Screening and Risk Assessment of Systemic Sclerosis Associated Pulmonary Arterial Hypertension

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Declaration

I, Hossam Fayed, confirm that the work presented in this thesis is my own. Information that has been derived from other sources has been clearly indicated and referenced within my thesis. The thesis presented is the one on which I expect to be examined.

Signed

Printed Name
Hossam Fayed
Date 23/1/2021

To Dr Moustafa Fayed

I hope I made you proud

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Abstract

Pulmonary arterial hypertension (PAH) is a common complication of systemic sclerosis (SSc) with high morbidity and mortality. There is potential benefit of early diagnosis and initiation of therapy, thus a strong rationale for active screening programs. Several screening algorithms have been suggested including the DETECT algorithm that was introduced in 2013.

Regular risk assessment of PAH is necessary to reassure those at lower risk, to assess the efficacy of intervention and to identify those where escalation of therapy is required. Comprehensive prognostic evaluation and risk assessment is therefore recommended in the most recent ESC/ERS guidelines for PH. The REVEAL study group also proposed a renewed REVEAL 2.0 risk prediction calculation tool. Most validation work to date of these models has focused on the idiopathic pulmonary hypertension population or mixed populations including modest numbers with connective tissue disease related PAH.

Microvesicles (MVs) are submicron vesicles that are released by different cell types into the circulation when they become activated or undergo apoptosis. The role of MVs as a potential biomarker in PH (for screening and risk assessment) has been previously proposed.

The hypothesis of this work is that there remains a potential to improvise on the current screening and risk assessment models for PAH in SSc patients, and that there

is a potential role of MVs in screening and risk assessment of PAH in patients with SSc.

The first objective was to try to demonstrate the impact of the current screening program on the early detection of PAH in SSc patients. The second objective was to validate, examine the reproducibility and to compare the different current risk assessment models in patients with SSc-PAH. The final objective was to examine the role of MVs in screening and risk stratification of SSC PAH.

We demonstrated that despite screening there has not been significant change in the demographics or the haemodynamic and risk profiles of patients diagnosed with SSc-PAH in the Royal Free pulmonary hypertension unit between 2006 and 2018. On comparison of different risk assessment tools driven from the ESC risk assessment and REVEAL models in the same group of patients, we found that all risk scores have clear discriminatory value at both baseline and on first follow up of SSc-PAH patients. The full REVEAL 2.0 risk score was the most accurate method in prognostication particularly at baseline as demonstrated by the highest AUC. Inclusion of non-modifiable factors does, however, appear to limit its value for serial risk assessments. The ESC based models not only provide a similar (albeit slightly less) discriminatory value, but also provide feedback on the impact of response to therapeutic intervention and may be the most useful in clinical practice.

In the fourth chapter, we found that in the recruited 33 patients with SSc-PH and 26 patients without PH, MVs were not shown to be effective biomarkers for the early detection of SSc-PAH. Total MV and the characterised MV subpopulations from

endothelial, smooth muscle or platelet origin failed to differentiate PH from non-PH in the studied population, irrespective of the site of sampling. The role of MVs in risk prediction was also limited in the studied population. However, the study maybe limited by the relatively more advanced SSc disease in comparison to the typical population in other screening studies. An interesting finding in this study was the significant gradient between the peripheral arterial and peripheral venous counts of some of the MVs (the total, PECAM1+/CD42- and ICAM1+/PECAM1-) in the SSc-PH patients. To our knowledge, this is the first report of such a finding.

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Abbreviations

6MWD	6-minute walking distance
ACEi	Angiotensin converting enzyme inhibitors
APS	Antiphospholipid syndrome
AUC	Area under the curve
BMI	Body mass index
BNP	Beta natriuretic peptide
CAD	Coronary artery disease
CI	Cardiac index
CKD	Chronic kidney disease
CMR	Cardiac magnetic resonance
CO	Cardiac output
COMPERA	Comparative, Prospective Registry of Newly Initiated Therapies for Pulmonary Hypertension
CRP	C reactive protein
CTD	Connective tissue disease
CTD-PAH	Connective tissue disease associated PAH
CTEPH	Chronic thromboembolic pulmonary hypertension
CW	Capillary wedge
CW	Capillary wedge occluded pressure
DETECT	Early, Simple and Reliable Detection of Pulmonary Arterial Hypertension in Systemic Sclerosis
DLCO	Diffusion lung capacity
DM	Diabetes mellitus
dSSc	Diffuse systemic sclerosis
eGFR	Estimated glomerular filtration rate
EGPA	Eosinophilic granulomatosis with polyangiitis
ERA	Endothelin receptor antagonist
ERS	European respiratory society
ESC	European society of cardiology
estPASP	Estimated pulmonary artery systolic pressure-estpasp
FA	Femoral artery
FEV1	Forced expiratory volume in 1 second
FV	Femoral vein
FVC	Forced vital capacity
GP9	Glycoprotein IX
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HRCT	High resolution CT scanning
HTN	Systemic hypertension
ICAM1	Intracellular cell adhesion molecule
ICD	Implantable cardiac defibrillator
ILD	Interstitial lung disease
IPAH	Idiopathic pulmonary hypertension
IVSD	Inferior vena cava diameter
lSSc	Limited systemic sclerosis
LV	Left ventricle
mPAP	Mean pulmonary artery pressure
MVs	Microvesicles

NG2	Neural glial antigen 2
NT-Pro BNP	N terminal pro beta natriuretic peptide
PA	Pulmonary artery
PAH	Pulmonary arterial hypertension
PAP	Pulmonary artery pressure
PDGFRβ	Platelet-derived growth factor receptor beta
PECAM1	Human platelet endothelial cell adhesion molecule
PFT	Pulmonary function test
PH	Pulmonary hypertension
PPP	Platelet poor plasma
pre-PH	Precapillary pulmonary hypertension
PM	Polymyositis
PS	Phosphatidylserine
PVOD	Pulmonary veno-occlusive disease
PVR	Pulmonary vascular resistance
RA	Rheumatoid arthritis
RAP	Right atrial pressure
REVEAL	Registry to Evaluate Early and Long-term PAH Disease Management
RHC	Right heart catheterisation
ROC	Receiver operating characteristic
RV	Right ventricle
SD	Standard deviation
SLE	Systemic lupus erythematosus
sPAP	Systolic pulmonary artery pressure
SSc	Systemic sclerosis
SSc-PAH	Systemic sclerosis associated pulmonary arterial hypertension
SSc-PH	Systemic sclerosis associated pulmonary hypertension
SVO2	Mixed venous oxygen saturation
TV	Tricuspid regurgitant jet peak velocity
V/Q	Ventilation and perfusion
WHO-FC	WHO functional class assessment
WSPH	World Symposium in Pulmonary Hypertension
WU	Wood’s units

Chapter 1 An introduction to Pulmonary Hypertension in Systemic Sclerosis

Pulmonary hypertension (PH) can be simply defined as elevated pressures in the pulmonary circulation and can be caused by multiple pathologies [1] [2]. By contrast pulmonary arterial hypertension (PAH) is relatively rare affecting around 1/15,000 of the UK population [3] and is a term used to describe patients with precapillary PH due to vasculopathy of the precapillary pulmonary arterioles. PH is also a common complication of systemic sclerosis (SSc) and it is a leading cause of SSc-related morbidity and mortality. A problem that clinicians often face is the late diagnosis of SSc related PH (SSc-PH) that can contribute to the higher morbidity and mortality related to this condition. Furthermore, PH different types often coexist and can contribute to the worse outcome.

1.1 Current definition and proposed changes

PH has been defined as a mean pulmonary artery pressure of greater than or equal to 25mmHg (mPAP $\geq$ 25mmHg). Pulmonary Arterial Hypertension (PAH) is the term used to describe patients with precapillary PH due to vasculopathy of the precapillary pulmonary arterioles. However, it is now recognised that this figure (25mmHg) was arbitrarily chosen as clearly distinct from the normal level (mPAP 14mmHg). Thus, important changes in the definition and subtyping of PH was suggested at the 6th World Symposium in Pulmonary Hypertension (WSPH) in 2018, some of which will have profound implications for epidemiology and indeed interpretation of previous work in this field [4]. Over the past few years evidence has accumulated to suggest that

an mPAP >20mmHg is pathological, and it is now proposed to change the definition of PH to a mPAP >20mmHg, and that of precapillary pulmonary hypertension(pre-PH), to a mPAP >20mmHg with a pulmonary vascular resistance (PVR) of ≥3 Wood's units (WU).

