité devenait non significative (p=0.071) après inclusion du continent dans le modèle statistique.

**Discussion.** La qualité méthodologique des études inclues est variable, mais n'explique pas l'importance de l'hétérogénéité observée. L'obtention d'une hétérogénéité plus faible lors de l'inclusion des études de forte qualité QUADAS peut être expliquée par une perte de puissance statistique (faible nombre d'études).

**Conclusion.** Cette méta-analyse, incluant la description de notre nouvelle cohorte, confirme l'importante variation inter-centres de la prévalence des anticorps anti-ARN polymérase III dans la ScS. La prévalence mondiale globale est de 11% (IC95% : 8-14). Les facteurs géographiques sont significativement associés avec la prévalence, soulignant l'implication probable du terrain génétique et des facteurs environnementaux. L'hétérogénéité observée entre les études demeure néanmoins en grande partie inexpliquée.

**II.3.3 Article** *(Arthritis Rheumatol 2014;66(2):407-417)*

**Prevalence of anti-RNA polymerase III antibodies in systemic sclerosis: new data from a French cohort, systematic review and meta-analysis**

**Running head:** Prevalence of ARA in systemic sclerosis

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**Key words:** systemic sclerosis – anti-RNA polymerase III antibodies – meta-analysis – systematic review – prevalence

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**ABSTRACT**

**Objectives.** Studies assessing the prevalence of anti-RNA polymerase III antibodies (ARA) in systemic sclerosis (SSc) have yielded a wide range of results. We described a new SSc cohort tested for ARA and performed a systematic review and meta-analysis to assess the worldwide prevalence of ARA and potential factors of variability.

**Methods.** Seropositivity for ARA was evaluated in a French cohort of SSc patients. A systematic review of the literature was carried out in PubMed and EmBase. Meta-analysis was performed using available data on prevalence, clinical characteristics of SSc patients and assays used for ARA testing.

**Results.** One hundred and thirty-three French SSc patients were tested for ARA, leading to a local prevalence of 6-9%. Thirty studies representing a total population of 8437 SSc patients were included in the meta-analysis. The prevalence of ARA was highly variable ranging from 0 to 41%. The overall pooled prevalence of ARA was 11% (95% confidence interval [95% CI]: 8-14) but heterogeneity was high among studies ($I^2$ 93%, p<0.0001). Geographical factors such as continents and countries partially explained this heterogeneity and correlated with the prevalence. No other baseline SSc characteristics significantly correlated with the prevalence.

**Conclusion.** Our new cohort and meta-analysis of the literature confirmed that ARA prevalence in SSc is variable between centers with a pooled prevalence of 11% (95% CI: 8-14). Geographical factors were significantly associated with prevalence, underlying the probable implication of genetic background and environmental factors. The heterogeneity among studies remained largely unexplained.

Systemic sclerosis (SSc) is a chronic connective tissue disorder characterized by vascular involvement, fibrosis and auto-immunity. Autoantibodies (aAbs) against multiple cellular components are used in daily practice as disease biomarkers for both positive diagnosis and prognosis. The most common antinuclear aAbs found in SSc are anti-centromeres (ACA), anti-topoisomerase I (ATA) and anti-RNA polymerase III antibodies (ARA) (1). Although ARA were identified in 1993 (2-3), data about their clinical impact were limited until the availability of ELISA test kits (4,5). Since then, many studies (2-4,6-17) assessing the prevalence and clinical association of ARA in SSc have been published. Although there is global consensus on the clinical association between ARA, diffuse cutaneous form and renal crisis (1,18), important and unexplained discrepancies in the prevalence of ARA are still present. For example, ARA have been found in 8% in a USA cohort and 16% in Canadian patients (19,20). In Japan, prevalence was estimated at 5% in the first description, and 11% in 2009 (2,15). In Europe, important variations exist between countries: 3-9% in France or Italy, and 20-22% in both Denmark and the United Kingdom (12,14,16,21,35). The aims of our study were: (i) to assess in a new French cohort the prevalence of ARA and (ii) to perform a systematic review and meta-analysis of published cohorts in order to assess the worldwide prevalence of ARA and potential factors explaining the observed heterogeneity.

## PATIENTS AND METHODS

### The new French cohort of SSc patients

Patients and clinical features

One hundred and thirty-three consecutive and unselected patients with SSc, evaluated between May and November 2009 in our Department were retrospectively studied. Patients had to fulfill the following inclusion criteria: age > 18 years; SSc according to the American College of Rheumatology criteria and/or LeRoy and Medsger criteria (22,23); and were classified as having diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc) or limited SSc (lSSc) according to LeRoy and Medsger (23). Age of disease onset was defined as age at the first non-Raynaud's symptom. Interstitial lung disease (ILD) was defined by the presence on HRCT of at least one usual sign of SSc-associated ILD, i.e. sub pleural pure ground glass opacities and/or interstitial reticular pattern with or without traction bronchiectasis and/or honeycomb cysts. Pulmonary hypertension (PHT) was diagnosed on right heart catheterization (mean pulmonary arterial pressure higher than 25 mmHg) and considered as SSc-associated precapillary pulmonary arterial hypertension if the pulmonary capillary wedge pressure was lower than 15 mmHg. Heart involvement was considered in case of ECG, echocardiography (systolic or diastolic dysfunction) or cardiac Magnetic Resonance Imaging abnormalities associated with symptoms (malaise or syncope, dyspnea, chest pain, palpitations). Renal crisis was defined by acute renal insufficiency or accelerated severe systemic hypertension with proteinuria (24). Myositis was diagnosed if there was muscular weakness associated with raised CPK level, electromyography, muscle MRI or muscular biopsy abnormalities.

Laboratory methods

Identification of ANA specificities was performed as part of routine clinical care using

both specific immunofluorescence patterns on HEp-2 cells and luminex approach (Bio-Plex 2200, Bio-Rad® Laboratories Ltd, CA) for anti-topoisomerase I antibodies (ATA), anti-centromere antibodies (ACA), anti-U1-RNP, anti-SSA/Ro and anti-SSB/La antibodies. Anti-PM/Scl antibodies were identified by immunodot (EUROLINE Systemic Sclerosis (Nucleoli) Profile (IgG), Euroimmun AG, Luebeck, Germany). Anti-mitochondrial antibodies (AMA) were identified by usual immunofluorescence patterns on rat liver kidney and stomach tissues (Bio-Rad®) and immunodot method (Euroimmun AG). Each serum was tested for ARA by two different ELISA assays, according to the manufacturers instructions: QUANTA Lite™ RNA Pol III (INOVA Diagnostics, San Diego, CA, USA, cut-off 20 UI/mL) and EliA™ RNA Pol III Well (ThermoFisher, Phadia® Laboratory Systems, cut-off 10 UI/mL). Calibration and controls samples were performed in duplicate. A positive result was confirmed in each case by a new assay.

In accordance with French legislation, written information was provided and consent was obtained for each patient. The study was conducted in accordance with the recommendations of the Helsinki Declaration and complied with the requirements of the French 'Commission Nationale Informatique et Libertés' (n° DC-2008-642).

**Systematic review and meta-analysis**

The PRISMA statement (Preferred Reporting Items for Systematic reviews and Meta-Analyses) was used as a guide to conducting the review and analysis (25).

Data sources and searches

Two reviewers (VS and DL) independently performed electronic searches on PubMed and EmBase, including all records (articles, conference abstracts) published until April 2012 (**Figure 1A**). We used combinations of the terms "systemic sclerosis",

"scleroderma", "anti-rna polymerase iii", "dna-directed rna polymerases" and "autoantibodies". We adapted the search strategy to the specificities of each database. The reference lists of the retrieved papers were searched to identify additional relevant publications.

Study selection

Studies that met the following criteria were included: 1. Reporting original data, in English or French; 2. Inclusion of patients ≥ 18 years old; 3. Diagnosis of SSc; 4. More than 30 SSc patients tested for ARA. Studies that failed to provide sufficient information for the data analysis were excluded. Two reviewers (VS and DL) independently screened the titles and abstracts of the articles retrieved and applied the selection criteria to identify relevant material to be read in full. The reviewers' selections were compared and, in case of disagreement, decisions were taken by consensus. The reviewers read independently the complete articles and applied the selection criteria to determine whether the studies would be included in the meta-analysis. The selections were again compared and decisions taken by consensus in case of disagreement. As first selection of studies retrieved some overlapping cohorts for a given center assessed during the same period, we chose to include in this case one study per center, which contained the highest number of patients. References that studied two or more cohorts were included if extraction of data for each cohort was feasible. In this case, each cohort was analyzed as an independent cohort. Multicenter studies were excluded if participating centers had published single cohort papers, which were already included. In other words, for each center, only one source of information was analyzed in order to avoid duplicate data.

Assessment of study quality and risk of bias

VS and DL independently assessed the quality of the studies (risk of bias) using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool (26). In keeping with the guidelines provided in the QUADAS-2 user manual (27), items were modified for this study (**supplementary table 1 and Figure 1B**). Item “Was a case-control design avoided?” in the domain 1 “Patient selection” was omitted. In the domain 2 “Index test”, items “Were the index test results interpreted without knowledge of the results of the reference standard?” and “If a threshold was used, was it pre-specified?” were substituted by “Was the method of antibody determination described?” because we included articles regardless of the technique of ARA testing. We omitted the domain 3 “Reference standard” with the items “Is the reference standard likely to correctly classify the target condition?” and “Were the reference standard results interpreted without knowledge of the results of the index test?”. For the domain 4 “Flow and timing”, we omitted item “Was there an appropriate interval between index test and reference standard?”, substituted item “Did all patients receive the same reference standard?” by “Were all patients tested for ARA?”. According to the QUADAS-2 manual, we assessed all articles “high”, “low” or “unclear” for each items (**supplementary table 1 and Figure 1C**).

Data extraction

Relevant data were extracted from the selected studies using a standard form that included information about the following items: continent, country, center, ethnic origin of the patients, study design, recruitment period, mid-cohort year, length of follow-up, disease duration, sex ratio, cutaneous form (percentage of diffuse forms), age of patients, percentage of patients with interstitial lung disease, percentage of patients with scleroderma renal crisis, method of antibody determination, kit used for ELISA, substrate used for IP, number of SSc patients tested for ARA, number of SSc patients

positive for ARA.

**Statistical analyses**

For the new French cohort analysis, results are expressed as the mean ± SD for continuous variables and as numbers with percentages for categorical variables. XLSTAT™ software (Addinsoft®, Paris, France), was used to perform statistical analyses. Comparisons between patients were conducted using the Student's *t* test for continuous variables normally distributed, Mann-Withney *U* test for those non-normally distributed and either the chi-square test or Fisher's exact test for discrete variables. The results were regarded as significant when the P-value was < 0.05. The R coefficient of correlation was estimated by Spearman method.

For the meta-analysis, we calculated weighted–pooled summary estimates of prevalence of ARA. Analyses were performed if at least two studies evaluating the same outcome could be combined. For each meta-analysis, the methods of Der Simonian and Laird were used. Accordingly, studies were considered as a random sample from a population of studies. Heterogeneity was quantified with a $\chi^2$ heterogeneity statistic and by means of $I^2$ for each analysis. A random effect model was used to combine data. The overall effect was estimated by a weighted average of individual effects, with weights inversely proportional to variance in observed effects. Freeman Tukey transformation was used. Meta-regression was performed to assess impact of continent, countries, types of test (ELISA or IP), manufacturer of ELISA, substrate of IP, year of publication, mean age of patients, proportion of male, percentage of Caucasians, percentage of Asians, percentage of Africans, disease subtype (dcSSc versus lcSSc), mean disease duration, proportion of interstitial lung disease, proportion of renal crisis. All analysis was performed using R software and metafor package (28).

## RESULTS

### Data from the cohort study

Clinical and biologic features

Clinical and biological characteristics of the 133 SSc patients are summarized in **Table 1**. Thirty-six patients (27%) had dcSSc and 97 (73%) had lcSSc – of which 3 had limited SSc. ATA were found in 17/36 (47%) dcSSc and in 14/97 (14%) lcSSc patients. ACA were found in 67/97 (69%) lcSSc patients.

ELISA assays for ARA

PHADIA test was positive in 8/133 (6%) SSc patients (4 dcSSc, 4 lcSSc). INOVA test was positive in 12/133 (9%) SSc patients (7 dcSSc, 5 lcSSc). Titers were 20 ± 8 AU/mL vs 45 ± 37 (p=0.143) (PHADIA) and 72 ± 54 vs 56 ± 48 (p=0.876) (INOVA) in patients with lcSSc and dcSSc respectively. PHADIA and INOVA tests were both positive in 7 patients, of whom one was also ATA positive. Tests showed discordance in 6 patients: one patient (lcSSc – ACA positive) was weakly positive with PHADIA only (14.5 AU/mL); five patients (3 dcSSc and 2 lcSSc) were positive with INOVA only (one with anti-U1RNP antibody and 4 with unidentified ANA). Titers were low for 4 out of these 5 patients (ranging between 23 and 34 AU/mL) but one patient (lcSSc) had a high titer of 150 AU/mL. Tests were compared on positivity agreement (Cohen's $\kappa$=0.68) and on titers amplitude ($R$=0.729, $p<0.0001$).

### Systematic review

Database search results

Using defined keywords, 1961 references were retrieved by electronic search on PubMed and EmBase databases. Searching through the references did not return any additional material. After reading the titles and abstracts, 99 articles were selected for

complete reading. 69 articles were assessed for eligibility and 39 articles were then excluded, essentially because of duplicate data (flow chart is presented in **Figure 1A**). Ultimately, 30 studies (5-14,16,17,20,21,29-43), including the present one, representing a total population of 8437 adult patients with SSc were included in the meta-analysis (see **Supplementary table 1** for characteristics and **Figure 1C** for results of QUADAS-2 assessment).

Overall prevalence of ARA and its determinants

The overall pooled prevalence of ARA was 11% (95% confidence interval [95% CI]: 8-14), with a high degree of heterogeneity ($I^2$ 93%, p<0.0001). We assessed whether geographic factors, characteristics of ARA testing methods and clinical characteristics of patients could explain the observed heterogeneity between studies. **Table 2** summarizes the results of all meta-regressions.