The evidence supporting this change comes from diverse sources, relying heavily on evidence gathered in connective tissue disease (CTD) associated PAH (CTD-PAH). First, analysis of the normal pulmonary pressures in over 1000 individuals has shown that the normal mPAP is 14±3.3mmHg, so 20.6 mmHg represents a PA pressure 2 standard deviations above normal [5]. Second, two studies have demonstrated a substantial risk of progression of PH among patients with scleroderma and an mPAP >20mmHg, with around 1/3 progressing to an mPAP ≥25mmHg over 3 years [6 7]. Third, patients with a substantial burden of residual clot in the pulmonary arteries have significant effort limitation despite "normal" resting pressures and are improved by surgery or balloon pulmonary angioplasty [8].

The implications of this new definition may not be straight forward to interpret. In the DETECT study (Early, Simple and Reliable Detection of Pulmonary Arterial Hypertension in Systemic Sclerosis) of 466 patients at increased risk of PH, 145 (31%) had an mPAP ≥ 25mmHg (of whom 87 had PAH). Of the 321 with a mPAP < 25, 79 had an mPAP of 21 – 24, potentially expanding the number with PAH substantially [9]. However, on analysing this population, lung disease or left heart disease was present in over half the patients and therefore the most likely explanation; among the remainder only 36 had an mPAP of 21 – 24mmHg of whom only 5 had a PVR ≥ 3WU.

To date this is simply a consensus proposal and has yet to be formally adopted by any guidelines committee, so for the foreseeable future, the definition of PH remains a mPAP ≥25mmHg, with this recommendation as simply a potential cause of controversy.

In WSPH, considerable clarification on the issue of left heart disease associated PH has also been proposed. It is now clear that patients with PH due to left heart disease are very unlikely to benefit from advanced therapies and in some cases are clearly worsened by such treatment [10]. In recognition of this it is no longer recommended that referral to a PH centre should be undertaken where the diagnosis is clear [10]. This includes most patients with reduced systolic function (ejection fraction < 40%), significant valve disease and heart failure with preserved ejection fraction (as evidenced by substantial left ventricular hypertrophy, left atrial enlargement or doppler parameters, or is associated with a clear clinical phenotype such as obesity, hypertension or diabetes patients. It is, however, recognised that in the setting of CTD, modest left heart abnormalities can co-exist with pulmonary vasculopathy [10].

Less progress has been made on the issue of PH associated with lung disease; here it is merely recognised that in the presence of substantial disease, mild PH should not be treated with advanced therapies [11]. The recommendation is that in the setting of lung disease in patients with CTD, this should either be mild (<20% lung fibrosis, < 5% emphysema by volume) or where more severe, this should be documented as stable. While there has to be clear worsening of effort tolerance and that the mPAP should exceed 35mmHg with a PVR of ≥ 4WU, before PAH treatment is trialled. In general,

we can expect a haemodynamic response, without clinical benefit if lung PH is being treated rather than PAH [11].

One point of relevance to CTD-PAH is the importance of gas transfer (DLCO or TLCO) in identifying patients with pulmonary veno-occlusive disease (PVOD) and lung disease associated PH [11]. A DLCO of $\leq$ 50% is strongly associated with both these conditions, thus helping to differentiate PAH from PVOD and lung disease associated PH. Unfortunately, since gas transfer is also reduced in CTD PAH, especially SSc-PAH, this threshold is not useful in CTD-PAH. Among this population a gas transfer $\leq$30% is, however, more suggestive of a process other than a vasculopathy driving the elevation of pulmonary pressure [12].

1.2 Epidemiology of Scleroderma-associated Pulmonary Hypertension (SSc-PH)

The reported prevalence of PH in scleroderma is variable dependent on the method used in diagnosis. While the more recent studies all have cardiac catheterisation in their diagnostic criteria, more historical cohorts were less rigorous and just relied on echocardiography to make the diagnosis, thus bringing into question the accuracy of the screening approach used. Other factors that can make the collective interpretation difficult is including symptomatic patients only or a more active screening approach and the duration of scleroderma in the population studied. Recent studies have all included right heart catheter for diagnosis, however, the screening barrier has varied as has detailed reporting of the compliance with screening protocol. As discussed above, the change in definition of PH at the recent WSPH will likely only have a modest impact on the prevalence.

Study	Population	Disease Duration	Screening Protocol	lSSc PH %	dSSc PH %	lSSc PAH %	dSSc PAH %
Vonk (2009) [13]	654	9.1yr	Various	10%		?	?
Avouac (2010) [14]	1165	13yr	TV>2.8m/s DLCO<50% Dyspnoea ?Cause	5.2%	6.3%	4.5%	1.4%
Nihtyanova (2014) [15]	398	13yr	TV>2.5m/s; DLCO<50% or fall 20%; Dyspnoea ?Cause	24%	18%	17%	10%
Coghlan* (2014) [9]	408	11.4yr	RHC			36%*	16%*
Vonk (2009) [13]	1636	14.6yr	sPAP > 40; DLCO<50% with FVC>85% or fall >20%; Dyspnoea ?cause			12.7%	10.1%

Table 1.1 Prevalence of SSc PH and PAH in recent studies.

*Coghlan et al (DETECT study) preselected high risk subgroup (DLCO < 60%), all patients were catheterised – the whole study comprised 466 patients, but the detailed report excluded 12% of patients with PH but not PAH – the breakdown in terms of limited versus diffuse was not reported.

**Morrisroe et al (ASIG) reported the prevalence of PH at 14.2% without a breakdown by disease subtype.

Larger more recent studies suggest a prevalence of 10% or more among populations with a disease duration exceeding 10 years. Both Nihtyanova et al [15] and Morrisroe et al [16] have reported the incidence of 1 – 2% per annum of PH among their populations. PAH seem to be more common in the limited SSc (lSSc) population with slightly more PH with no PAH in those with diffuse SSc (dSSc). In the DETECT study using catheterisation in all patients is clearly the only completely reliable study to date, although two thirds of SSc patients had a DLCO >60% and were thus excluded from the study. This threshold was chosen on the basis of the findings in the Itineraire study that PAH was much less frequent in that population (prevalence 1.2% v 8%) [17].

1.3 Screening for PAH in SSc patients

It has been reported that up to 20% of SSc patients can be asymptomatic at the time of PAH diagnosis (Hinchcliff, Fischer, Schiopu, & Steen, 2011 and Chung et al., 2014). However, PAH is a major cause of morbidity and mortality in SSc, with a meta-analysis study reporting pooled 1-,2- and 3-year survival in incident SSc PH to be 81%, 64% and 52%, respectively [20].

An early systematic detection of PAH in SSc patients would allow early intervention that could potentially lead to a reversing of the pathophysiological process or at least a slowing of its progression. The latter is implied by a study who reported improved survival in SSc-PAH patients that were diagnosed through an active screening program compared to those in routine clinical practice [21]. The EARLY study also reported potential benefit of bosentan treatment in patients with milder PH symptoms[22]. The significant prevalence rate of PH in SSc patients, lack of symptoms

earlier on in the disease process, the high morbidity and mortality from PH and the potential benefit from early intervention with a more widely available therapeutic options, provide a strong rationale for active screening programs.

Several screening algorithms that combine clinical, pulmonary function tests (PFTs) and chemical biomarkers in addition to echocardiogram have been suggested. The ESC/ERS 2009 guidelines provide an algorithm which was updated in the latest 2015 version [2]. The DETECT algorithm was put forward in 2013 following a large prospective and multicentre study in SSc patients in a cohort with higher risk of PAH (>3 years of SSc diagnosis and diffusion lung capacity (DLCO) <60% predicted) [9]. Another cohort analysis of 419 patients with SSc at risk for PAH reported that the use of echo alone failed to diagnose PAH in up to 31 percent of patients, over half of whom were captured by PFTs (pulmonary function tests) [23]. The combination of echo and PFTs improved the negative predictive value complementing each other's role in diagnosis of PAH [23].

It is widely accepted that screening should start with PFTs; If DLCO 60 – 80%, it is recommended that a multimodal approach is taken combining FVC/DLCO > 1.8 and/or NT-proBNP if more than double normal. For those with normal DLCO, echocardiography criteria using the ESC/ERS table (combining tricuspid velocity or estimated pulmonary artery systolic pressure-estPASP) with evaluation of the right heart for signs of elevated afterload [24].