*Geographical influence*

Meta-regression revealed a significant association between continent and ARA prevalence (p=0.008, **Table 2**), suggesting that the continent could explain a part of the heterogeneity. When continent was entered as a moderator, residual heterogeneity between studies was still significant (p<0.0001). Meta-analysis stratified by continent showed a prevalence of 7% (95% CI 5-9) for Asia, 9% (95% CI 6-13) for Europe and 14% (95% CI 8-21) for North America. Prevalence for Oceania was 15% (95% CI 12-19) and South America 41% (95% CI 31-52) but both were based on single studies (**Figure 2**). Meta-regression also revealed a significant correlation between countries and prevalence (p<0.0001; **Table 2**). Residual heterogeneity was still highly significant (p<0.0001) after including country as a moderator. Meta-analysis stratified by countries showed that heterogeneity was also still important within some countries like USA and

France while ARA prevalence was more homogeneous in some other countries like Japan or Italy (**Figure 3**).

*Characteristics of ARA testing*

Meta-regression did not reveal a significant association between the types of test (ELISA or IP) and ARA prevalence (p=0.14). For ELISA, meta-analysis showed a prevalence of 13% (95% CI 9-19) but heterogeneity was present. Within studies using ELISA test, meta-regression showed a trend for the correlation between the manufacturer (MBL, INOVA, PHADIA and in-house) and prevalence (p=0.06). However, there was no association between manufacturer and prevalence after inclusion of the geographical variable in the model (p=0.14). For IP, the prevalence was 7% (95% CI 5-9).

*Characteristics of the patients included in studies*

Meta-regressions revealed no other factors significantly associated with prevalence (**Table 2**): year of publication (p=0.77), mid-cohort year (p=0.62), age of patients (p=0.98), gender (p=0.36), ethnicity (percentage of Caucasians: p=0.33, percentage of Asians: p=0.12; percentage of Africans: p=0.22), disease subtype (p=0.83), disease duration (p=0.50), prevalence of interstitial lung disease (p=0.25) or of renal crisis (p=0.80).

*Influence of studies quality on ARA prevalence and its determinants*

As quality bias is an important concern in meta-analyses, we performed again all the previous analyses on the highest quality studies. By selecting the 13 studies (5-7,10,12-14,35,37,41,42 and the present one) rated “yes” for the items 1, 2 and 4 of

QUADAS-2 (see patients and methods), we found an overall pooled prevalence of 9% (95% CI 7-12) with a lower heterogeneity ($I^2$: 73%). Meta-regression still showed that continents were associated with ARA prevalence (p=0.0013). When continents were entered as moderator in the model, residual heterogeneity became non significant (p=0.071).

## DISCUSSION

The main results of our study were as follows: (i) The prevalence of ARA was 6-9% in our new cohort of 133 French patients; (ii) the ARA prevalence in the existing literature including more than 8000 patients from various countries worldwide is highly variable ranging between 0 and 41%; (iii) the overall pooled prevalence of ARA was 11% (95% CI 8-14) but there was a marked heterogeneity between studies only partially explained by the geographical origin and not by the baseline characteristics of patients.

Our study provides data to estimate the frequency of ARA in SSc patients. Estimation of the worldwide prevalence of ARA in SSc is challenging because published studies are of different sample sizes and methodology. These results are represented in **Figure 4**. The estimated pooled prevalence of ARA was 11% (95% CI 8-14%), in keeping with estimation of Koenig *et al.* in 4672 patients from different countries (44). As a comparison, prevalence of ACA was estimated at 26% in the review of Koenig et al. and 33% in the 2003 ACR guidelines for antibodies testing in SSc; prevalence of ATA between 20 and 29% (44,45). Anti-fibrillarine (U3-RNP) antibodies are found in 4-10% of SSc patients, anti-U1RNP antibodies in 6%, anti-PM/Scl in 4-11%, anti-Th/To antibodies in 2-5% (18). ARA are often mutually exclusive of ATA and ACA. ARA are therefore one of the most frequent antinuclear antibodies in SSc after ACA and ATA. Moreover, ARA are known to be associated with some clinical characteristics carrying a bad prognosis in SSc such as diffuse cutaneous form and renal crisis (1,18,19). Altogether, these data and our results on the high prevalence of ARA favor a systematic ARA testing in SSc patients without ATA and ACA.

The considerable variation of prevalence of ARA in the literature was not surprisingly associated with a high heterogeneity between studies included in our meta-analysis. Some hypotheses were worth testing to explain this heterogeneity: geographical origin of patients, type of assays and baseline characteristics of SSc. First, the most obvious

variation of the prevalence of ARA appears to be the geographical origin. Our study demonstrates this suggestion by showing that: 1. Location of the studies partially explained heterogeneity. 2. A significant correlation between continent and ARA prevalence by meta-regression with, for example, a lower prevalence in Europe than in North America (9% vs. 14%). Moreover, within continents, prevalence was also significantly associated with countries. The explanations of these variations between continents and even between countries are not well established. Interestingly, observational studies have suggested that SSc manifestations can be variable between patients from different countries. Analysis of the EUSTAR (EULAR Scleroderma Trials and Research) group database showed a large variability of disease presentation in Europe (46). Theses differences could be ascribed to genetic or environmental factors, which could influence ARA prevalence (47-49). As an example, Liu and co-workers have recently generated a composite interferon-inducible chemokine score that correlated with the interferon gene signature (50). This score correlated with the SSc activity and organ involvement (lung, skin, muscle). Interestingly, it was negatively associated with the presence of ARA, suggesting that antibody patterns could be associated with specific genetic background and different cytokine patterns. Whether other genetic background or cytokine production patterns could explain the variation of ARA prevalence among continents deserve further studies.

We could not explain variability in prevalence of ARA by ethnicity in our whole analysis. However, two recent studies have shown that ARA were less prevalent in patients of African origin than in white patients (51,52). In a sensibility analysis, we reran the model including these 2 studies; heterogeneity between centers still remained highly significant (data not shown). These observations highlight several points. Firstly, definitions of ethnicity can be different between studies and ethnic groups are often

heterogeneous. Secondly, ethnicity data were missing for several studies and have therefore not been extensively included in the meta-regression. Indeed, only five studies included patients of African origin and 3 out of them included less than 10% of such patients.

In studies comparing ELISA and IP for the diagnosis of SSc versus others CTDs, ELISA was reported to be more sensitive and IP to be more specific (4,5,13,14). In our study, ELISA assay led to a higher prevalence of 13% (95% CI 9-19) vs. 7% (95% CI 5-9) for IP in the whole population, but meta-regression did not reveal a significant association between the types of test (ELISA or IP) and prevalence. When the type of assay was included in the model as a moderator, the heterogeneity remains very significant. The impact of the type of assay on heterogeneity between studies is therefore probably negligible

ARA have been associated with diffuse cutaneous form and renal crisis (1,19,44). ILD seems to be less prevalent in this group of patient (19). Therefore one might expect that the variability in ARA prevalence could influence the clinical findings in case series of SSc patients reported from different countries. In our study, we searched whether a different proportion of these clinical manifestations in each study could account for heterogeneity in the reported prevalence rates. Disease subtype, disease duration, ILD or renal crisis frequencies were not found to explain heterogeneity between studies. In other words, the variation of ARA prevalence between studies was not explained by differences in the percentage of patients with diffuse SSc, renal crisis or ILD at baseline in the included studies.

Our meta-analysis has several limitations. Firstly, as a systematic review, we found a broad range of quality in methodological design of studies. We chose to include the highest number of studies providing data from various centers rather than to select only studies with the highest methodological quality. For each center, only one source

of information was then included, in order to avoid duplicate inclusion of patients. As we showed, when selecting the 13 studies with the highest quality, we found similar results with a lower heterogeneity. The fact that residual heterogeneity became not significant after entering continents as moderator in the model should be read cautiously as reducing the number of studies could have reduced the power of the study**.** Secondly, the number of studies and size of samples studied are very different between centers. Our first aim was to estimate the more accurate worldwide prevalence. Then, for the selection of studies, we took into account each center, regardless of the sample size. Weighted-pooled summary estimates allowed us to consider the sample size effect. Nevertheless there are discrepancies for some countries between population size of the country and the sample size. For example, estimation of prevalence in South America is based on one study of 85 patients from Brazil and prevalence from a country like China is estimated on 92 patients. Thirdly, some articles were not designed for the estimation of prevalence of ARA but provided sufficient information to estimate it. In a few studies, the total number of patients tested for ARA (denominator) was uncertain. We have extrapolated this number from the total number of patients analyzed for antinuclear antibodies. Therefore prevalence of ARA may have been underestimated in these studies (29-32, 36, 38, 43). To address this issue, we provided the quality evaluation of the studies in **supplementary table 1**. Moreover, when we ran again the analysis on the most robust studies, we found a close prevalence of 9% (95% CI 7-12). Finally, some studies have used ELISA assays from different manufacturers, or did not provide the cut-off used. We were therefore not able to assess the impact of the cut-off value on prevalence.

In conclusion, this study provided a new estimation of ARA prevalence in a SSc French center. The systematic review and meta-analysis highlighted known discrepancies between centers around the world and provided an overall pooled prevalence of ARA

of 11% (95% CI: 8-14). Geographical factors (continents and countries) partially explained this heterogeneity suggesting the probable implication of genetic background and/or environmental factors. No other baseline characteristic was found to be significantly associated with ARA prevalence.

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## TABLES

### Table 1: Characteristics of the patients included in this study

| | SSc patients (n=133) | | | | |
|---|---|---|---|---|---|
| | **N** | **dcSSc (n=36)** | **N** | **lcSSc (n=97)*** | **p** |
| *Demographic characteristics* | | | | | |
| Sex, *female*† | 36 | 22 (61) | 97 | 90 (93) | **< 0.0001** |
| Age, *years*‡ | 36 | 56.6 (43.4-69.9) | 97 | 64.5 (51.0-78.0) | **0.002** |
| Ethnic origin | 36 | - Caucasian (n=32)<br>- African (n=3)<br>- Asian (n=1) | 97 | - Caucasian (n=92)<br>- African (n=5) | |
| *Clinical characteristics* | | | | | |
| Age of onset disease§, *years*‡ | 35 | 46.4 (32.1-60.7)‡ | 84 | 50.3 (36.3-64.3)‡ | 0.114 |
| Duration of disease§, *years*‡ | 35 | 10.0 (4.9-15.2) | 84 | 13.9 (5.6-22.1) | **0.01** |
| Pulmonary arterial hypertension† | 36 | 4 (11) | 97 | 21 (22) | 0.167 |
| Interstitial lung disease† | 36 | 26 (72) | 97 | 31 (32) | **< 0.0001** |
| Digital ulceration† | 36 | 22 (61) | 97 | 33 (34) | **0.005** |
| Joint involvement† | 36 | 15 (42) | 97 | 29 (30) | 0.2 |
| Myositis† | 36 | 12 (33) | 97 | 6 (6) | **0.0002** |
| Esophageal involvement† | 36 | 30 (83) | 97 | 82 (85) | 0.866 |
| Renal crisis† | 36 | 4 (11) | 97 | 1 (1) | **0.019** |
| Heart involvement† | 36 | 3 (8) | 97 | 6 (6) | 0.703 |
| Overlap syndromes | 36 | Sjögren's syndrome (n=5) | 97 | Sjögren's syndrome (n=23), PBC (n=10), SLE (n=2) | |
| *Immunological characteristics* | | | | | |
| ANA specificities | 36 | ATA (n=17)<br>• Isolated ATA (n=15)<br>• And anti-PM/Scl (n=1)<br>• And anti-Ro/La (n=1)<br>Anti-PM/Scl (n=3)<br>Anti-Ro/La (n=3) | 97 | ACA (n=67)<br>• Isolated ACA (n=59)<br>• And AMA (n=6)<br>• And anti-Ro/La (n=2)<br>ATA (n=14)<br>• Isolated ATA (n=13)<br>• And AMA (n=1)<br>Anti-PM/Scl (n=1)<br>Anti-U1RNP (n=4)<br>AMA (n=1) | |
| - Unidentified | | 12 | | 10 | |
| - Negative | | 1 | | 0 | |

N: number of patients with available data, SSc: systemic sclerosis, dcSSc: diffuse cutaneous SSc, lcSSc: limited cutaneous SSc, SLE: systemic lupus erythematosus, PBC: primary biliary cirrhosis, ATA: anti-topoisomerase-I antibodies, ACA: anti-centromeres antibodies, AMA: anti-mitochondrial antibodies, ARA: anti-RNA polymerase III antibodies

*: 2 of whom with limited SSc, †: number (%), ‡: mean (standard deviation), §: first non-Raynaud's symptom

**Table 2: Results of meta-regression between characteristics of cohorts and ARA prevalence.** The residual heterogeneity after inclusion of each factor in the analysis was significant with P value <0.0001.

| | **Moderated P value** |
|---|---|
| Continents | **0.0077** |
| Countries | **< 0.0001** |
| Types of test (ELISA, IP, not specified) | 0.1438 |
| Manufacturers of ELISA kit | 0.0624 |
| Year of publication | 0.7716 |
| Mid-cohort year | 0.6170 |
| Age of patients | 0.9769 |
| Sex ratio | 0.3641 |
| Ethnicity (Caucasian) | 0.3314 |
| Ethnicity (Asian) | 0.1207 |
| Ethnicity (African) | 0.2246 |
| Disease subtype | 0.8322 |
| Disease duration | 0.4992 |
| Interstitial lung disease | 0.2516 |
| Scleroderma renal crisis | 0.8010 |

## FIGURES

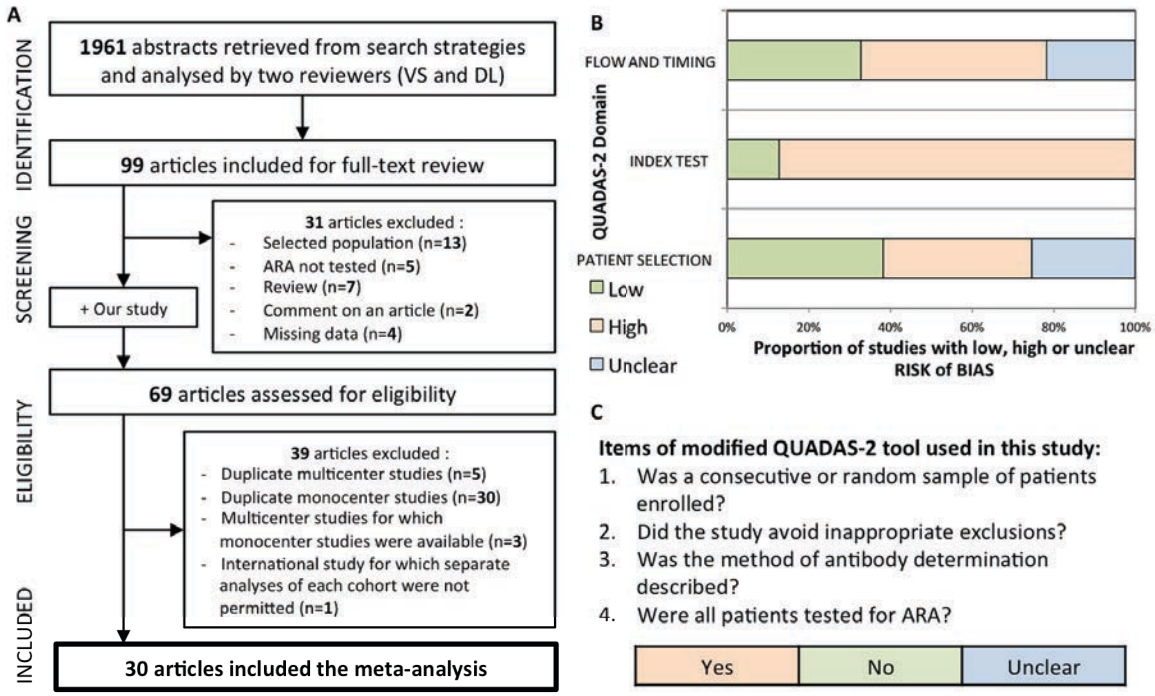


**Figure 1: A:** Flow chart. **B:** Items of modified QUADAS-2 tool used in this study. **C:** Results of QUADAS-2 methodological assessment: proportion of studies with low, high or unclear risk of bias.

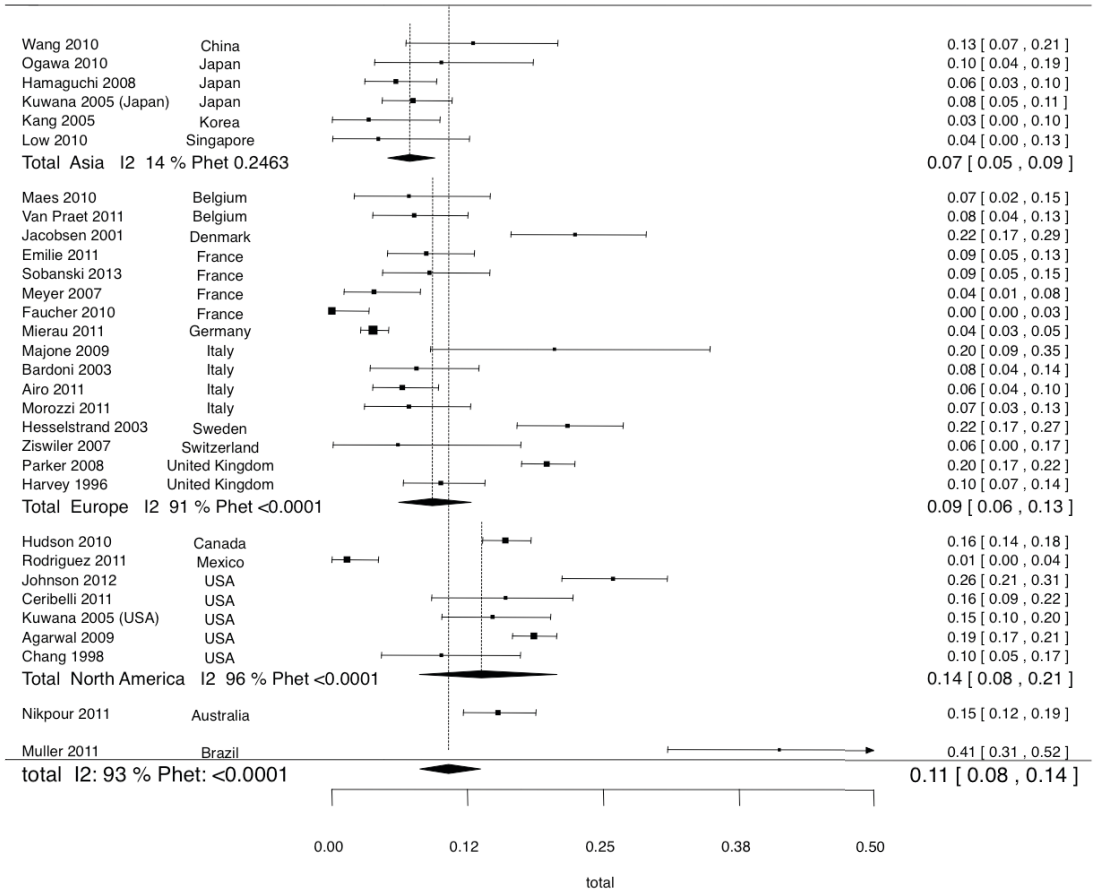


**Figure 2:** Forest plots of the prevalence of ARA in SSc patients represented by the pooled prevalence in the whole population and separated between continents. Each square represents an individual prevalence, the size of the square being proportional to the weight given to the study. The lines represent the 95% CI for the point estimate in each study. The diamonds represent the combined prevalence. Phet = P value for heterogeneity.

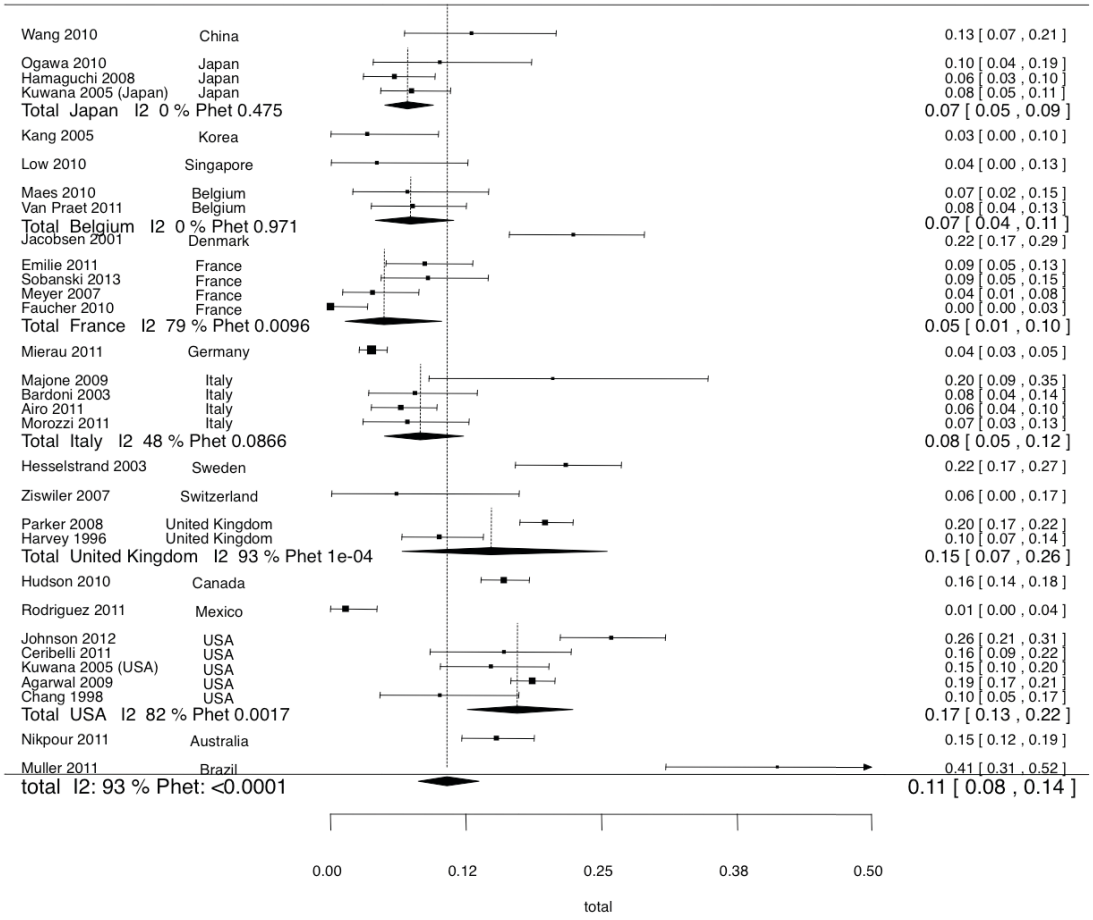


**Figure 3:** Forest plots of the prevalence of ARA in SSc patients represented by the pooled prevalence in the whole population and separated between countries. Each square represents an individual prevalence, the size of the square being proportional to the weight given to the study. The lines represent the 95% CI for the point estimate in each study. The diamonds represent the combined prevalence. Phet = P value for heterogeneity.

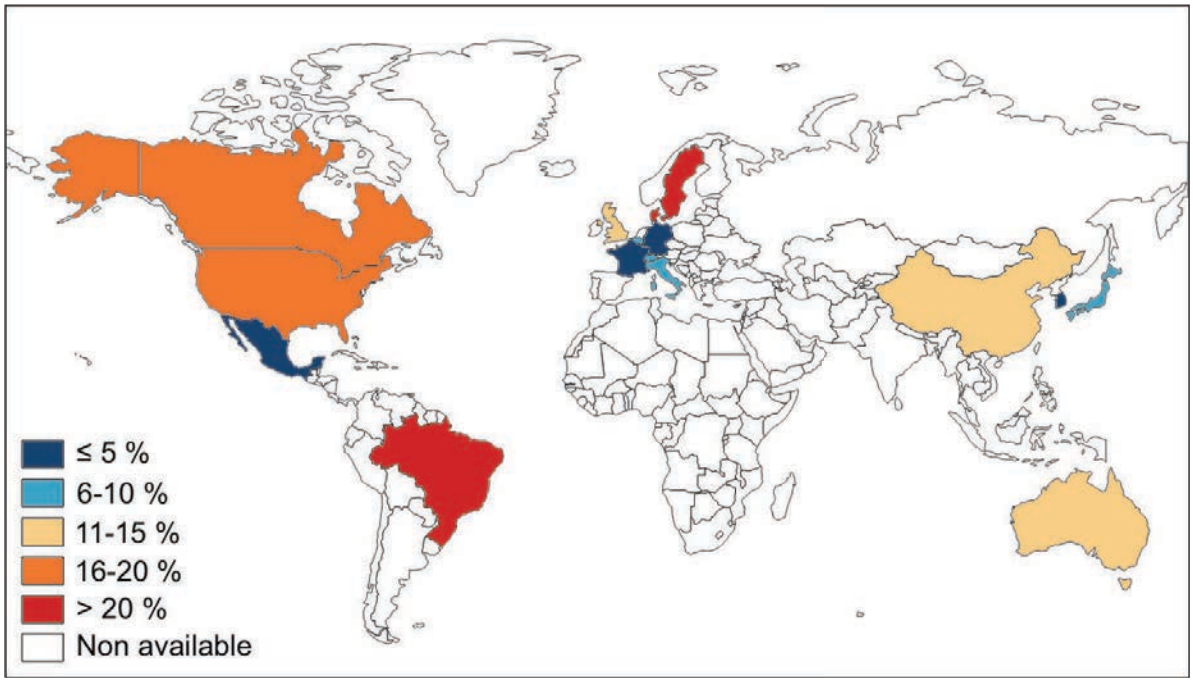


**Figure 4:** Estimation of worldwide prevalence of ARA in SSc patients according to the meta-analysis.

**Supplementary table 1:** Characteristics of the studies included in the meta-analysis and results of methodological quality assessment using the QUADAS-2 tool.

Items of modified QUADAS-2 tool used in this study:

1. Was a consecutive or random sample of patients enrolled?
2. Did the study avoid inappropriate exclusions?
3. Was the method of antibody determination described?
4. Were all patients included in the study tested for ARA?

According to the QUADAS-2 manual, each item was assessed “yes”, “no” or “unclear”:

| | |
|---|---|
| | Yes |
| | No |
| | Unclear |

| **Author, year (ref.)** | **Country *Ethnic origin of the patients*** | **Study design (time interval)** | **Length of follow-up or duration of disease** | **Method of ARA determination – Cut-off for ELISA** | **No. of SSc patients positive/tested for ARA** | **QUADAS** 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|---|---|
| Agarwal *et al*, 2009 (29) | USA *Caucasian 76%, African-American 13%, Hispanic 7%* | Patients selected from a registry (1986-2009) | NS | ELISA (MBL) | 261/1402† | | | | |
| Airo *et al*, 2011 (8) | Italy *Caucasian 100%* | Cohort of consecutive patients with at least 2 visits (1988-2010) | Median = 9.8 years | ELISA (MBL) | 17/262 | | | | |
| Bardoni *et al*, 2003 (6) | Italy *NS* | Cohort of consecutive patients followed-up at least 2 years (1995-1999) | Median = 4.87 years | IP (HeLa) | 9/115 | | | | |
| Ceribelli *et* | USA | All | NS | IP (K562) | 18/119 | | | | |

| | | | | | |
|---|---|---|---|---|---|
| *al*, 2011 (7) | *NS* | patients from a registry | | | |
| Chang *et al*, 1998 (13) | USA *Caucasian 100%* | Cohort of consecutive patients (NS) | lcSSc : 11.7 yr (1-35) dcSSc : 9.5 yr (1-27) | IP (HeLa) | 9/89 |
| Emilie *et al*, 2011 (12) | France *NS* | Cohort of consecutive patients | NS | ELISA (INOVA) | 17/195 |
| Faucher *et al*, 2010 (14) | France *NS* | Cohort of consecutive patients | Mean duration of disease = 13 years | ELISA (PHADIA) – 15 U/mL<br>ELISA (INOVA) – 20 U/mL<br>IP (HeLa - confirmation) | 3/50<br>0/50<br>1/50 |
| Hamaguchi *et al*, 2008 (30) | Japan *Asian 100%* | NS | 7.1 ± 4.3 yr* | IP (K562) | 12/203† |
| Harvey *et al*, 1996 (17) | UK *NS* | NS | NS | IP (K562) | 25/249 |
| Hesselstrand *et al*, 2003 (31) | Sweden *Caucasian (all but one)* | Cohort of consecutive patients (1983-1998) | 7.0 ± 4.5 yr* | ELISA | 60/276† |
| Hudson *et al*, 2010 (20) | Canada *Caucasian 90%* | Patients from a registry (2004-2009) | Disease duration 11.0 ± 9.5 yr* | ELISA (INOVA) | 168/1048 |
| Jacobsen *et al*, 2001 (21) | Denmark *NS* | Patients selected from a prospective cohort (NS) | NS | ELISA – 20 U/mL | 39/174 |
| Johnson *et al*, 2012 (32) | USA *NS* | Patients selected from a cohort (by year) | NS | NS | 81/313† |
| Kang *et al*, 2005 (11) | Korea *Asian 100%* | NS (NS) | Duration of disease = 3.9 ± 4.3 yr* | IP (K562) | 2/59 |
| Kuwana *et al*, 2005 (5) | Japan *Japanese 100%* | Patients randomly selected from computer databanks | NS | IP (HeLa)<br>ELISA – 4.2 U/mL | 17/265<br>20/265 |
| Kuwana *et al*, 2005 (5) | USA (South Carolina) *Caucasian 68%, African* | Patients randomly selected from computer databanks | NS | IP (HeLa)<br>ELISA – 4.2 U/mL | 30/196<br>29/196 |