Where the DLCO is $\leq$60%, the evidence-based approach is to use the DETECT algorithm, which combines independently predictive parameters to optimise

identification of those with PAH. For this to work effectively, at least 5 of the following 6 parameters need to be measured: ECG axis; antibody status; presence or absence of telangiectasia; serum urate; NT-proBNP and FVC/DLCO. The value of repeating the DETECT score on multiple occasions is not known, so the default at present is to monitor on follow up and to assess the FVC/DLCO and NT-proBNP levels as above. The consensus is to monitor the variable aspects of the DETECT protocol (ECG axis, urate, NT-proBNP and DLCO) for significant changes and re-catheterise if these suggest significant disease progression [25].

The main drawback of these screening methods is the need for a higher number of invasive investigations (i.e. right heart catheterisation- RHC), which is not without risk, especially if this has to be repeated on frequent intervals (e.g. yearly). Hence, there is a need for a non-invasive biomarker that can add to the positive predictive value of the screening tool, increase the compliance to the screening tool both by clinicians and patients while minimising the risk of using an invasive method.

1.4 Diagnosis and classification of PH in SSc

The diagnosis of PH usually begins following clinical instinct and suggestive changes in echocardiographic features. The purpose of the diagnostic workup when PH is suspected is to confirm the presence of PH and then to classify according to aetiological, functional and haemodynamic parameters. The diagnosis of PAH or chronic thromboembolic PH (CTEPH) should be established in specialist centres before consideration of specific targeted therapies.

Typical symptoms of PH include progressive exertional breathlessness and lethargy, and in more advanced cases, syncope or pre-syncope on exertion due to reduced cardiac output and angina with right ventricle (RV) hypertrophy. As RV failure advances ankle oedema, abdominal swelling due to hepatomegaly and ascites followed by cachexia, develop in patients. As these symptoms are non-specific the diagnosis of PH is often delayed or missed completely. Therefore, PH should be considered as soon as more common causes like left heart and lung disease are ruled out. This is even more important in patients who have risk factors associated with the development of PAH, namely a family history, CTD (especially scleroderma), HIV, portal hypertension or history of drug or toxin intake that is known to cause PH. General physical signs which generally only appear later in the course in advanced pulmonary hypertension, include raised venous pressures and hepatomegaly (can be pulsatile) with tricuspid regurgitation, peripheral oedema and ascites due to right ventricular failure and cold peripheries due to reduced cardiac output.

PH is most often suspected on the basis of echocardiography. Typically, PH can be considered likely if the tricuspid regurge velocity (TV) exceeds 2.7m/s, which typically gives an estimated systolic pulmonary artery pressure 35 – 40mmHg). Despite this prediction, one has to understand the limitation of echocardiography in diagnosis of PH as it can lead to both under or overdiagnosis[2]. While invasive cardiac catheterisation (RHC) remains the gold standard in diagnosis of PH, further tests are almost always needed to further classify PH into the different groups including PAH and others (table 1.2) [2].

Among the tests required to fully differentiate the different groups:

- Assessment of the pattern and severity of pressure elevation and its relationship to flow (cardiac catheterisation with flow determined by thermodilution, direct Fick or cardiac magnetic resonance (CMR)).
- Cardiac imaging including echocardiography or CMR.
- Lung parenchyma imaging with high resolution CT scanning (HRCT).
- Lung perfusion imaging to rule out thromboembolic disease (ventilation/perfusion scanning and/or pulmonary angiography);
- Full lung function testing (including volumes and gas transfer);

Other tests may include assessment for portal hypertension and serological analysis for HIV, liver disease, autoimmune screen and others if suspected (e.g. for schistosomiasis).

Thus, PH diagnostic work up to now is extensive as it requires ruling out all the other potential pathologies that can cause PH before one can reach a reliable diagnosis. That said, there is often an overlap between two or more of these pathologies.

In SSc, almost all the different types of PH can exist (table 1.2). PAH, or PH associated with left heart or lung disease are all common. Thus, the above-mentioned diagnostic work up still applies in order to classify PH associated with SSc. In most studies SSc-PAH has been reported to be the most common form of PH but there are exceptions, with left heart disease [26] or PVOD reported as more common in other studies [27]. On the other hand, lung disease can be under-reported, so its true incidence is unknown.

An observation noted is that PAH is more common in SSc patients with severe PH while mild PH is more likely to have no pre-capillary component [7].

Left heart disease is thought to be extremely common in SSc [28], but this is largely dependent on the diagnostic method used. Fluid loading for example, is a highly sensitive yet unproven technique [26], and if used, it is possible to attribute significantly higher number of SSc PH as post-capillary disease. When more widely accepted assessments are used, less than 15% of SSc PH appears to be due to left heart disease[9]. PVOD is also thought to be more common in SSc patients (if compared to other forms of PH). For example, CT data from patients referred to transplant after failing therapy, had a prevalence approaching 50% in people that have been labelled as having SSc-PH [27] and about 15% in another study for those who have been labelled as SSc-PAH [29].

The extent of lung disease associated PH in SSc is possibly under reported because the less likely of this being diagnosed in people with extensive lung disease including lung fibrosis and obstructive airway disease including small airway disease. A standard approach is to consider anyone with a DLCO of < 40% as having a lung disease contribution to PH. With respect to pulmonary fibrosis, < 20% pulmonary fibrosis extent on HRCT is thought not likely to cause PH [30], unless of course it is associated with emphysema [31], and that if the extent of emphysema exceeds 5%, then it is difficult to label someone as having a pure vasculopathy.

Unlike in antiphospholipid syndrome or systemic lupus erythematosus (SLE), CTEPH is relatively uncommon in SSc. Table 1.2 outlines that different forms of PH associated

with CTD in general, but with specific reference to the various forms of PH associated with SSc.

Finally, the recommended new definition of PAH (mPAP 21 – 24, with a PVR $\geq$ 3WU) may have implication on the distribution of the different groups of PH in SSc. About 30% of this previously labelled as “borderline PH” have been demonstrated to progress to standard PH over 5 years [6]. However, a significant proportion will progress to Group 2 or 3 PH rather than PAH, which may cause concerns in the management of this population [7].

WHO group	Group 1	Group 2	Group 3	Group 4	Group 5
Type	PAH	Post-capillary PH	Hypoxia or lung disease associated PH	Thrombo-embolic PH	Uncertain mechanism or multifactorial
Pathology	Vasculopathy affecting pulmonary arterioles (<200μm)	Elevated left atrial pressure with/without secondary pulmonary venous and arterial changes	Alveolar destruction or hypoxic vasoconstriction with/without secondary vasculopathy	Vascular occlusion usually intraluminal	Multiple contributory pathologies
Typical	SSc, SLE,	RA, SSc	Sarcoid, DM, PM, SSc	APS, SLE,	Sarcoid
Unclear frequency	MCTD, Sarcoid, DM/PM, Sjogren's	DM/PM, EGPA		Behçet's disease	SSc
Rare	RA			SSc	

Table 1.2 Diagnosis and WHO classification of PH in connective tissue disease.

SSc systemic sclerosis, SLE systemic lupus erythematosus, RA rheumatoid arthritis, PM polymyositis, DM dermatomyositis, APS antiphospholipid syndrome, EGPA eosinophilic granulomatosis with polyangiitis.

1.5 Management of SSc PH

Different types of SSc PH are associated with significantly worse morbidity and mortality. Regardless of the SSc PH subtype, supportive measures are usually the first step in the management. This can range from diuresis to manage fluid status, oxygen therapy (hypoxic patients in particular), pulmonary rehabilitation to improve the effort tolerance and psychological support [2]. Other important supportive measures have to be tailored in special circumstances. For example, in pregnancy advice on contraception earlier in the disease process and even consideration of termination in severe PH. Peri-operative management of PH patients should aim to ensure optimal management of right ventricular function, using spinal anaesthesia where possible, and often with invasive monitoring of central venous pressure [32,33]. Early mobilisation post operation is essential with aggressive management of any complications, especially infection.

1. SSc PAH

The role of advanced therapies in SSc PAH has been reported to have less beneficial response if compared to idiopathic pulmonary hypertension (IPAH) [34], with less improvement in 6-minute walking distance (6MWD) and quality of life benefits and more frequent side effects. In a meta-analysis published by Rhee and colleagues looking at individual patient level data from the pivotal trials, the magnitude of benefit was reported to be lower in CTD PAH, although benefit in terms of 6MWD was the same in SSc PAH as in IPAH [35]. The difference being that among those diagnosed with IPAH, patients experienced an increase in 6MWD with treatments,

while in SSc PAH, those receiving placebo still had a substantial reduction in 6MWD. However, more recent studies came to slightly different conclusions as the magnitude of benefit, in terms of reduction of morbidity/mortality events or clinical failure end-points, was essentially similar between the CTD/SSc PAH and the IPAH patient populations [36]. Among those receiving combination therapy in these clinical trials, event rates were almost identical between IPAH and CTD/SSc PAH populations. Further, while adverse event rates were higher in the CTD/SSc PAH patients there was no disproportionate impact of therapy on these rates. In the GRIPHON trial, [37] while those on Selexipag had a significant burden of prostanoid side effects, the relative burden of side effects was identical between active treatment and placebo patients whether they had IPAH or CTD PAH. In the AMBITION trial [38], the relative side effect burden was equally reduced in the combination therapy arm (Ambrisentan plus Tadalafil), irrespective of the underlying diagnosis. A comprehensive list of pivotal trials where sub-analysis for the SSc PAH population has been performed and was recently published by our group [39].

Therefore, it is safe to conclude that therapy is beneficial in SSc PAH and that combination therapy is necessary in most patients with SSc PAH as the majority of patients have an intermediate or high-risk status.

2. Post Capillary SSC PH

The priority in the management of postcapillary PH is in general on reversible causes. For example valvular heart disease should be managed surgically where

appropriate[40]. Aortic stenosis in SSc often progresses rapidly [41] and valve replacement significantly reduces left heart filling pressures.

Heart failure with preserved ejection fraction (HFpEF) is more commonly associated with post capillary PH than with the reduced ejection fraction (HFrEF). Diuresis has an important and proven role in its management [42,43] while NT-proBNP is a useful biomarker to monitor the response to an intervention. Implantable PA pressure monitoring provides a useful monitoring tool to optimise left sided filling pressures [44]. Additionally, the standard management of HFrEF including ACE inhibitors and beta blockers should be pursued (carvedilol tends to be tolerated even in the presence of Raynaud's phenomenon).

SSc often affects the myocardium and can cause persistent troponin positive 'myocarditis' that can lead to systolic heart failure in a small proportion of SSc patients. Immunosuppression with cyclophosphamide or mycophenolate has a role in the management of such patients[45].

In the presence of a significant 'scar' burden (regional late gadolinium enhancement on CMR) then the implantation of implantable cardiac defibrillator (ICD) (+/- cardiac resynchronisation therapy in the presence of wide QRS complex on the ECG and severe LV systolic impairment) should be considered as sudden death is prominent in this population [46].

3. Lung PH in CTD

PH is most commonly associated with pulmonary fibrosis in SSc and emphysema can occur even in the absence of smoking in SSc [31]. Pulmonary fibrosis is frequently associated with PH in SSc and is associated with a poorer outcome [11]. The mainstay of management is mainly supportive with underlying management of hypoxemia and pulmonary rehabilitation as well as dealing with fluid overload.

Advanced therapies have no convincing evidence of efficacy. However, all randomised trials for management of PAH have included patients with mild lung disease [2] (e.g. FVC60-80% if no extensive lung disease on HRCT) and thus would have included patients with mild fibrosis, emphysema and combined fibrosis and emphysema. In the setting of lung PH there is some suggestion of benefit in terms of haemodynamics [11], but no convincing evidence of symptomatic or effort tolerance benefit. In advanced lung disease there is very little evidence for advance therapies and a clear risk of worsening hypoxemia. In lung fibrosis, there is clear evidence of worse outcomes[47] [48] and bosentan was clearly ineffective in idiopathic pulmonary fibrosis [49].

4. Pulmonary Veno-occlusive Disease (PVOD)

PVOD is a severe form of PAH that is characterised by very poor response to advanced PAH therapies and an extremely poor prognosis. SSc patients are known to have higher prevalence of PVOD [27] and the first suspicion of such a condition is when there is severe hypoxaemia or low gas transfer (<50%) [50]. Diagnosis is then confirmed by the HRCT signs of interlobular septal thickening, centrilobular ground glass

shadowing and lymphadenopathy [51]. Management usually focuses on diuresis, limiting advanced therapies (prostanoids in particular) and consideration of transplant [52].

1.6 Risk Assessment in SSc PH

Regular risk assessment is a cornerstone in the management of PH. The main advantages of risk stratification include the ability to identify those at highest risk and reassure those at lower risk, and to assess the efficacy of interventions with escalating of therapy as appropriate or referral for lung transplantation.

Comprehensive prognostic evaluation and risk assessment is recommended in the most recent ESC/ERS guidelines for PH [2]. The proposed multi-parameter approach includes clinical, exercise, biomarker, and haemodynamic components. Most of the proposed variables and cut-off values were based on expert opinion[2]. Low, intermediate and high risk were defined with predicted one-year mortality of <5%, 5-10% and >10%, respectively. These figures are crude estimates and were based mainly on variables identified in studies on patients with IPAH. Several groups have applied different methods to their registries in order to create a usable clinical tool for patient monitoring. The Swedish group [53], the COMPERA registry group[54] and the French group [55] used the proposed cut-offs and applied them to their respective registries but with a different calculation method. All these methods have shown good prognostication ability. Of particular relevance is the French system which is based on three non-invasive criteria (functional class 1 or 2, 6MWD > 440m and BNP

<50ng/L or proBNP <300ng/L). The presence of at least one low risk criterion is associated with an excellent 5 year survival [56].

Another popular risk assessment scheme is the original multiparameter score (the REVEAL score) which is very comprehensive and provided an accurate prognostication method [57]. However, it has been considered by some clinicians as too cumbersome for routine use and concerns have been raised that it included many components that were not amenable to modification by changes in therapeutic strategy (e.g. age, PAH subtype, co-morbidities). More recently, Benza and colleagues have proposed a simplified version of the REVEAL risk prediction calculation (REVEAL 2.0) and compared it head to head with European calculation methods on their registry [58].

SSc-PAH is phenotypically unique and carries one of the worst prognosis amongst different aetiologies of PAH, including IPAH and other forms of CTD-PAH. Most validation work to date has focused on the IPAH patient population or mixed populations including modest numbers with connective tissue disease related (CTD)-PAH. However, the John Hopkin's group applied the ESC/ERS risk calculation methods on their registry of 151 patients with SSc-PAH and demonstrated that an abbreviated version of the guideline risk assessment tool was accurate in predicting survival in one year in incident PH [59]. This particular study's main drawback was missing data particularly at follow up. The French group applied their method (the number of low risk criteria) on their respective registry of SSc PAH patients [1] and demonstrated only modest ability of baseline line data to discriminate risk at one year although was slightly better at the time of first follow up. There has been no specific work to date

to validate the REVEAL score in SSc PAH or to compare it to the ESC-ERS guidelines risk assessment tool.

1.7 Biomarkers in SSc PH and the potential role of microvesicles

Biomarkers have an important role throughout the different phases of management of PH, including screening, diagnosis and risk assessment. Several serum biomarkers have been developed and tested over the years including, for example, biomarkers for vascular dysfunction (e.g. asymmetric dimethylarginine [60], endothelin-1 [61] and von Willebrand factor [62]), markers of inflammation (e.g. C-reactive protein [63], interleukin-1 [64] and chemokines [65]). However, none of these biomarkers have made it to routine clinical practice except markers of myocardial stress (e.g. natriuretic peptides or cardiac troponins) and markers of secondary organ damage (e.g. serum creatinine) [66,67]. In particular, BNP/NT-Pro BNP has been proven to be highly valuable in daily practice, being well validated in predicting outcomes so is widely included in clinical trials and guidelines for risk assessment. Despite their usefulness, BNP/NT-Pro BNP are markers of right ventricular failure, so they are markers which show up late in the pathological process of PH.

Microvesicles (MVs) are submicron vesicles that are released by different cells into the circulation upon cell activation or apoptosis [68, 69]. MVs have membrane receptors and contain proteins that are related to the parent cells from which they were derived. They avidly expose phosphatidylserine (PS) on their membranes, and the calcium-dependent phospholipid, annexin V+ , which has high affinity for PS [68]. By

targeting cell specific antigens or combination of antigens, we can identify their cellular origins using flow cytometry.

The role of MVs as a potential biomarker in PH has been previously proposed. Amabile and colleagues found that levels of endothelial and leukocyte MVs were elevated in patients with precapillary PH in comparison to control subjects [70]. In addition, levels of PECAM(+) and VE-cadherin(+), but not E-selectin(+) endothelial MVs predicted hemodynamic severity of the disease. Further work by the same group also shown that there was an association of another endothelial subpopulation of MVs (CD62+) with adverse outcomes [71]. Similar findings were reported by Bakouboula and colleagues et al. who showed that elevated levels of endoglin+ (CD 105+) MVs were elevated in PAH patients compared with control subjects [72]. They also found higher levels in patients with more advanced disease (functional class III or IV and 6MWD <380m). More recently, a study found that patients with SSc-PAH had elevated levels of CD144+ endothelial MVs compared to either SSc patients with no PH or healthy controls [73]. Thus, MVs may be of clinical value as a screening tool in SSc patients and considered as potential biomarkers to indicate inflammatory status, extent of vascular remodelling and/or tissue damage, important pathophysiological features of PAH.