| | | | | | |
|---|---|---|---|---|---|
| | *American 28%, Hispanic 3%, and Asian 1%* | | | | |
| Low *et al*, 2010 (33) | Singapore *Asian 89%* | NS | NS | LIA | 2/46 |
| Maes *et al*, 2010 (9) | Belgium *NS* | Samples from hospital serum data bank | NS | ELISA (INOVA) – 20 U/mL | 5/70 |
| Majone *et al*, 2009 (34) | Italy *NS* | NS | NS | ELISA (MBL) | 8/39 |
| Meyer *et al*, 2007 (35) | France *Caucasian 91%, African 5%* | Cohort of consecutive patients (1975-2002) | 6.0 ± 6.1 yr* | IP (NS) | 5/127 |
| Mierau *et al*, 2011 (36) | Germany *NS* | Cohort of consecutive patients (2004-2007) | NS | IP | 33/863† |
| Morozzi *et al*, 2011 (37) | Italy *Caucasian 100%* | Cohort of consecutive patients (2007-2008) | Disease duration = 8.1 ± 4.7 yr* | ELISA (MBL) | 8/112 |
| Müller *et al*, 2011 (38) | Brazil *Caucasian 78%, Black 4%, mixed 17%* | NS (2007-2009) | NS | ELISA (INOVA) – 20 U/mL ELISA (MBL) – 28 U/mL | 35/85† |
| Nikpour *et al*, 2011 (10) | Australia *Caucasian 94.3%, Asian 3.9% and Aboriginal-Islander 1.8%* | Prospective cohort (2008-2009) | 1.9 ± 0.8 yr* | ELISA (INOVA and MBL) | 69/451 |
| Ogawa *et al*, 2010 (39) | Japan *Asian 100%* | NS | Disease duration ranged from 3.0 to 8.3 yr | ELISA and/or IP | 7/69 |
| Parker *et al*, 2008 (16) | UK *NS* | Samples from hospital serum data bank | NS | ELISA (INOVA) – 20 U/mL and IP | 202/1018 |
| Rodriguez *et al*, 2011 (40) | Mexico *Mexican Mestizo* | Patients selected from a registry (2007- | Disease duration = 10.9 ± 9.5 yr* | ELISA (INOVA) | 2/139 |

| | | | | | |
|---|---|---|---|---|---|
| | | 2009) | | | |
| Van Praet *et al*, 2011 (41) | Belgium *Caucasian 99%, African 1%* | Cohort of consecutive patients | Duration of Raynaud phenomenon = 10 ± 12 yr* | IP (K562) | 11/145 |
| Wang *et al*, 2010 (42) | China *Asian 100%* | Cohort of consecutive patients | NS | NS | 12/92 |
| Ziswiler *et al*, 2007 (43) | Switzerland *NS* | Cohort of consecutive patients (1990-2000) | NS | NS | 2/33† |

*mean ± standard deviation, †uncertain number of patients tested for ARA (denominator extrapolated from the total number of patients analysed for antinuclear antibodies), NS: not specified, ELISA: enzyme-linked immunosorbent assay, IP: immunoprecipitation, LIA: line immunoassay

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# Prevalence of Anti–RNA Polymerase III Antibodies in Systemic Sclerosis

## New Data From a French Cohort and a Systematic Review and Meta-Analysis

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***Objective.*** **Studies assessing the prevalence of anti–RNA polymerase III (anti–RNAP III) antibodies in systemic sclerosis (SSc) have yielded a wide range of results. The aim of the present study was to describe a new SSc cohort tested for presence of anti–RNAP III and perform a systematic review and meta-analysis to assess the prevalence of anti–RNAP III in patients worldwide and the potential factors of variability.**

***Methods.*** **Seropositivity for anti–RNAP III was evaluated in a French cohort of SSc patients. A systematic review of the literature was carried out in PubMed and EMBase. Meta-analysis was performed using available data on prevalence, clinical characteristics of SSc patients, and the types of assays used for anti–RNAP III testing.**

***Results.*** **One hundred thirty-three French SSc patients were tested for anti–RNAP III, and a prevalence of 6–9% was found in these patients. Thirty studies representing a total population of 8,437 SSc patients were included in the meta-analysis. Prevalence of anti–RNAP III in this population was highly variable (range 0–41%). The overall pooled prevalence of anti–RNAP III was 11% (95% confidence interval 8–14), but heterogeneity was high among studies ($I^2$ = 93%, $P$ < 0.0001). Geographic factors such as continent or country of study origin partially explained this heterogeneity and correlated with the prevalence. No other baseline SSc characteristics were significantly correlated with the prevalence of anti–RNAP III.**

***Conclusion.*** **Data on our new cohort and our meta-analysis of the literature confirmed that anti–RNAP III prevalence in SSc varies among centers. Geographic factors were significantly associated with prevalence, which underscores the probable implication that genetic background and environmental factors play a role. Heterogeneity among studies remained largely unexplained.**

Systemic sclerosis (SSc) is a chronic connective tissue disorder characterized by vascular involvement, fibrosis, and autoimmunity. Autoantibodies against multiple cellular components are used in daily practice as disease biomarkers for both positive diagnosis and prognosis. The most common antinuclear autoantibodies found in SSc are anticentromere (ACA), anti–topoisomerase I (anti–topo I), and anti–RNA polymerase III (anti–RNAP III) antibodies (1). Although

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## II.4 Anticorps anti-U1RNP et HTAP des connectivites

### II.4.1 Introduction

L'HTAP est une cause majeure de morbi-mortalité chez les patients souffrant de connectivites. La ScS est la connectivite présentant la prévalence la plus élevée d'HTAP (environ 10%) dont le pronostic est le plus sévère. En effet, la médiane de survie des patients sclérodermiques souffrant d'HTAP se situe autour de 3 ans. Les données sont plus rares concernant la prévalence d'HTAP dans les autres connectivites telles que le lupus érythémateux systémique (LES) ou la connectivite mixte (CM, aussi appelée syndrome de Sharp), mais celle-ci est probablement inférieure. Le pronostic des malades atteints d'HTAP compliquant un LES ou une CM semble meilleur que dans la ScS. Les raisons de cette différence de pronostic ne sont pour le moment que très partiellement comprises.

Les facteurs pronostiques des patients atteints d'HTAP associée à une connectivite les plus étudiés concernent les données hémodynamiques et la tolérance à l'exercice. Il existe peu de données à l'heure actuelle sur la place des auto-anticorps en tant que facteurs pronostiques de survie. Alors que les anticorps les plus fréquemment rencontrés dans la ScS (anticorps anti-centromère, anticorps anti-Scl70) ne semblent pas associés à la mortalité, l'impact de certains anticorps moins fréquents n'a pas été évalué. Parmi ceux-ci, les anticorps anti-U1RNP présentent des caractéristiques intéressantes. En effet, ils sont rencontrés au cours des principales connectivites associées à l'HTAP, mais à des fréquences différentes et sont associées au sein des connectivites à des manifestations différentes. Ainsi, les anticorps anti-U1RNP sont présents chez 2 à 14 % des patients avec ScS, 20 à 40 % des patients avec LES et, par définition, tous les patients avec CM. De plus, il a été suggéré que la présence des anticorps anti-U1RNP pourrait être un facteur prédictif d'atteinte pulmonaire dans

le lupus, en particulier d'hypertension pulmonaire. Dans la ScS, bien que les anticorps anti-U1RNP soient classiquement associés avec une maladie moins sévère, plusieurs études ont relevé une association avec l'HTAP.

A notre connaissance, aucune étude ne s'est intéressée au rôle des anticorps anti-U1RNP en tant que facteur pronostique de survie dans l'HTAP des connectivites. Ce travail avait donc pour objectifs (i) de comparer les caractéristiques cliniques, fonctionnelles et hémodynamiques des patients avec ou sans anticorps anti-U1RNP ; (ii) d'analyser la survie des patients en étudiant le rôle pronostique des anticorps anti-U1RNP dans l'HTAP des connectivites, et en particulier l'HTAP associée à la ScS.

### II.4.2 Résumé

**Contexte :** L'hypertension artérielle pulmonaire (HTAP) est une complication sévère des connectivites. Le rôle des auto-anticorps en tant que facteurs pronostiques est encore largement méconnu. Cette étude visait à étudier les caractéristiques et la survie des patients souffrant d'HTAP associée aux connectivites et présentant des anticorps anti-U1RNP.

**Méthodes :** Tous les patients avec HTAP associée aux connectivites étaient inclus prospectivement. Les données cliniques, immunologiques et la mortalité étaient ajoutées secondairement à la base de données constituée. Les caractéristiques cliniques et hémodynamiques étaient comparées dans deux groupes constitués selon la présence ou l'absence des anticorps anti-U1RNP. Ces anticorps étaient ensuite analysés en tant que facteurs pronostiques potentiels de survie dans l'HTAP associée aux connectivites, et plus particulièrement la ScS.

**Résultats :** 342 patients avec HTAP associée aux connectivites étaient inclus, dont 36 (11 %) avec anticorps anti-U1RNP. Les patients avec anticorps anti-U1RNP étaient plus jeunes au moment du diagnostic d'HTAP et leur tolérance à l'exercice était meilleure que les patients ne présentant pas ces anticorps. Les paramètres hémodynamiques étaient similaires entre les deux groupes. Parmi les patients avec HTAP associée aux connectivites, la présence d'anticorps anti-U1RNP était un facteur protecteur significatif de mortalité en analyse univariée (HR 0.34 [intervalle de confiance à 95% : 0.18-0.65] ; p<0.001). En analyse multivariée, la présence d'anticorps anti-U1RNP était associée à une meilleure survie (HR 0.44 [IC 95% : 0.20-0.97] ; p=0.043) indépendamment de l'âge, du sexe, des paramètres fonctionnels, respiratoires et hémodynamiques. Pour l'HTAP associée à la ScS, les résultats étaient similaires mais l'association entre la présence d'anticorps anti-U1RNP et la survie

n'atteignait pas la significativité en analyse univariée (HR 0.47 [IC 95% : 0.22-1.02] ; p=0.055) et multivariée (HR 0.47 [IC 95% : 0.20-1.11] ; p=0.085).

**Conclusion :** La présence d'anticorps anti-U1RNP était associée avec des caractéristiques cliniques différentes des autres patients avec HTAP associée aux connectivites ou à la ScS, mais ne semblait pas influencer les paramètres hémodynamiques. Les analyses de survie suggéraient que la présence d'anticorps anti-U1RNP pouvait être un facteur protecteur de mortalité chez les patients avec HTAP associée aux connectivites ou à la ScS.

**II.4.3 Article** *(Arthritis Rheumatol 2016;68(2):484-493)*

# Characteristics and survival of patients with anti-U1RNP antibodies in connective tissue disease associated pulmonary arterial hypertension

**Running head:** Anti-U1RNP antibodies in CTD-PAH

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**ABSTRACT**

**Objectives:** Pulmonary arterial hypertension (PAH) is a severe complication of connective tissue diseases (CTDs). This study aimed to study the clinical and hemodynamic characteristics and survival of patients with anti-U1RNP antibodies in CTD-PAH, with a focus on systemic sclerosis (SSc)-PAH.

**Methods:** We implemented a prospective database that included CTD-PAH patients with clinical, autoantibody and mortality data. We compared clinical and hemodynamic characteristics accordingly to anti-U1RNP antibodies status. We then assessed whether anti-U1RNP antibodies could be a prognostic factor in CTD-PAH with a focus on SSc-PAH.

**Results:** A total of 342 CTD-PAH patients were studied, of whom 36 (11%) were anti-U1RNP antibodies positive. Patients with anti-U1RNP antibodies were younger and less functionally impaired than anti-U1RNP negative patients in CTD- and SSc-PAH. Hemodynamic parameters were similar between anti-U1RNP positive and negative patients. In CTD-PAH, anti-U1RNP positivity was associated with a decreased mortality in univariable analysis (HR 0.34 [95% CI: 0.18-0.65]; p<0.001). In multivariable analysis, anti-U1RNP was also associated with a decreased mortality (HR 0.44 [0.20-0.97]; p=0.043), independently of age, sex, functional parameters, lung involvement and hemodynamic. In SSc-PAH, results were similar although the association between anti-U1RNP positivity and survival did not reach significance in univariable (HR 0.47 [0.22-1.02]; p=0.055) and multivariable analysis (HR 0.47 [0.20-1.11]; p=0.085).

**Conclusion:** Anti-U1RNP positivity was associated with distinct clinical characteristics and survival in CTD- and SSc-PAH. While hemodynamic parameters were similar

between anti-U1RNP positive and negative patients, our results suggest that anti-U1RNP positivity could be a protective factor of mortality in CTD-PAH and SSc-PAH.

**Key words:** anti-U1RNP antibodies – pulmonary hypertension – systemic sclerosis – systemic lupus erythematosus – mixed connective tissue disease

## INTRODUCTION

Pulmonary arterial hypertension (PAH) is a leading cause of morbidity and mortality in patients with connective tissue diseases (CTDs) (1-4). Systemic sclerosis (SSc) is the CTD with the higher prevalence of PAH (around 10%) and the worse prognosis, as a recent meta-analysis estimated the 3-yr overall survival at 56% for patients with SSc and PAH (SSc-PAH) (1,5-7). In other CTDs like systemic lupus erythematosus (SLE) or mixed connective tissue diseases (MCTD), there are less robust data on the PAH prevalence but it is very probably lower than in SSc (3,4). The prognosis of SLE/MCTD-associated PAH (SLE/MCTD-PAH) is also better than in SSc-PAH with a 3 yr-overall survival between 74-88% in SLE-PAH and 63-64% in MCTD-PAH (8,9). There is no clear explanation for this difference in survival between CTD-PAH (10,11). Among the prognosis factors of SSc-PAH, a lot attention has been made on hemodynamics and exercice tolerance (NYHA functional class and 6 min walk test) (12). Data are much more limited concerning the potential of autoantibodies as prognostic factors in SSc-PAH. Among the few studies assessing this role, anticentromere or antitopoisomerase antibody positivity did not influence outcome (1,13). Anti-U1RNP antibodies are another important candidate as prognosis factor in SSc- and CTD-PAH. Anti-U1RNP antibodies are shared by CTDs characterized by different prevalence of PAH and prognosis. Indeed, anti-U1RNP antibodies are found in 2-14% of SSc patients, 20-40% of SLE patients and, by definition, in 100% of MCTD patients (14,15). Some studies have suggested an association between anti-U1RNP antibodies and the occurrence of pulmonary damage in SLE patients (16) and especially pulmonary hypertension (17-19). In SSc, although anti-U1RNP antibodies are usually associated with a milder disease (15), several studies have suggested an

association with PAH (20,21).