In work underpinning this research, Professor Clapp's team [74] has characterised by fluorescence-activated cell sorting (FACs), subpopulations of MVs staining with markers for endothelial cells (PECAM-1+/CD41-/PDGFRβ-), smooth muscle cells (PDGFRβ+/NG2+/PECAM1-), platelets (CD42a+) and leukocytes (e.g. CD16) in the

venous blood of patients with severe PAH. This suggests that MVs could therefore be a potential *in vivo* surrogate of vascular remodelling and inflammatory status.

1.8 Conclusion

SSc PH encompasses a varied and unique spectrum of pathologies with significant morbidity and mortality. Screening, diagnostic work up and risk stratification still poses several challenges to the practicing clinician and there is a room for improvement that potentially would improve the outcome from such a devastating condition. Novel biomarkers including microvesicles may have a role to play in screening and risk assessment.

1.9 Hypothesis and objectives

The hypothesis of this work is that there remains a potential to improvise on the current screening and risk assessment models for PAH in SSc patients, and that there is a potential role of MVs in screening and risk assessment of PAH in patients with SSc.

The first objective was to try to demonstrate the impact of the current screening program on the early detection of PAH in SSc patients. The second objective was to validate, examine the reproducibility and to compare the different current risk assessment models in patients with SSc-PAH. The final objective was to examine the role of MVs in screening and risk stratification of SSC PAH.

Chapter 2 Impact of Routine Screening on Detection, Severity and Outcome of Pulmonary Arterial Hypertension in Systemic Sclerosis.

2.1 Introduction

Pulmonary arterial hypertension (PAH) is a common complication of systemic sclerosis (SSc) with an estimated prevalence of 10-15% [17, 75, 76] and it is a leading cause of death [77, 78, 79]. It has been reported that up to 20% of SSc patients can be asymptomatic at the time of PAH diagnosis [18, 19]. However, PAH is major cause of morbidity and mortality in SSc. A meta-analysis reported pooled 1-,2- and 3-year survival to be 81%, 64% and 52% respectively, in incident SSc PH [20].

In theory, an early systematic detection of PAH in SSc patients would allow early intervention that would allow reversing pathophysiological processes or at least slow its progression. Indeed improved survival in SSc-PAH patients that were diagnosed through an active screening program compared to those in routine clinical practice has been reported [21]. In addition, the EARLY study reported potential benefit of bosentan treatment in patients with milder PH symptoms [22].

Thus, the high prevalence rate, non-specific symptoms and high morbidity and mortality from PAH, as well the potential benefit from early intervention with the development more widely available therapeutic options, provides a strong rationale for active screening programs.

Several screening algorithms that combine clinical, pulmonary function tests (PFTs) and chemical biomarkers in addition to echocardiogram, have been suggested. The ESC/ERS 2009 guidelines have adopted an algorithm which was updated in 2015, and remains the current version [2]. The DETECT (Early, Simple and Reliable Detection of Pulmonary Arterial Hypertension in Systemic Sclerosis) algorithm was developed in 2013 from a large prospective and multicentre study in SSc patients with higher risk of PAH (>3years of SSc and DLCO <60%) [9].

The objective of this study was to examine the impact of a screening program on the early detection of SSc-PAH. We looked at demographics, clinical and haemodynamic data of serial patients diagnosed with SSc-PAH before and after the application of an active screening program in a large national PH referral centre with special interest in CTD-PAH.

2.2 Patients & Methods

All newly diagnosed adult patients with SSc-PAH that are ⩾18 years are prospectively enrolled in the Royal Free Pulmonary Hypertension Service Registry. The current study included newly diagnosed patients between January 2006 and January 2018. Precapillary PH was identified with right heart catheterisation (RHC), where patients had a mean pulmonary artery pressure (mPAP) of ≥25 and pulmonary capillary wedge pressure (PCWP) of ≤15mmHg pulmonary vascular resistance (PVR) ≥3WU. Significant lung disease was excluded using both lung function testing (FVC >60%, FEV1 >50%, FEV1/FVC >70%) and CT chest assessment for interstitial lung disease ILD (<20% parenchymal involvement), emphysema (<5%) or ≥2 features compatible with pulmonary venous occlusive disease (PVOD). Left heart disease was excluded on echocardiography (LVEF < 50%, significant valvular disease, IVSD > 1.2cm, left atrial area > 20cm^2). Chronic thromboembolic disease (CTEPH) was excluded using V/Q scanning followed if positive or inconclusive by pulmonary angiography.

Patients with disproportionate precapillary PH (mPAP >35mmHg, PVR >5WU), were also considered to have PAH if identified lung disease had been demonstrated as stable for more than 2 years on HRCT and lung function testing. The presence of modest diastolic abnormalities on echocardiography (e.g. isolated left atrial enlargement), was permissible if all other data supported a precapillary cause.

SSc was diagnosed based on the contemporary American College of Rheumatology/European League Against Rheumatism criteria [80]. Patients with left heart disease were classified as WHO pulmonary hypertension (PH) group 2, while

patients with significant lung disease on high resolution CT chest (HRCT) or on pulmonary function test (PFTs) were classified as group 3 and patients with significant thromboembolic disease on angiography were classified as group 4. Patients with WHO group 2, 3, and 4 PH were all excluded from this analysis.

1. Data extraction

Patients identified as having an initial diagnosis of SSc PAH were identified from the Royal Free Hospital database (1.4.1), for each patient a retrospective notes review was undertaken to extract clinical and investigational data, therapy instituted and response to therapy after 6 months.

2. Identification of co-morbidities

On review of the notes and at the time of the initial assessment, co-morbidities present at baseline (were recorded. Included in the analysis were patients diagnosed with ILD (not severe enough to be classified as group 3 as per the aforementioned criteria), chronic kidney disease (CKD, eGFR<60ml/min), coronary artery disease (CAD), diabetes mellitus (DM), systemic hypertension (HTN) or obesity (BMI>30). were documented and included in the analysis.

3. Baseline and follow-up assessments

At baseline, subjects had WHO functional class assessment (WHO-FC), 6-minute walking distance (6MWD) and NT-Pro BNP measured and then had conventional cardiac catheterisation to obtain full haemodynamic assessment.

4. Long-term outcome

Patient vital status was assessed on the 30/1/2020 by interrogation of the NHS Spine database. The duration of follow up for surviving patient was based on the last patient contact.

5. Ethics

Data were collected as part of a national audit and all identifiable data were anonymised prior to analysis. In line with the standard practice at the start of the project, the local Research and Development department and the Ethics committee advised that this is an audit of standard practice and formal approval was not required.

6. Statistical analysis

Data were extracted from the registry database and were analysed using SPSS software (IBM, version 26). Continuous variables were expressed as mean ±SD if normally distributed or median ± quartile range if distribution was skewed. Categorical variables were presented as number and percentage. Each variable's data was plotted on histogram and Kolmogorov–Smirnov test was used to test for normal distribution. Comparison between groups was done using simple t-test (if normally distributed) or Mann-Whitney U test (if not normally distributed) for numeric data and Chi-square test was used for categorical data. Survival time was calculated between the date of diagnosis and last follow up or death and then truncated at 5 years; log-rank test was used for comparison as well as Cox-regression analysis to calculate proportional hazards ratios.

2.3 Results

	Pre-2013 N= 164	Post 2013 N=141	p Value
Age (years ±SD)	62.5 (±12.46)	63.6 (±11)	0.403
Females n. (%)	142 (86.6%)	123 (87.2%)	0.876
Comorbidities (ILD, CKD, CAD, DM, HTN or obesity)			
3 or more	10 (6.1%)	11 (7.8%)	
1 or 2	92 (56.1%	75 (53.2%)	
No other significant comorbidities	62 (37.8%)	55 (39%)	0.078
Interstitial lung disease (ILD)	41 (25%)	43 (30.5%)	0.284
Chronic kidney disease (CKD)	4 (2.4%)	13 (9.25)	0.01*
Coronary artery disease (CAD)	23 (14%)	27 (19.1%)	0.228
Systemic Hypertension (HTN)	68 (41.5%)	30 (21.3%)	<0.001*
Diabetes Mellitus (DM)	14 (8.5%)	9 (6.4%)	0.478
Obesity	5 (3%)	1 (0.7%)	0.142
Pulmonary Function tests			
FVC <70%	9 (6.7%)	11 (20.4%)	<0.006*
FEV1 <70%	21 (19.6%)	18 (33.3%)	0.055
DLCO <40%	67 (50%)	29 (55.8%)	0.48
DLCO <60%	120 (89.6%)	50 (96.2%)	0.15
WHO FC			
I	2 (1.2%)	0	
II	15 (9.1%)	7 (5%)	
III	124 (75.6%)	118 (83.7%)	
IV	23 (14%)	16 (11.3%)	0.203
6MWD mean ±SD	246(±140)	241 (±169)	0.806
NT-proBNP median (IQR)	697.00(231-3107)	1091 (233-3490.5)	0.148
RAP median (IQR)	7 (5-10)	8 (6-10.75)	0.12
mPAP median (IQR)	40 (30-57)	43.5 (33.25-51)	0.073
CO median (IQR)	4.5 (3.65-5.4)	4.5 (3.7-5.42)	0.888
CI median (IQR)	2.68 (2.19-3.13)	2.57 (2.25-3.1)	0.928
PVR median (IQR)	495.24 (307.69-800)	605.45 (349.14-827.35)	0.232
SVO_2 median (IQR)	68 (60-73)	68 (59.25-77)	0.537
ESC risk group			
Low risk	36 (22%)	22 (15.6%)	
Intermediate risk	104 (63.4%)	95 (67.4%)	
High risk	24 (14.6%)	24 (17%)	0.356
Early treatment strategy			
Monotherapy	110 (74.3)	59 (43.8%)	
Dual Combination therapy	44 (29.7%)	79 (57.2%)	
Triple therapy	2 (1.3%)	0	
IV monotherapy	2 (1.3%)	0	<0.001*

Table 2.1: Baseline characteristics of the two patient groups. Pre-2013 are patients diagnosed between January 2006 and December 2012 and post-2013 are those diagnosed between January 2013 and January 2018.

Data presented as mean ± SD or median with interquartile range. WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; RAP: right atrial pressure; mPAP mean pulmonary artery pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; SvO_2: mixed venous oxygen saturation. Comparison between groups was done using simple t-test (if normally distributed) or Mann-Whitney U test (if not normally distributed) for numeric data and Chi-square test was used for categorical data.

1. Baseline characteristics

The baseline characteristics are described in table 1. Three-hundred and five patients were diagnosed with SSc-PAH in the Royal Free Pulmonary Hypertension National Unit between 2006 and 2018. Of these, 164 patients were diagnosed pre-2013 (January 2006 - December 2012) and 141 patients diagnosed post-2013 (January 2013 - January 2018). Average demographics were similar at presentation, where the average age was 62.5 and 63.6 years in the pre-2013 and post-2013 groups, respectively, and of these, a similarly high percentage (87%) were females in both groups. The number of comorbidities distribution was also similar between the two groups except that chronic kidney disease (CKD) was significantly more common post-2013 (13 vs 4, p-value 0.01) and systemic arterial hypertension, being almost twice more prevalent in the pre-2013 group. Interstitial lung disease was prevalent in both groups, with over a quarter of the patients being diagnosed with this type of lung disease but was not statistically different between groups.

Pulmonary function tests results were similar in both groups except that the post 2013 group of patients were more likely to have worse lung volumes; FVC<70% was significantly worse. Diffusion lung capacity (DLCO) was similar reflecting a higher risk population in both groups as just over a half of both groups had less than 40% of expected DLCO.

Non-invasive assessments at baseline included WHO functional class (WHO-FC), 6-minute walking distance test (6MWD) and NT-pro beta natriuretic peptide levels (NT-proBNP). There was no statistical difference between both groups in terms of WHO-

FC, with the absolute majority of both groups presenting with WHO-FC III or IV (≥89%), although the proportion of the post-2013 group that presented with WHO-FC I or II was about half of these in the pre-2013 group. 6MWD was similar in both groups (246 ± 140 metres in pre-2013 and 241±169 metres in post-2013 group (p-Value= 0.806). NT-proBNP levels were higher in the post-2013 group (median of 1091 ng/L quartile range of 233-3490.5 ng/L) than the pre-2013 group (median of 697.00 and quartile range of 231-3107 ng/L), but this was not statistically significant.

Index cardiac catheterisation measurements were compared (right atrial measurements; RAP, mean pulmonary artery pressure; mPAP, cardiac output; CO, cardiac index; CI, pulmonary vascular resistance; PVR and mixed venous oxygen saturation; SVO_2) and both groups had very similar measurements, except that the group diagnosed from 2013 onwards trended towards a higher mPAP (43.5mmHg vs. 40mmHg) and higher PVR (605.45vs. 495.24 mmHg/L.min.m^2) but this was not statistically significant.

The non-invasive (WHO-FC, 6-MWD and NT-proBNP) and haemodynamic measurements (RAP, CI and SvO_2) were used to calculate the ESC guidelines risk score as described previously by Kylhammmar et al. (table 2.2) [53]. It was noted that higher proportion of patients in the post-2013 were in the higher risk categories than the pre 213 group (84.4% vs. 78%) but this was found not to be statistically significant (p value 0.356).

ESC/ERS 2015 guidelines risk categories			
Variable	**Low**	**Intermediate**	**High**
WHO FC	I-II	III	IV
6MWD m	>440	165 – 440	<165
NT-proBNP ng/L	<300	300–1400	>1400
RAP mmHg	<8	8–14	>14
CI L/min/m2	>_ 2.5	2.0–2.4	< 2.0
SvO_2 %	>65%	60–65%	<60%

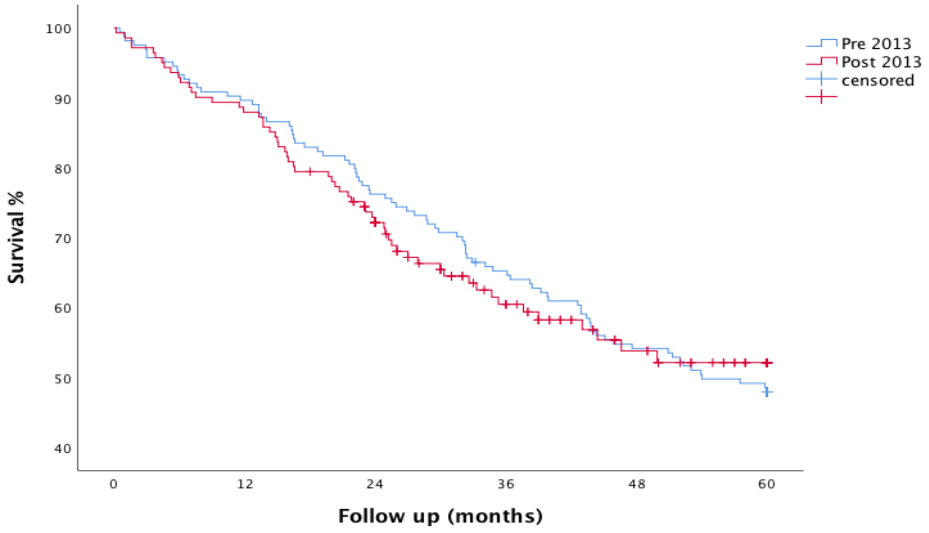
Table 2.2: Selected variables from the ESC/ERS 2015 guidelines for different risk categories.

WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; RAP: right atrial pressure; CI: cardiac index; SvO_2:mixed venous oxygen saturation.

Early treatment strategy was significantly different with the post-2013 cohort, with almost twice the number of patients more likely to be started on early dual combination therapy rather than monotherapy ($p < 0.001$).

2. Survival of both groups

Comparing the long term survival of both groups with follow up truncated at 5 years, demonstrated no statistical difference between the two groups (post 2013 group, 1-, 3- year and 5-year survival was 87.9%, 60.4% and 52.1%, respectively and the pre-2013 group, 1-, 3- year and 5-year survival 89.6%, 65.2% and 49%, respectively) with a log rank p value of 0.869. Applying Cox regression analysis of proportional hazard, and adjusting for ESC risk score at baseline, there was no significant difference on the predicted survival if someone got diagnosed pre or post 2013, whilst the ESC risk category at baseline was a highly significant predictor of survival as expected.



		1 year	2 year	3 year	4 year	5 year	Log rank
Pre 2013	n=164	147 (89.6%)	125 (76.2%)	106 (65.2%)	87 (54.1%)	77 (49%)	0.869
Post 2013	n=141	124 (87.9%)	97 (72.2%)	59 (60.4%)	34 (54.8%)	15 (52.1%)	

Figure 2.1: 5-year Kaplan-Meier survival analysis of both groups. Survival time was calculated between the date of diagnosis and last follow up or death and then truncated at 5 years. Log-rank test was used for comparison.