To date there are no studies focusing on the role of anti-U1RNP antibodies as prognosis factors in CTD-PAH. This study aimed to fill this gap and study the clinical and hemodynamic characteristics and survival of patients with anti-U1RNP antibodies in CTD-PAH, with a focus on SSc-PAH.

## METHODS

### Cohort of patients and PH diagnosis

The Royal Free Hospital (RFH) Pulmonary Hypertension (PH) database included prospectively all patients who underwent at least one right heart catheterization (RHC) between January 1st 1998 and December 31st 2012. It contains hemodynamic parameters for each RHC: right atrial pressure (RAP), pulmonary capillary wedge pressure (PCWP), mean pulmonary artery pressure (mPAP), mean aortic pressure (mAoP), cardiac index (CI), pulmonary vascular resistances (PVR), arterial oxygen saturation (SaO2) and venous oxygen saturation (SvO2).

According to the guidelines for PH diagnosis (22,23), PH was defined as a mPAP ≥ 25mmHg by RHC at rest without raised cardiac output. Post-capillary PH was defined as PH with a PCWP > 15mmHg. Patients with PH and an elevated PCWP or left ventricular end-diastolic pressure (LVEP) > 15mmHg were considered to have PH secondary to left heart disease (PH-LHD). Patients with pre-capillary PH (PCWP ≤ 15mmHg) were divided into two groups: PH-ILD (PH associated with interstitial lung disease) for patients with a forced vital capacity (FVC) less than 70% predicted and/or ILD extent above 20% on high-resolution CT-scan (HRCT) (24); and PAH (no ILD or ILD with FVC % predicted ≥ 70% and extent on HRCT ≤ 20%).

### CTD diagnosis

The type of CTD was defined at the time of PH diagnosis. Patients were diagnosed with SSc if they fulfilled the American College of Rheumatology criteria (25) and/or LeRoy and Medsger criteria (26), and classified as having diffuse (dcSSc), limited cutaneous (lcSSc) or limited SSc (lSSc) form according to LeRoy and Medsger (27). SLE were diagnosed according to usual criteria (28,29). In cases of overlap between

SSc and SLE, patients were entered into the SSc group. As previously described (30), MCTD was defined in patients without full criteria for a definite CTD and fulfilling at least one of three most commonly used criteria sets of MCTD: Sharp's criteria set, Kasukawa and co-workers or Alarcón-Segovia and Villareal.

**Immunological tests**

Autoimmune serology was extracted from the clinical database or chart records (data were missing in 31 patients). Identification of ANA specificities (anti-topoisomerase I antibodies (ATA), anti-U1RNP, anti-SSA/Ro, anti-SSB/La and anti-Jo1 antibodies) was performed as part of routine clinical care using both specific immunofluorescence patterns on HEp-2 cells substrate (Bio-Diagnostics Ltd, Upton-upon-Severn, UK) and counter immunoelectrophoresis as previously described (31). Anti-centromere antibody (ACA) was identified by characteristic staining pattern on HEp-2 cell substrate. Anti-double stranded DNA (dsDNA) antibodies were identified by a commercially available ELISA method (Thermo Fisher Scientific, Immunodiagnostics, Uppsala, Sweden).

**Other measurements**

Other variables were retrospectively implemented into the PH database. Survival data were retrieved from clinical letters or United Kingdom NHS (National Health Service) database (data were censored at 1$^{st}$ March 2013 for analysis). Demographic data, date of disease onset (defined as age at the first non-Raynaud's symptom), pulmonary function tests (FVC % predicted value and DLCO [diffusion capacity of the lung for carbon monoxide] % predicted value), WHO functional class (FC), 6 minute walking distance (6MWD) were retrieved from letters, PH and/or SSc local databases. This study was approved by the Royal Free Hospital local ethics committee (London-

Hampstead NRES Reference Number 6398).

**Statistical analyses**

Continuous variables were described using mean and standard deviation (SD) and compared using Kruskal-Wallis test. Categorical variables were described using number and percentage (%) and compared using Fisher exact test. Survival estimates were performed by Kaplan-Meier analyses with comparisons performed by log-rank test.

Multiple Cox proportional hazards regression models examined factors associated with survival. A first non-adjusted model was performed to study survival according to anti-U1RNP positivity. Then two adjusted models were built: (A) model adjusted on age and sex because significant differences were observed between groups; (B) model adjusted on functional parameters, lung involvement (FVC % predicted value, WHO FC) and hemodynamic parameters (RAP, PCWP, mPAP and CI). Proportional hazards hypothesis was verified for each model. Analyses were performed for the entire population of CTD-PAH (SSc-, SLE- and MCTD-PAH) and then only for SSc-PAH. For the SSc-PAH population, models were also adjusted on the cutaneous subtype (lcSSc vs. dcSSc).

Sensitivity analyses were conducted: (i) because patients with overlap between SSc and SLE were entered into the SSc group, we studied whether exclusion of these patients (n=5) modified results of the Cox regression analyses; (ii) anti-U1RNP status and the type of CTD (SSc, SLE, MCTD) were strongly associated, precluding adjustment on the later (non-convergence of Cox regression models). Consequently we assessed whether adjusting on the type of CTD (SSc, no SSc) with or without excluding MCTD modified the results.

Statistical analyses were performed using R Software version 3.1.2 (32). A p value less than 0.05 was taken as significant throughout.

## RESULTS

### Study population

On 2250 patients who underwent a RHC for a suspicion of pulmonary hypertension, 1013 had been diagnosed previously as having a CTD (SSc, SLE or MCTD). Pulmonary hypertension was confirmed in 626/1013 CTD patients. Among them, 342 CTD patients had pre-capillary PH of group 1 (PAH), and constituted our study population (**Figure 1A**).

As shown in **Figure 1B**, the prognosis was significantly different between CTDs. The 3- and 5-year survival rates from PAH diagnosis were 63% and 43% for SSc-, 86% and 85% for SLE-, 100% and 100% for MCTD-PAH, respectively (p<0.001).

Thirty-six out of 342 (11%) CTD-PAH patients had anti-U1RNP antibodies: 14 with SSc, 10 with SLE, 2 with an overlap SSc/SLE and 10 with MCTD (**Figure 1C**).

### Anti-U1RNP antibodies in CTD-PAH

*Clinical and hemodynamic characteristics*

Comparisons between anti-U1RNP positive and negative CTD-PAH patients (**Table 1**) showed that anti-U1RNP positive patients were younger (45.3 ± 14.2 versus 61.9 ± 11.8 years; p<0.001) and had a lower CTD duration at PH diagnosis (9.8 ± 8.8 vs. 14.0 ± 10.3 years; p=0.040). Anti-U1RNP positive patients were less functionally impaired as shown by a larger proportion of patients in WHO FC I-II vs. III-IV (39% vs. 22%; p=0.031) and a higher 6MWD (352 ± 109 vs. 258 ± 131 meters; p=0.006). The mean DLCO was higher in the anti-U1RNP positive group (49.1 ± 9.9 vs. 42.6 ± 14.4%; p=0.004). Hemodynamic parameters were similar except for a lower mAoP (94.0 ± 19.9 vs. 101.9 ± 17.8 mmHg; p=0.025) and a higher SaO2 (95.5 ± 2.6 vs. 93.9 ± 4.0%;

p=0.020) in the anti-U1RNP positive group.

*Survival analysis*

Kaplan-Meier analysis showed that patients with anti-U1RNP antibodies had a better survival than anti-U1RNP negative patients (**Figure 2A**). The 5-year and 10-year survival rates were 78% and 59% in the anti-U1RNP positive group versus 44% and 23% in the anti-U1RNP negative group, respectively (p=0.001).

Cox regression analyses were performed to highlight predictors of mortality in CTD-PAH (**Table 2**). In univariable analysis, anti-U1RNP positivity was associated with a better survival (hazard ratio (HR) 0.34 [95% CI: 0.18-0.65]; p<0.001). Besides anti-U1RNP positivity, sex, age at PH diagnosis, WHO FC, 6MWD, DLCO % predicted, RAP, PCWP, mPAP, CI, PVR, SaO2 and SVO2 were significantly associated with mortality. There was a trend for a negative association between FVC % predicted and mortality (p=0.054). There was no association between CTD duration at PH diagnosis or mAoP and mortality. In multivariable analysis, anti-U1RNP positivity remained negatively associated with mortality in both models: model A including anti-U1RNP positivity, age at PH diagnosis and sex (HR 0.54 [0.28-1.05]; p=0.067) and model B including anti-U1RNP positivity, age at PH diagnosis, sex, WHO FC, FVC % predicted and hemodynamic parameters (HR 0.44 [0.20-0.97]; p=0.043).

**Focus on SSc-PAH**

*Clinical and hemodynamic characteristics*

In SSc-PAH, anti-U1RNP positive patients were younger at PAH diagnosis (54.4 ± 12.8 vs. 62.7 ± 11.3 years; p=0.012), had a higher mean DLCO (48.6 ± 10.9 vs. 41.9 ± 13.9 %, p=0.031) and a higher proportion of patients in WHO FC I-II vs. III-IV (50%

vs. 21%; p=0.020) than anti-U1RNP negative patients (**Table 1**). There was no difference in the proportion of dcSSc between anti-U1RNP positive and negative patients. Hemodynamic parameters were similar, except for a trend in a lower RAP (7.6 ± 7.2 vs. 8.2 ± 4.7 mmHg; p=0.089) in anti-U1RNP positive patients.

*Survival analysis*

Survival analysis showed a trend for a better survival in anti-U1RNP positive patients (p=0.055; **Figure 2B**). The 5-year and 10-year survival rates were 71% and 36% in the anti-U1RNP positive group versus 41% and 20% in the anti-U1RNP negative group, respectively. Cox regression analyses were performed using a similar methodology than in CTD-PAH (**Table 3**). In univariable analysis, there was a trend towards a positive association between anti-U1RNP positivity and a better survival (HR 0.47 [0.22-1.02]; p=0.055). Besides anti-U1RNP positivity, sex, age at PH diagnosis, WHO FC, 6MWD, FVC % predicted, DLCO % predicted, RAP, PCWP, mPAP, CI, PVR, SaO2 and SVO2 were significantly associated with mortality. CTD duration at PH diagnosis and cutaneous form of SSc were not significantly associated with mortality. In multivariable analysis, anti-U1RNP positivity remained negatively associated with mortality in both models but did not reach significance: model A (HR 0.58 [0.27-1.25]; p=0.164); model B (HR 0.47 [0.20-1.11]; p=0.085).

**Sensitivity analyses**

Results of the Cox regression analyses in CTD-PAH yielded similar results with the same and constant trend for anti-U1RNP positivity to be associated with a better survival when we reran the models (**Figure 3**): (i) by excluding SSc/SLE overlap patients (HR for anti-U1RNP positivity in model B: 0.49 [0.22-1.08]; p=0.075); (ii) by

adjusting on the type of CTD (SSc vs. non-SSc) (HR 0.52 [0.23-1.15]; p=0.107); (iii) by adjusting on the type of CTD and cutaneous form of SSc (dcSSc vs. lcSSc vs. non-SSc) (HR 0.53 [0.24-1.19]; p=0.124); (iv) by excluding the MCTD patients and adjusting on SSc vs. SLE (HR 0.55 [0.25-1.22]; p=0.140); (v) by excluding the MCTD patients and adjusting on cutaneous form of SSc (dcSSc vs. lcSSc vs. SLE) (HR 0.56 [0.25-1.26]; p=0.160).

**DISCUSSION**

The main results of our study are as follows: 1) the survival was significantly different between the CTDs (SSc, SLE and MCTD) associated with PAH, in accordance with previous reports, 2) in the population of CTD-PAH, anti-U1RNP positivity was significantly associated with several clinical characteristics and a better survival in univariable and multivariable analysis, and 3) in the population of SSc-PAH, results were similar although the association between anti-U1RNP positivity and survival missed the statistical significance in univariable (p=0.055) and multivariable analysis (p=0.085).

Survival analyses showed that prognosis of SSc-PAH was poor in our population with a 3- and 5-year survival rates were of 63% and 43%, respectively. This is in keeping with the results of a recent meta-analysis of survival studies in SSc-PAH, showing a 3 year-survival of 56% (95% CI: 51-61) (1). Recent data from REVEAL (Registry to Evaluate Early and Long-term Pulmonary Arterial Hypertension Disease Management) showed a 3-year survival in CTD-PAH (SSc represented about 2/3 of the cohort) of 57% and a 5-year survival of 44% (33). Regarding SLE-PAH, we found a 3-year and 5-year survival rates of 86%. This is similar to the 3-year survival of 74% shown in a UK cohort and 88% in a Chinese cohort (8,9). In our study, survival in MCTD-PAH was 100% at 5 years. This result should be interpreted with caution, as only a small number (n=10) of patients were included. However, it confirms that prognosis in MCTD-PAH might be better than SSc-PAH. Chung et al. found a 1-year survival rate of 88% (34) and Condliffe et al. found a 3-year survival rate of 63% (8) in this population.