	P Value	HR	95% CI
Pre-2013 vs 2013 onwards	0.775	1.051	0.749-1.475
ESC risk score at baseline	<0.001	2.580	1.944-3.426

Table 2.3: Cox regression model to calculate proportional hazards ratio (HR) adjusting for the ESC risk category.

2.4 Discussion

We can summarise the overall findings of our study and say that there was no significant difference in patient demographics over the study period before and after 2013 although CKD was more common in the post-2013 group of patients, while systemic hypertension was less common. WHO-FC and NT-proBNP had tendency to be worse in the post-2013 group but this was not statistically significant. Some of the haemodynamic parameters (mPAP and PVR) were worse in the post-2013 but found not to be statistically significant. These findings were also reflected on the distribution of the ESC risk categories across the two groups with more patients in the higher risk categories among the more contemporary group of patients, but similarly this was not statistically significant. These findings seemingly contradict the hypothesis that the introduction of an active screening program leads to the detection of lower risk patients (i.e. earlier diagnosis in their disease process). On the other hand, this may have had an effect on the increasing number of patients that were diagnosed in our centre which rose (on average) to 28 new SSc-PAH patients yearly since 2013 compared to 23 prior to 2013. However, other unaccounted factors may have caused this growth in numbers.

Interestingly, despite treatment strategies across the two groups reflecting the change in practice with the adoption of upfront-dual oral combination therapy becoming significantly more common since 2013, this did not translate into improved survival rate in the post 2013 patient cohort. This could be explained by the trend in

slightly worse baseline parameters, although the current study was not designed to examine the effect of the different treatment strategies on survival. Such a study would need an individual adjustment for risk category which was beyond the scope of the current analysis.

An optimal screening method should rely on non-invasive tools that are both reproducible and cost effective and most importantly, has to demonstrate a high negative predictive value [2]. Of these non-invasive tools, PFTs, NT-proBNP and echocardiography, are the most commonly used. In SSc-PAH, the most recent ESC/ERS guidelines of 2015 have highlighted the role of these tests, but only resting echocardiography has class I recommendation in asymptomatic SSc patients, replacing the 2009 guidelines, which recommended echo only in symptomatic patients. A combined approach including biomarkers, PFTs and echocardiography had a class IIa recommendation.

The algorithm proposed in the DETECT study [9], includes a stepwise detection approach with simple clinical and biological assessments in step 1 (history of telangiectasias, PFTs, ECG and serum ACA, NT-proBNP and urate). Step 2 is a referral for echocardiography to determine if cardiac catheterisation is required. In this study, the approach was associated with a high overall sensitivity (96%) but with a reduced specificity of 48%. In the DETECT study, applying the proposed approach has led to a significant reduction of the number of false negatives, only 4% had missed PAH diagnosis versus 29% if the ESC/ERS 2009 guidelines were applied. The main limitations of this approach are the substantial need for RHC (62%) in the higher risk patients pre-defined in the study of SSc for more than 3 years and DLCO<60%. While

such procedure is safe in experienced hands, with a very low complication rate, they can lead to significant anxiety among patients, especially if needs to be done repeatedly. Another limitation is the lack of evidence that this approach is useful in SSc patients with DLCO>60%, particularly as the reported incidence of SSc is only 1.2% in this population, so the application of such approach will likely reduce specificity further.

In a prospective study of 195 consecutive unselected SSc patients, a Belgian group screened them using either ESC/ERS guidelines recommendations, DETECT algorithm or both combined [81]. The combined approach reduced the need for RHC and increased its positive predictive value. While the DETECT algorithm on its own was more sensitive for with borderline PAH than the other methods, this group would be of particular interest to study further given the recent recommendations to the change of the haemodynamic definition of PAH to include [82].

One of the strengths of this analysis is that it shows serial complete data set of all patients newly diagnosed PAH in patients with SSc with demonstration of demographics, haemodynamic data and their respective risk profile at the time of diagnosis of PAH. It illustrates the change of these profiles over a 12-year period with more adoption of active screening program.

Nevertheless, this study has a significant limitation, the DETECT landmark paper was published in 2013. Thus, the choice of 2013 as a cut off for the pre and post adoption of a new active screening program. This is based on an assumption rather than an established date after which scleroderma physicians has started using such programs.

However, the temporal span over which this study extends should reflect the overall recognition and adoption of the active screening programs.

2.5 Conclusion

There still remains a strong rationale for active screening of PAH in SSc patients, as the prognosis in patients remains relatively worse despite advances in therapeutic options. The limitations in the current screening programs may explain why we have not been able to detect more patients in the lower risk categories. Further development of these programs in order to overcome their shortfalls is thus urgently needed. Non-invasive biomarkers may offer hope to achieve the aim of earlier diagnosis of PAH by enhancing the usefulness of these screening programs further.

Chapter 3 Risk Assessment in Scleroderma Associated Pulmonary Arterial Hypertension. Validation and comparison of different risk assessment models.

3.1 Introduction

Pulmonary arterial hypertension (PAH) is characterised by sustained pulmonary vasoconstriction and remodelling of the small blood vessels in the pulmonary circulation leading to a substantive increase in pulmonary vascular resistance and progressive right ventricular (RV) dysfunction. Although recent registry data suggest improvement in outcomes, PAH still carries a high morbidity and mortality burden [83]. Regular risk assessment is necessary to reassure those at lower risk, to assess the efficacy of intervention and to identify those where escalation of therapy or referral for lung transplantation is required.

Comprehensive prognostic evaluation and risk assessment is recommended in the most recent ESC/ERS guidelines for pulmonary hypertension [2]. The proposed multi-parameter approach includes clinical, exercise, biomarkers and haemodynamic components. Most of the proposed variables and cut-off values were originally based on expert opinion[2]. Low, intermediate and high risk have been defined with predicted one-year mortality of <5%, 5-10% and >10%, respectively. These figures are crude estimates and based on variables mainly identified in studies of idiopathic pulmonary arterial hypertension (IPAH). Several groups have applied different methods to their registries in order to create a usable set of clinical tools for patient monitoring. The Swedish group [53], the COMPERA registry [54] and French group [55] used cut-offs

proposed in the ESC/ERS guidelines and applied to their respective registries employing different calculation methods. The original multi-parameter score (the REVEAL score) has been considered by some clinicians as too cumbersome for routine use and concerns raised that it included too many parameters that are not amenable to modification by changes in therapeutic strategy (e.g. age, PAH subtype, co-morbidities). More recently, Benza and colleagues proposed a newer and a simplified versions of the REVEAL risk prediction calculation (REVEAL 2.0) and compared it head to head with European calculation methods on their registry [58]. Most validation work to date has focused on the IPAH population or mixed populations including modest numbers with connective tissue disease related (CTD)-PAH.

Scleroderma associated (SSc)-PAH is phenotypically unique and carries one of the worst prognosis amongst the different aetiologies of PAH. Although rare in the general population, PAH is relatively common in SSc (estimated prevalence 7–12%) and is a leading cause of morbidity and mortality in patients with his disease [20, 84]. It is important to know which risk scoring tool is optimal for this particular patient population and whether the poor outcome is driven by identified modifiable risk factors associated with PAH in general.

The objectives of the current study were to:

1. Provide validation of the discriminatory ability of the different risk prediction models in a unique population with SSc-PAH.
2. Examine the reproducibility of risk scores on incident SSc-PAH patients at baseline and at the time of first reassessment.

3. Compare the different proposed risk prediction models and identify the optimal risk scoring system for patients with SSc-PAH.
4. Examine the effect of modification of serial risk scores on patients outcome.

3.2 Patient characteristics & methods

All newly diagnosed adult patients with SSc-PAH that are ⩾18 years are prospectively enrolled in the Royal Free National Pulmonary Hypertension Service Registry. The current study population includes incident SSc-PAH patients between January 2006 and January 2018. Precapillary PH was identified on the cardiac catheterisation (RHC): (mean pulmonary artery pressure (mPAP) of ≥25 and PCWP of ≤15mmHg pulmonary vascular resistance (PVR) ≥3 Wood units). SSc was diagnosed based on the contemporary American College of Rheumatology/European League Against Rheumatism criteria [80].