Among the characteristics differentiating these CTDs, the positivity of anti-U1RNP

antibodies is a major element. Anti-U1RNP antibodies are shared by CTDs characterized by different features: 2-14% of SSc patients, 20-40% of SLE patients and, by definition, in 100% of MCTD patients (14,15). Therefore we first focused on CTD-PAH and compared anti-U1RNP positive vs. negative patients. Patients with anti-U1RNP antibodies were younger and had a lower CTD duration at PH diagnosis. These differences might be due to a higher proportion of SLE or MCTD patients in the anti-U1RNP positive group. Chung et al. showed in the REVEAL cohort that SLE-PAH patients were younger than SSc-PAH (45.5 ± 11.9 vs. 61.8 ± 11.1 years; $p<0.0001$) (34). Condliffe et al. found similar results (42.0 ± 12.9 vs. 63.9 ± 10.5 years; $p<0.001$) (8). These values are comparable to what we found in anti-U1RNP positive and negative groups (45.3 ± 14.2 vs. 61.9 ± 11.8 years; $p<0.001$). In a cohort of 70 Japanese CTD-PAH patients, SLE or MCTD were younger than SSc patients at PH diagnosis. MCTD had the lowest time interval between CTD onset and PH diagnosis (35).

In our study, anti-U1RNP positive patients were less functionally impaired as shown by a higher proportion of patients in WHO FC I or II and a higher 6MWD and had a higher mean DLCO. Again, these differences might be due to a majority of SLE- or MCTD-PAH in the anti-U1RNP positive group. Condliffe et al. showed that SLE-PAH had higher 6MWD and mean DLCO than SSc-PAH, but there was no difference in term of WHO FC (8). Chung et al. found a higher mean DLCO in SLE-PAH than in SSc-PAH. There was no significant difference for 6MWD and WHO FC (34).

Hemodynamic parameters were similar except for a lower mAoP and a higher SaO2 in the anti-U1RNP positive group. In the studies comparing hemodynamic values between SSc- and SLE-PAH, no differences were found for RAP, mPAP, CI, PVR,

SvO2 (8,34,35). Only PCWP was significantly different between SSc- and SLE-PAH in the REVEAL cohort (34). Interestingly, despite a similar hemodynamic severity, anti-U1RNP positive patients had a better survival than those who were negative. Moreover, multivariable analyses showed that anti-U1RNP positivity were associated with survival, independently of age, sex, functional impairment and hemodynamic severity in CTD-PAH. These results highlight a possible serological homogeneity carried by anti-U1RNP antibodies between the different CTD-PAH, with an impact on disease characteristics and survival.

We then assessed whether these findings were similar inside a selected CTD. Among our SSc-PAH population, we found comparable characteristics (younger patients in anti-U1RNP positive group, less functionally impaired with a similar hemodynamic severity). Anti-U1RNP positivity remained associated with a better survival (HR were similar than in CTD-PAH group but did not reach significance). Overall these results are consistent with a unique phenotype and a different prognosis of anti-U1RNP positive patients in CTD- and SSc-PAH.

Anti-U1RNP antibodies bind to U1 small nuclear ribonucleoprotein autoantigen (U1snRNP), a complex that is involved in splicing heterogeneous nuclear RNA into mRNA (15). In SSc, anti-U1RNP antibodies are usually associated with overlap syndromes and are more frequent among lcSSc patients compared to those with dcSSc (36-38). Patients with anti-U1RNP antibodies tend to be younger at SSc diagnosis with a less severe skin involvement and uncommon renal involvement. Puffy hands, Raynaud's phenomenon, arthritis and myositis are commonly seen (15,38,39). Nevertheless, although anti-U1RNP antibodies have been classically associated with a milder disease (15), several studies have suggested an association with PAH in SSc

(20,21,40,41). In SLE, anti-U1RNP antibodies, Raynaud's phenomenon and antiphospholipid antibodies have been associated with PAH (18,19,42,43). In a cluster analysis of MCTD patients, Szodoray et al. have shown a cluster strongly associated with PH. This cluster presented a higher frequency of swollen hands, Raynaud's phenomenon, livedo reticularis and secondary anti-phospholipid syndrome (44).

The exact mechanisms of PAH in CTD remain elusive. Chow et al. have suggested that anti-U1RNP antibodies in SLE could confer a vasculopathy similar to SSc and that antiphospholipid antibodies could lead to a thromboembolic process (17). Histologic studies of pulmonary arteries in MCTD-PAH patients showed intimal hyperplasia, hypertrophic media, plexiform lesion and locally formed microthrombi. These features are similar to those found in SSc- or SLE-PAH. Vegh et al. found a higher frequence of anti-endothelial cell antibodies and higher serum thrombomodulin and von Willebrand factor antigen concentrations suggesting endothelial cell activation. Interestingly, anti-U1RNP antibodies levels were higher in MCTD patients with PAH than in those without PAH (45). Thus anti-U1RNP antibodies might be a hallmark of a distinct phenotype in CTD-PAH.

Previous studies have suggested that immunosuppressive therapy in SLE- or MCTD-PAH could improve survival in responding patients (10,11). Therefore patients with SLE- or MCTD-PAH might have received more frequently an immunosuppressive therapy than SSc-PAH in which this treatment has not proved efficacy (10). However, one of the results highlighted here is that SSc-PAH patients with anti-U1RNP are different than SSc-PAH patients without anti-U1RNP. Whether or not immunosuppressive treatment could be efficient in SSc-PAH with anti-U1RNP antibodies deserves further studies.

To the best of our knowledge, this study is the first to compare hemodynamic data and survival in a subgroup of CTD-PAH patients characterized by a serological homogeneity. However this work has some limitations. First, this was a single-center study analysing a selected population of patients referred to a PAH referral centre. Nevertheless, we included all consecutive patients with SSc-, SLE- or MCTD-PAH referred to our centre during a 14-year period. This design allows a valuable analysis of the patients' characteristics and outcomes. Moreover, hemodynamic parameters were entered into the database at the time of the RHC resulting in a very limited number of missing data and robustness of hemodynamic variables. Second, due to the retrospective design of clinical variables implementation, we were unable to collect precise data on specific treatment for PAH (especially immunosuppressive therapies) or data on causes of death. MCTD patients can present clinical symptoms suggestive of SSc (i.e. swollen fingers, digital ulcers, oesophageal dysmotility etc.). Studying whether the existence of SSc manifestations in MCTD patients could have a role in the prognosis evaluation would have been of interest. Unfortunately, specific detailed organ involvement of CTD patients was not gathered. Finally, although this is one of the largest cohorts of CTD-PAH patients, we lacked the statistical power to confirm the association in SSc-PAH patients because of the small number of patients with anti-U1RNP antibodies positive.

In conclusion, our study confirms that survival is significantly different between SSc-, SLE- and MCTD-PAH. Anti-U1RNP antibodies positivity is associated with distinct clinical characteristics and survival in CTD- and SSc-PAH. Although hemodynamic parameters were similar between anti-U1RNP positive and negative patients, anti-U1RNP positivity was negatively and independently associated with mortality in CTD-

PAH. In SSc-PAH, survival analyses suggested a negative association between anti-U1RNP antibodies and survival. These results highlight the clinical need for a better characterization of CTD-PAH phenotypes, especially in therapeutic studies.

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## TABLES

**Table 1:** Comparison of clinical and hemodynamic characteristics between anti-U1RNP positive and negative patients.

| | CTD-PAH | | | | SSc-PAH | | | |
|---|---|---|---|---|---|---|---|---|
| | **N** | **Anti-U1RNP positive (n=36)** | **Anti-U1RNP negative (n=306)** | **p** | **N** | **Anti-U1RNP positive (n=16)** | **Anti-U1RNP negative (n=292)** | **p** |
| Age at PH diagnosis, *years* | 342 | 45.3 ± 14.2 | 61.9 ± 11.8 | **< 0.001** | 308 | 54.4 ± 12.8 | 62.7 ± 11.3 | **0.012** |
| Sex, *female* | 342 | 31 (86) | 261 (85) | > 0.999 | 308 | 14 (88) | 248 (85) | > 0.999 |
| Cutaneous form of SSc, *diffuse* | | | | | 292 | 2 (13) | 37 (13) | > 0.999 |
| CTD duration at PH diagnosis, *years* | 179 | 9.8 ± 8.8 | 14.0 ± 10.3 | **0.040** | 162 | 11.9 ± 10.6 | 14.2 ± 10.3 | 0.386 |
| Follow-up, *years* | 338 | 5.6 ± 4.1 | 3.7 ± 2.9 | **0.010** | 305 | 5.7 ± 4.2 | 3.6 ± 2.8 | **0.032** |
| FVC, *% predicted* | 277 | 87.8 ± 12.6 | 93.4 ± 17.8 | 0.166 | 255 | 92.7 ± 13.2 | 93.4 ± 17.6 | 0.871 |
| DLCO, *% predicted* | 272 | 49.1 ± 9.9 | 42.6 ± 14.4 | **0.004** | 250 | 48.6 ± 10.9 | 41.9 ± 13.9 | **0.031** |
| WHO FC I-II | 318 | 13 (39) | 62 (22) | **0.031** | 286 | 7 (50) | 58 (21) | **0.020** |
| vs. III-IV | | 20 (61) | 223 (78) | | | 7 (50) | 214 (79) | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 6MWD, *meters* | 92 | 352 ± 109 | 258 ± 131 | **0.006** | 67 | 321 ± 174 | 248 ± 130 | 0.428 |
| RAP, *mmHg* | 338 | 7.9 ± 6.0 | 8.1 ± 4.7 | 0.273 | 305 | 7.6 ± 7.2 | 8.2 ± 4.7 | 0.088 |
| PCWP, *mmHg* | 333 | 10.0 ± 2.4 | 10.3 ± 3.3 | 0.644 | 302 | 9.4 ± 2.1 | 10.2 ± 3.2 | 0.250 |
| mPAP, *mmHg* | 342 | 39.9 ± 12.2 | 40.3 ± 12.7 | 0.889 | 308 | 36.8 ± 14.2 | 39.9 ± 12.4 | 0.116 |
| mAoP, *mmHg* | 301 | 94.0 ± 19.9 | 101.9 ± 17.8 | **0.025** | 270 | 100 ± 19 | 102 ± 18 | 0.606 |
| CI, $L.min^{-1}.m^{-2}$ | 308 | 2.6 ± 0.8 | 2.7 ± 0.7 | 0.484 | 277 | 2.7 ± 1.0 | 2.7 ± 0.7 | 0.767 |
| PVR, $dynes.s.cm^{-5}$ | 323 | 644 ± 471 | 615 ± 432 | 0.584 | 292 | 646 ± 640 | 607 ± 417 | 0.579 |
| SaO2, *%* | 303 | 95.5 ± 2.6 | 93.9 ± 4.0 | **0.020** | 274 | 95.2 ± 3.4 | 93.8 ± 4.0 | 0.134 |
| SvO2, *%* | 314 | 67.7 ± 8.1 | 65.9 ± 9.9 | 0.506 | 283 | 67.9 ± 8.0 | 65.8 ± 9.9 | 0.693 |

*Definition of abbreviations:* PH: pulmonary hypertension; CTD: connective tissue disease; FVC: forced vital capacity; DLCO: diffusion capacity of the lung for carbon monoxide; WHO FC: world health organization functional class; 6MWD: 6-minute walking distance; RAP: right atrial pressure; PCWP: pulmonary capillary wedge pressure; mPAP: mean pulmonary arterial pressure; mAoP: mean aortic pressure; CI: cardiac index; PVR: pulmonary vascular resistances; $SaO_2$: arterial oxygen saturation; $SvO_2$: mixed venous oxygen saturation
Continuous variables were summarized by the mean ± standard deviation and categorical variables by frequency (percentage).

**Table 2:** Predictors of mortality in CTD-PAH patients.

| | **Univariable** | **Multivariable** | |
|---|---|---|---|
| | | **Model A** | **Model B** |
| Variable | **HR (95% CI)** | **HR (95% CI)** | **HR (95% CI)** |
| Sex, *male vs. female* | 1.49 (1.03-2.17)* | 1.96 (1.33-2.88)*** | 2.07 (1.31-3.26)** |
| Age at PH diagnosis, *per year* | 1.04 (1.03-1.06)*** | 1.04 (1.03-1.06)*** | 1.05 (1.03-1.07)*** |
| CTD duration at PH diagnosis, *per year* | 1.00 (0.98-1.02) | | |
| Anti-U1RNP, *positive vs. negative* | 0.34 (0.18-0.65)*** | 0.54 (0.28-1.05)§ | 0.44 (0.20-0.97)* |
| FVC, *per %* | 0.99 (0.98-1.00)§ | | 0.99 (0.98-1.01) |
| DLCO, *per %* | 0.96 (0.95-0.97)*** | | |
| WHO FC, *III-IV vs. I-II* | 2.51 (1.65-3.80)*** | | 1.44 (0.86-2.42) |
| 6MWD, *per 100 m* | 0.56 (0.41-0.78)*** | | |
| RAP, *per mmHg* | 1.06 (1.03-1.09)*** | | 1.10 (1.04-1.16)** |
| PCWP, *per mmHg* | 0.91 (0.86-0.95)*** | | 0.85 (0.79-0.92)*** |
| mPAP, *per mmHg* | 1.03 (1.02-1.04)*** | | 1.00 (0.98-1.02) |
| mAoP, *per mmHg* | 1.00 (0.99-1.01) | | |
| CI, *per* $L.min^{-1}.m^{-2}$ | 0.53 (0.42-0.68)*** | | 0.91 (0.64-1.30) |
| PVR, *per 100 dynes.s.*$cm^{-5}$ | 1.12 (1.09-1.15)*** | | |
| SaO2, *per %* | 0.91 (0.88-0.94)*** | | |
| SvO2, *per %* | 0.94 (0.93-0.96)*** | | 0.98 (0.95-1.00)§ |

§p<0.10, *p<0.05, **p<0.01, ***p<0.001

**Table 3:** Predictors of mortality in SSc-PAH patients.

| | **Univariable** | **Multivariable** | |
|---|---|---|---|
| | | **Model A** | **Model B** |
| Variable | **HR (95% CI)** | **HR (95% CI)** | **HR (95% CI)** |
| Sex, *male vs. female* | 1.51 (1.03-2.21)* | 1.95 (1.31-2.89)*** | 1.94 (1.23-3.08)** |
| Age at PH diagnosis, *per year* | 1.03 (1.02-1.05)*** | 1.04 (1.02-1.05)*** | 1.04 (1.02-1.06)*** |
| Cutaneous form of SSc, *limited vs. diffuse* | 0.72 (0.47-1.12) | | |
| CTD duration at PH diagnosis, *per year* | 0.99 (0.97-1.01) | | |
| Anti-U1RNP, *positive vs. negative* | 0.47 (0.22-1.02)§ | 0.58 (0.27-1.25) | 0.47 (0.20-1.11)§ |
| FVC, *per %* | 0.99 (0.98-1.00)* | | 0.99 (0.98-1.01) |
| DLCO, *per %* | 0.97 (0.95-0.98)*** | | |
| WHO FC, *III-IV vs. I-II* | 2.50 (1.64-3.79)*** | | 1.41 (0.84-2.37) |
| 6MWD, *per 100 m* | 0.65 (0.46-0.93)* | | |
| RAP, *per mmHg* | 1.07 (1.04-1.10)*** | | 1.08 (1.02-1.14)** |
| PCWP, *per mmHg* | 0.91 (0.86-0.96)*** | | 0.86 (0.80-0.93)*** |
| mPAP, *per mmHg* | 1.04 (1.02-1.05)*** | | 1.01 (0.99-1.03) |
| mAoP, *per mmHg* | 1.00 (0.99-1.00) | | |
| CI, *per* $L.min^{-1}.m^{-2}$ | 0.48 (0.37-0.61)*** | | 0.89 (0.62-1.27) |
| PVR, *per 100* $dynes.s.cm^{-5}$ | 1.13 (1.10-1.17)*** | | |
| SaO2, *per %* | 0.92 (0.89-0.95)*** | | |
| SvO2, *per %* | 0.94 (0.93-0.96)*** | | 0.97 (0.95-1.00)* |

§p<0.10, *p<0.05, **p<0.01, ***p<0.001

## FIGURES

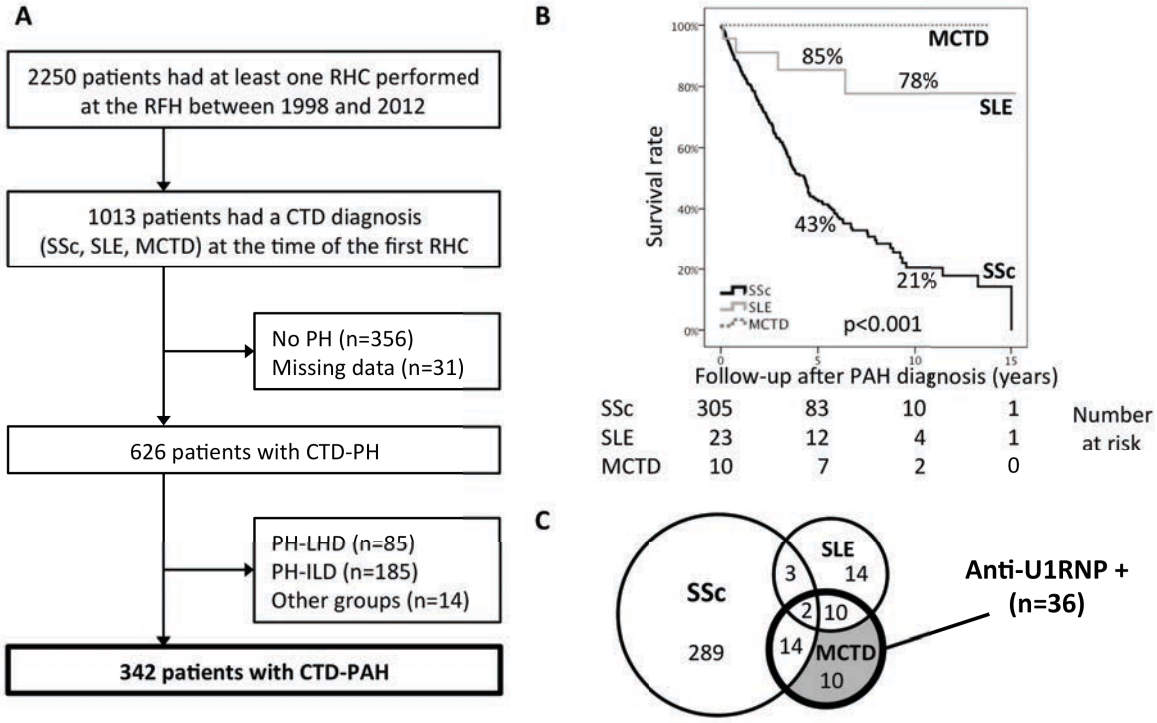
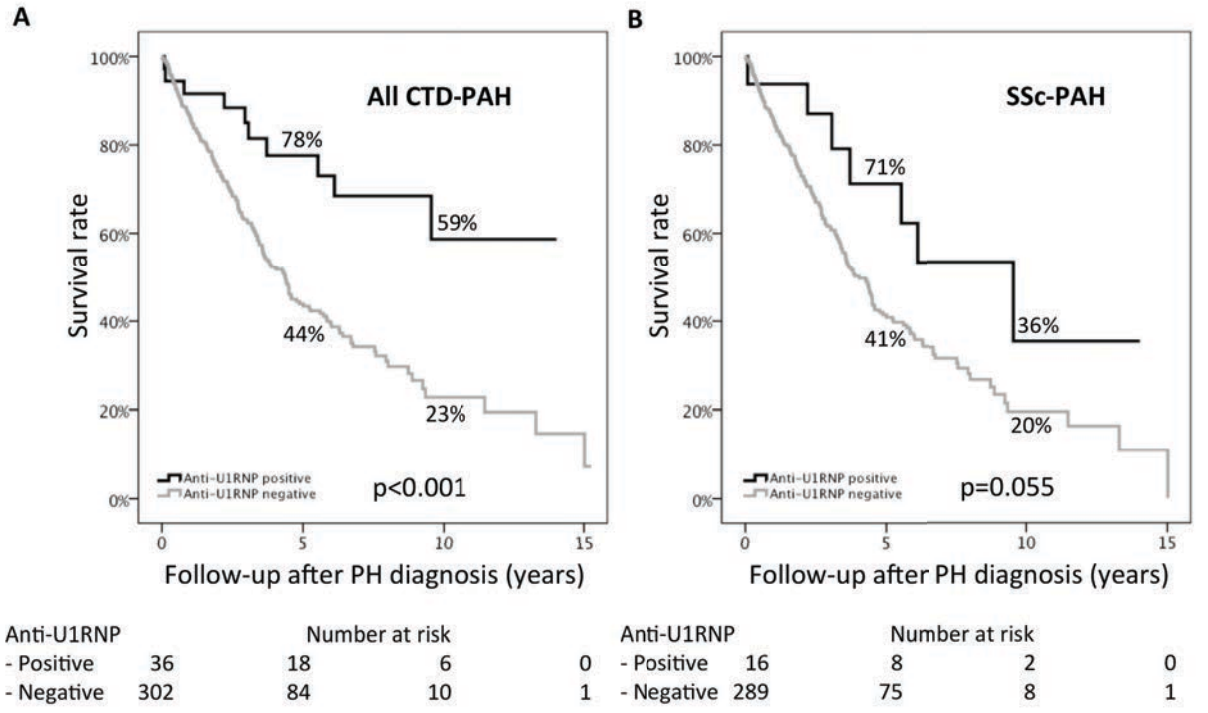



**Figure 1: A.** Patients included in this study. **B.** Kaplan-Meier curves of survival after PAH diagnosis. **C.** Venn diagram representing distribution of CTDs and anti-U1RNP positivity among the PAH population.




| Anti-U1RNP | | Number at risk | | |
|---|---|---|---|---|
| - Positive | 36 | 18 | 6 | 0 |
| - Negative | 302 | 84 | 10 | 1 |

| Anti-U1RNP | | Number at risk | | |
|---|---|---|---|---|
| - Positive | 16 | 8 | 2 | 0 |
| - Negative | 289 | 75 | 8 | 1 |

**Figure 2:** Kaplan-Meier curves of survival after PAH diagnosis. **A:** In all CTD-PAH patients. **B:** In SSc-PAH patients.

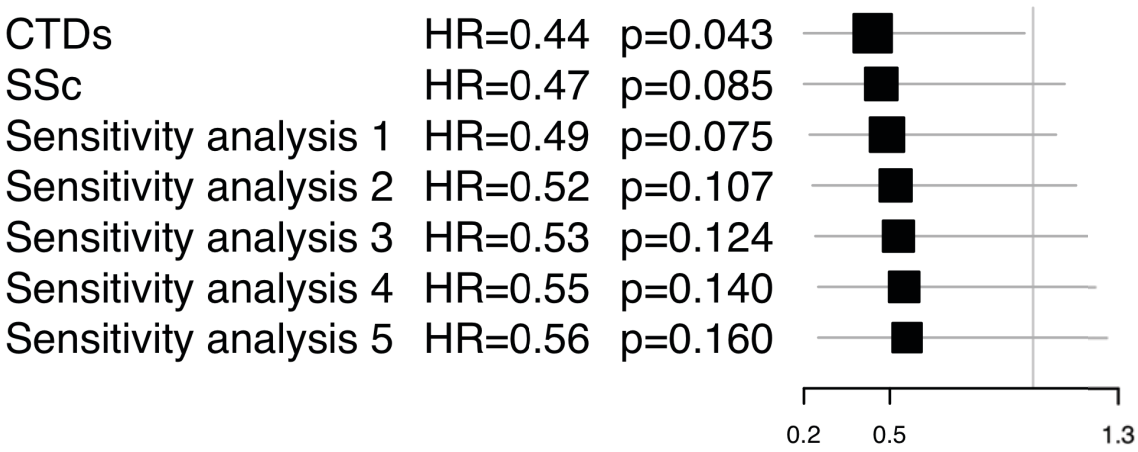



**Figure 3:** Results of sensitivity analyses: hazard ratios of survival for anti-U1RNP positivity.

# ANNEXES

## Annexe : Classification Criteria for MCTD (adapted from Cappelli et al. (14))

| **Sharp** (1987) | **Kasukawa et al.** (1987) | **Alarcón-Segovia et al.** (1987) |
|---|---|---|
| A. Major criteria<br>1) Myositis, severe<br>2) Pulmonary involvement<br>a. Diffusion capacity <70% of normal values<br>b. Pulmonary hypertension<br>c. Proliferative vascular lesions on lung biopsy<br>3) Raynaud's phenomenon or esophageal hypomotility<br>4) Swollen hands or sclerodactyly<br>5) Anti ENA ≥1:10,000 and anti-U1snRNP positive and anti-Sm negative<br><br>B. Minor criteria<br>1) Alopecia<br>2) Leukopenia<br>3) Anemia<br>4) Pleuritis<br>5) Pericarditis<br>6) Arthritis<br>7) Trigeminal neuropathy<br>8) Malar rash<br>9) Thrombocytopenia<br>10) Mild myositis<br>11) History of swollen hands | A. Common symptoms<br>1) Raynaud's phenomenon<br>2) Swollen fingers or hands<br><br>B. Anti-U1 snRNP antibody positive<br><br>C. Mixed symptoms<br><br>1. SLE-like findings<br>1) Polyarthritis<br>2) Lymphadenopathy<br>3) Facial erythema<br>4) Pericarditis or pleuritis<br>5) Leukopenia (<4000/mm3) or thrombocytopenia (<100,000/mm3)<br><br>2. SSc-like findings<br>1) Sclerodactyly<br>2) Pulmonary fibrosis, restrictive changes of lung (VC < 80%) or reduced diffusion capacity (DLCO < 70%)<br>3) Hypomotility or dilatation of esophagus<br><br>3. PM-like findings<br>1) Muscle weakness<br>2) Elevated serum levels of muscle enzymes (CPK)<br>3) Myogenic pattern on EMG | A. Serologic<br>Anti-U1 snRNP at a hemagglutination titer of ≥1:1600<br><br>B. Clinical<br>1) Edema in the hands<br>2) Synovitis<br>3) Myositis<br>4) Raynaud's phenomenon<br>5) Acrosclerosis |
| At least 4 major criteria plus anti-U1snRNP titer of at least 1:4000 (exclusion criterion: positivity for anti-Sm);<br>or 2 major criteria from among 1, 2, and 3 plus 2 minor criteria plus anti-U1 snRNP titer of at least 1:1000 | At least 1 of the 2 common symptoms plus positive for anti-U1 snRNP plus 1 or more of the mixed symptoms in at least 2 of the 3 disease categories | Serologic criterion plus at least 3 clinical criteria, including either synovitis or myositis |

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# Characteristics and Survival of Anti–U1 RNP Antibody–Positive Patients With Connective Tissue Disease–Associated Pulmonary Arterial Hypertension

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***Objective.*** **Pulmonary arterial hypertension (PAH) is a severe complication of connective tissue diseases (CTDs). This study aimed to investigate the clinical and hemodynamic characteristics and survival of anti–U1 RNP–positive patients with CTD-associated PAH, with a focus on systemic sclerosis (SSc)–associated PAH.**

***Methods.*** **We implemented a prospective database that included patients with CTD-associated PAH for whom there were clinical, autoantibody, and mortality data. We compared clinical and hemodynamic characteristics to anti–U1 RNP antibody status. We then assessed whether anti–U1 RNP antibodies could be a prognostic factor in CTD-associated PAH with a focus on SSc-associated PAH.**

***Results.*** **We studied a total of 342 patients with CTD-associated PAH, of whom 36 (11%) were anti–U1 RNP antibody positive. Anti–U1 RNP–positive patients were younger and less functionally impaired than were anti–U1 RNP–negative patients in CTD- and SSc-associated PAH. Hemodynamic parameters were similar in anti–U1 RNP–positive and anti–U1 RNP–negative patients. In CTD-associated PAH, anti–U1 RNP positivity was associated with decreased mortality in univariable analysis (hazard ratio 0.34 [95% confidence interval 0.18–0.65], $P < 0.001$). In multivariable analysis, anti–U1 RNP positivity was also associated with decreased mortality (hazard ratio 0.44 [95% confidence interval 0.20–0.97], $P = 0.043$) independently of age, sex, functional parameters, lung involvement, and hemodynamic parameters. Results were similar in SSc-associated PAH, although the association between anti–U1 RNP positivity and survival did not reach significance in univariable (hazard ratio 0.47 [95% confidence interval 0.22–1.02], $P = 0.055$) and multivariable (hazard ratio 0.47 [95% confidence interval 0.20–1.11], $P = 0.085$) analyses.**

***Conclusion.*** **Anti–U1 RNP positivity was associated with distinct clinical characteristics and survival in CTD- and SSc-associated PAH. While hemodynamic parameters were similar in anti–U1 RNP–positive and anti–U1 RNP–negative patients, our results suggest that anti–U1 RNP positivity could be a factor protecting against mortality in CTD- and SSc-associated PAH.**

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# PARTIE III – CONCLUSION GENERALE ET PERSPECTIVES

En conclusion, nous avons montré dans ce travail que les marqueurs immunologiques peuvent permettre de mieux cerner l'hétérogénéité de la ScS. Ainsi, l'analyse en cluster de la cohorte EUSTAR a confirmé qu'il existait des groupes homogènes de patients au-delà de la dichotomie diffuse/limitée. La présence d'une atteinte d'organe et les auto-anticorps étaient deux facteurs déterminants dans la classification des patients en sous-groupes. Parmi les différents systèmes de classification proposés, de nombreux travaux se sont intéressés à la place des auto-anticorps.

Le dosage des chaines légères libres sériques était corrélé à l'activité et la sévérité de la ScS ; il pourrait donc avoir une place dans la classification des patients. Cela témoigne également de l'implication du système immunitaire, en particulier le lymphocyte B, dans la physiopathologie de la ScS. Nous avons estimé la prévalence mondiale globale des anticorps anti-ARN polymérase de type III dans la ScS. Cette prévalence était très variable entre les centres. Les facteurs géographiques permettaient d'expliquer une partie de cette hétérogénéité, mettant en évidence l'implication de facteurs génétiques et/ou environnementaux. Enfin, la présence des anticorps anti-U1RNP était associée avec des caractéristiques cliniques et une survie différentes dans l'HTAP associée aux connectivites et en particulier la ScS. Ces résultats soulignent que l'HTAP n'est pas homogène au sein des connectivites et que la positivité des anticorps anti-U1RNP est associée à un phénotype particulier avec une meilleure survie. Ils soulèvent la question importante de savoir si en miroir de l'homogénéité sérologique, on pourrait envisager une homogénéité thérapeutique basée non pas sur la connectivite mais sur la positivité des anticorps anti-U1RNP.

Pour faire suite à nos travaux, diverses perspectives sont envisagées.

Tout d'abord, au sein de notre équipe de recherche nous étudions les liens entre auto-immunité et fibrose. En particulier, nous nous intéressons à la place du système humoral dans la physiopathologie de la ScS. Lors de mon Master Recherche puis durant cette Thèse, nous avons mis au point une technique d'identification d'auto-anticorps : l'immunoprotéomique bidimensionnelle en fluorescence (IPBDF) (Dutoit-Lefèvre et al., 2015). Durant la procédure, le substrat protéique est soumis à une iso-électrofocalisation permettant la séparation des protéines selon leur point isoélectrique. Puis une deuxième séparation a lieu, cette fois selon le poids moléculaire, à l'aide d'une électrophorèse. On réalise enfin un « immuno-blot » grâce au transfert des protéines depuis le gel vers une membrane, suivi de la réalisation des hybridations primaire (sérum du sujet ou anticorps sélectionné) et secondaire (anticorps anti-IgG humaines couplés à une peroxydase pour la révélation des complexes antigènes-anticorps sériques). Cette technique permet l'analyse des réactivités sériques d'un sujet grâce à l'acquisition simultanée de trois différentes cartes (**Figure 8**) :

- La carte protéique : le substrat (extrait de cellules Hep2) étant marqué par un fluorochrome
- La carte des réactivités dites « calibratrices » : il est possible de choisir des anticorps dirigés contre des cibles sélectionnées et d'analyser leurs réactivités
- La carte des réactivités sériques : révélation des complexes antigènes-anticorps sériques.

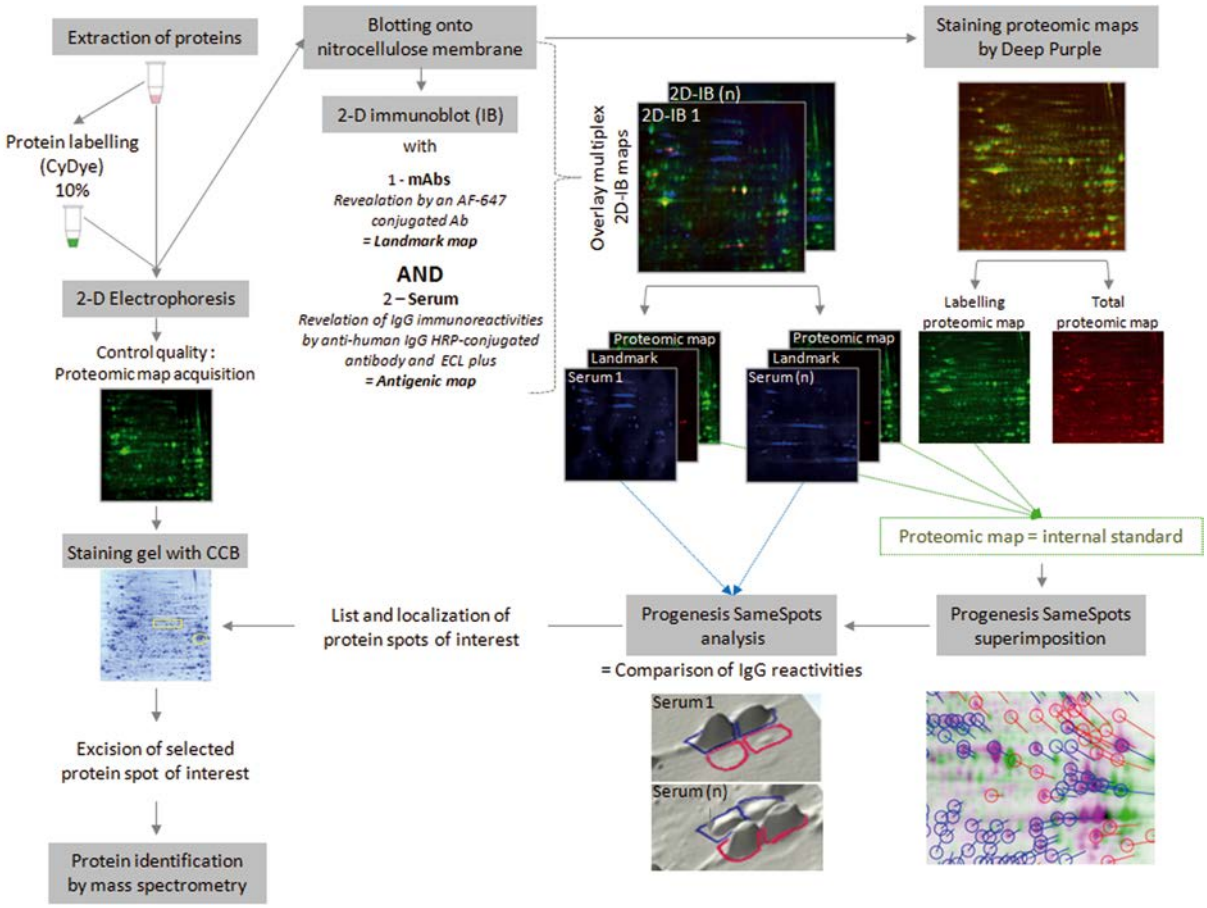


**Figure 8 :** Etapes de la technique d'immunoprotéomique bidimensionnelle en fluorescence (IPBDF), d'après Dutoit-Lefèvre et al., 2015

Grâce à une analyse informatique et humaine, il est possible de prendre en compte les déformations de gel inhérentes à la technique. Ainsi, on peut obtenir une superposition fiable des profils protéiques entre eux, suivie automatiquement d'une superposition des profils de réactivités. Cette méthode permet donc l'alignement des cartes de réactivité sélectionnées et leur comparaison afin de déterminer les spots de réactivités identiques ou discriminants entre les groupes.

Quarante gels ont donc été réalisés (16 patients avec dcSSc dont 8 avec anticorps anti-Scl70, 16 patients avec lcSSc dont 8 avec anticorps anti-centromère, 8 sujets sains). L'analyse informatique a mis en évidence 1363 spots protéiques, 527 ont été modifiés manuellement. L'alignement des cartes protéiques a été validé grâce à l'utilisation des anticorps « calibrateurs ». Sur les 1363 spots protéiques, 729 ont été

identifiés comme « antigéniques », c'est-à-dire porteurs d'une réactivité sérique au moins une fois. Les cartes de réactivités sériques de chacun des sujets ont ensuite été comparées deux à deux au sein des différents groupes constitués. Nous avons ainsi pu obtenir une carte de réactivité « globale » dans chacun des groupes. Puis chaque carte de réactivité individuelle a été comparée à la carte de réactivité globale du groupe concerné afin de déterminer la fréquence de présence de réactivité pour chacun des spots protéiques. Nous avons sélectionné 78 spots protéiques qui nous semblaient discriminants entre les groupes, permettant l'identification en spectrométrie de masse ORBITRAP de 392 protéines. Dix protéines ont été sélectionnées sur la base de leur pouvoir discriminant, de leur fréquence d'identification dans les spots protéiques et des données de la littérature. Nous cherchons actuellement à confirmer ces réactivités à l'aide d'une deuxième technique sur une population plus grande.

Ensuite, nous souhaitons étudier le rôle pathogénique potentiel des auto-anticorps. En effet, bien que les auto-anticorps représentent des biomarqueurs diagnostiques et pronostiques d'intérêt, leur rôle pathogénique demeure relativement peu connu. Il a été récemment montré que les Ig de patients atteints de ScS induisent une croissance et un état pro-fibrosant des cellules musculaires lisses vasculaires par le biais du récepteur de l'EGF ainsi que des modifications de l'expression des gènes pro-fibrosants (Arts et al., 2014). Nous déterminerons si les immunoglobulines des patients induisent un profil pro-fibrosant sur les fibroblastes dermiques normaux. Dans ce but nous mettrons en culture des fibroblastes normaux en présence d'IgG purifiées de patients ou de sujets sains. Le rôle des Ig purifiées sur le profil pro-fibrosant des fibroblastes sera étudié par différentes méthodes : étude de la prolifération cellulaire par RT-qPCR, test de migration des fibroblastes, mesure de la production de collagène par le dosage de l'hydroxyproline, étude de l'expression génétique impliquée dans la

fibrogénèse par RT-qPCR, étude des cytokines pro-inflammatoires par ELISA et étude protéomique par chromatographie liquide couplée à la spectrométrie de masse. Si les Ig des patients induisent un profil profibrosant, cela permettra de mieux comprendre la physiopathologie de la ScS et renforcera le rationnel quant à l'utilisation des traitements immunomodulateurs dans cette pathologie.

Au total, ce travail de Thèse apporte de nouveaux éléments pour la compréhension de l'hétérogénéité de la ScS, en suggérant que les marqueurs immunologiques tels que les auto-anticorps sont associés à des phénotypes cliniques particuliers, et ouvre de nouvelles perspectives à la fois sur le plan fondamental et appliqué.

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**Date de Soutenance : 20 septembre 2017 à 14h**

**Titre de la Thèse : Les auto-anticorps : marqueurs immunologiques de l'hétérogénéité de la sclérodermie systémique**

**Thèse – Doctorat d'Université – Sciences de la Vie – Immunologie - Lille 2017**

**Mots-clés : sclérodermie systémique – hétérogénéité – auto-anticorps**

## Résumé :

La sclérodermie systémique (ScS) est une connectivite associant atteinte vasculaire, auto-immunité et fibrose. Cette pathologie est associée à une morbi-mortalité importante, et les ressources thérapeutiques sont limitées. La physiopathologie de la ScS n'est que partiellement connue, mais il apparait que les liens entre le système immunitaire et la fibrose sont étroits. Ainsi, la ScS peut être considérée comme un modèle prototypique d'étude des liens immunité-fibrose. Il s'agit d'une maladie hétérogène, c'est-à-dire que les phénotypes cliniques présentés par les patients sont variables, rendant complexe l'établissement d'une classification des patients en groupes homogènes. Mieux comprendre cette hétérogénéité est un préalable indispensable à la constitution d'endotypes, permettant l'étude des mécanismes physiopathologiques propres à chacun d'entre eux. L'objectif de cette Thèse a été de mieux appréhender cette hétérogénéité clinique et d'étudier la place des marqueurs immunologiques, en particulier les auto-anticorps, en tant que biomarqueurs de cette hétérogénéité.

Le premier travail a été une classification sans a priori des patients de la cohorte européenne EUSTAR (European Scleroderma Trials and Research Group) par une analyse en cluster sur 24 variables sélectionnées (atteintes cliniques, auto-anticorps). Deux puis 6 groupes de patients homogènes ont été obtenus, dont la survie était significativement différente. Ce travail a suggéré qu'il existait des groupes homogènes de patients au-delà de la dichotomie historique forme cutanée diffuse vs. limitée. La présence d'atteintes viscérales et le statut des auto-anticorps apparaissent comme des éléments importants dans la constitution des groupes.

Le deuxième travail s'est intéressé au dosage des chaines légères libres sériques (serum free light chain : SFLC) dans la ScS. Le taux de SFLC est plus élevé chez les patients ScS que chez les contrôles et est associé à des paramètres de gravité de la maladie tels que le score de Rodnan, les scores d'activité, les pressions pulmonaires et la DLCO. Cette étude apporte des arguments supplémentaires pour évoquer la participation active des lymphocytes B à la physiopathologie de la ScS.

Nous avons ensuite réalisé une estimation de la prévalence des anticorps anti-ARN polymérase de type III dans notre cohorte de patients avec ScS avant d'inclure ces données dans une revue systématique avec méta-analyse. Ce travail a montré que la prévalence de ces anticorps était hétérogène entre les centres. Les facteurs potentiels pouvant expliquer une partie de cette hétérogénéité sont des facteurs géographiques, suggérant l'implication de facteurs génétiques et/ou environnementaux.

Le dernier travail a été dédié à l'hypertension artérielle pulmonaire (HTAP) des connectivites, en particulier de la ScS. A partir d'une cohorte de patients provenant du centre de référence de l'HTAP du Royaume-Uni, les anticorps anti-U1RNP ont été analysés en tant que marqueur pronostique. Ces anticorps sont associés de façon significative à une meilleure survie des patients avec connectivite et HTAP. Dans la ScS, on observe une tendance vers une meilleure survie également chez les patients porteurs de ces anticorps.

Les auto-anticorps constituent donc des biomarqueurs diagnostiques et pronostiques puissants dans la ScS. Ils permettent de mieux cerner l'hétérogénéité de cette pathologie, et devraient probablement être intégrés dans les futures classifications. Leur rôle pathogénique reste encore à démontrer. Les perspectives de notre travail sont d'identifier de nouveaux auto-anticorps et d'étudier leurs effets sur le fibroblaste, cellule effectrice centrale de la fibrose.

**Composition du Jury :**

**Président : Professeur David Launay**

**Rapporteurs : Professeur Brigitte Granel, Professeur Thierry Martin**

**Examinateur : Docteur Laurence Michel**

**Directeur de Thèse : Professeur Sylvain Dubucquoi**