Significant lung disease was excluded using both lung function testing (FVC > 60%, FEV1 > 50%, FEV1/FVC >70%) and CT chest assessment for ILD (<20% parenchymal involvement), emphysema (<5%) or ≥2 features compatible with pulmonary venous occlusive disease (PVOD)). Left heart disease was excluded on echocardiography (left ventricle ejection fraction <50%, significant valvular disease, interventricular systolic diameter >1.2cm, left atrial area >20cm^2) and on invasive haemodynamics.

Patients with disproportionate precapillary pulmonary hypertension (mPAP >35mmHg, PVR >5WU, PAWP ≤15mmHg and >20% HRCT lung involvement), were also considered to have pulmonary vasculopathy (PAH) if identified lung disease had

been demonstrated as stable for more than 2 years on HRCT and lung function testing (<5% fall in FVC). The presence of modest diastolic abnormalities on echocardiography (e.g. isolated left atrial enlargement), was permissible if all other data supported a precapillary cause.

Chronic thromboembolic disease (CTEPH) was excluded using V/Q scanning followed if positive or inconclusive by pulmonary angiography.

Patients with left heart disease were classified as WHO pulmonary hypertension (PH) group 2, patients with significant lung disease on high resolution CT chest (HRCT) or on Pulmonary Function Test (PFTs) were classified as group 3 and patients with significant thromboembolic disease on angiography were classified as group 4 all were excluded from this analysis.

1. Data extraction

Patients identified as having an initial diagnosis of SSc PAH were identified from the database, for each patient a retrospective notes review was undertaken to extract clinical and investigational data, therapy instituted and response to therapy after 6 months.

2. Identification of co-morbidities

Co-morbidities present at baseline (at the time of the initial assessment) were recorded. Interstitial lung disease (ILD, that is not severe enough to be excluded from

the study population and classified as group 3 as per the aforementioned criteria), chronic kidney disease (CKD, eGFR<60ml/min), coronary artery disease (CAD), diabetes mellitus (DM), systemic hypertension (HTN) or obesity (BMI>30) were documented and included in the analysis.

3. Baseline and follow up assessments

At baseline, subjects underwent a WHO functional class assessment (WHO-FC), had 6-minute walking distance (6MWD) and NT-ProBNP measured followed by a conventional right heart catheterisation to obtain full haemodynamic assessment of the patient. Non-invasive parameters were repeated at a follow up assessment that was held between 4 and 8 months from baseline. Cardiac catheterisation was also performed if clinically indicated.

All patients identified as having PAH at baseline were included in the study. Information on changes in diagnosis at follow up were also provided (e.g. patients with a normal wedge pressure at baseline, but elevated wedge pressure on therapy at first follow up – Group 2), unmasked during therapy (e.g. PVOD not evident on baseline HRCT, becoming manifest on therapy) or co-morbidities evolving during the course of follow up (e.g. progression of ILD).

4. Long term outcome

Patient vital status was assessed on 30/1/2020 by interrogation of the NHS Spine database. The duration of follow up for surviving patients was based on the last patient contact.

5. Ethics

Data were collected as part of a national audit and all identifiable data were anonymised prior to analysis. In line with the standard practice at the start of the project, the local Research and Development department and the Ethics committee advised that this is an audit of standard practice and formal approval was not required.

6. Statistical Analysis

Data were extracted from the registry database and were analysed using SPSS software (IBM, version 26). Each variable's data was plotted on histogram and Kolmogorov–Smirnov test was used to test for normal distribution. Continuous variables were expressed as mean ± SD if normally distributed or median and inter-quartile range (IQR) if distribution was skewed. Categorical variables were presented as frequencies and percentages. Changes between baseline and follow up were compared using paired t-test or Wilcoxon signed-rank test where appropriate for numerical data and Chi-square test for categorical data. Follow up time was

calculated between the date of diagnosis or date of 6-month follow-up and the last follow up or death but was truncated at 5 years.

Multivariable Cox regression models were fitted according to the different risk prediction models and the score calculated from the different cut offs (ESC/ERS 2015 guidelines, table 5) or (REVEAL 2.0, table 7). Kaplan-Meier survival analysis was calculated according to the different risk calculation models and log-rank test was used for comparison. Two methods were used for head to head comparison between the different models C-Statistics using area under the curve (AUC) and Bayesian Information Criterion (BIC)[85]. The model with the lowest BIC is preferred. BIC was calculated BIC= -2 log L + q ln(n), where q is the number of unknown parameters and n is the sample size.

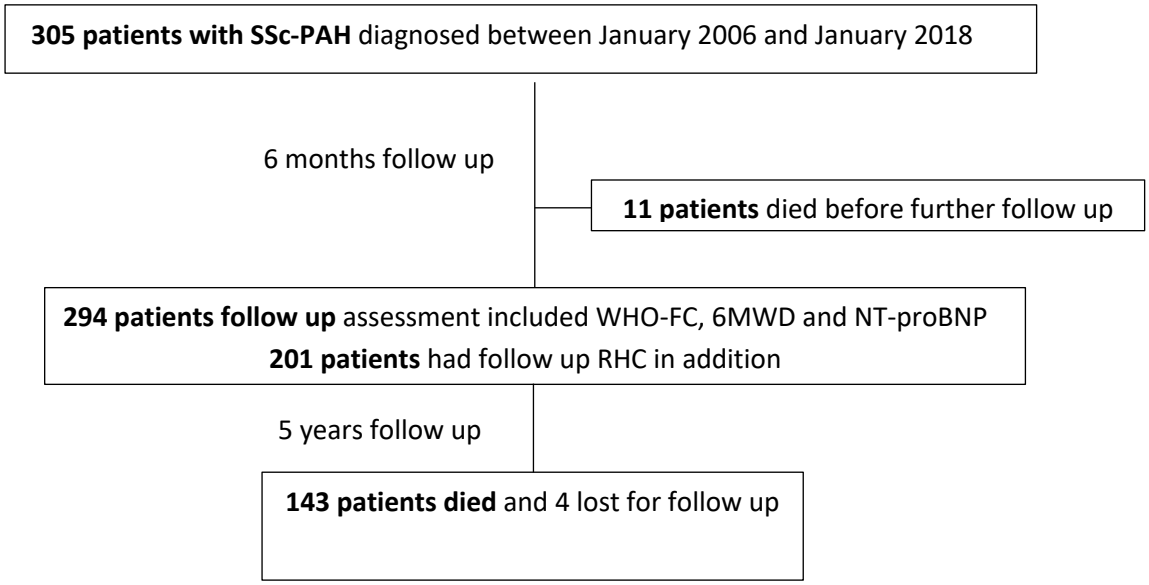


Figure 3.1: Flow chart of the study population.

SSc-PAH: systemic sclerosis associated pulmonary arterial hypertension; WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; RHC: right heart catheterisation.

3.3 Results

1. Population characteristics

We identified 305 patients that were included in our study (Figure 3.1). Analysis of follow up was truncated at 5 years and 94 patients (21%) were followed for at least 5 years. 143 patients died within the follow-up period and 21 patients died within the first 6 months. Patients' characteristics at baseline are given in table 1. The majority (86.9%) of these were females with mean age of 63 years and most had limited SSc (77%). Over half had at least one comorbidity (54.4%) while only 6.9% had at least three comorbidities. Whilst the most common comorbidity was systemic hypertension (98 patients), 83 patients (27.7%) had limited interstitial lung disease. During follow-up, other pathologies evolved to become the primary concern. For example, it was found that the severity of associated conditions had been underappreciated, with 25 patients having their PH diagnosis updated to other than WHO PH group 1. Within this group of patients, 7 patients were reclassified as group 3 and 8 patients had their diagnosis updated to PVOD.

During the study period, the initial treatment strategy was monotherapy (either an endothelin receptor antagonist or a phosphodiesterase type 5 inhibitor in just over half of all of patients (55.4%) or early combination therapy (PDEI+ERA) in 40.3% of patients. Only four patients were on IV prostanoid therapy as the initial treatment strategy.

Patients characteristics	No. (%)
Sex (n = 305)	
Female	265 (86.9%)
Age at diagnosis (years) (n = 305)	
Mean ± SD.	63 ± 12
Scleroderma subtype (n = 271)	
Limited SSc	209 (77%)
Diffuse SSc	31 (11%)
Overlap connective tissue disease	26 (10%)
SSc sine scleroderma	2 (0.7 %)
Comorbidities (ILD, CKD, CAD, DM, HTN or obesity)	
3 or more	21 (6.9%)
1 or 2	166 (54.4%)
No other significant comorbidities	118 (38.7%)
Interstitial lung disease (Only in 7 diagnosis changed)	83 (27.2%)
Chronic kidney disease	17 (5.6%)
Coronary artery disease	50 (16.4%)
Systemic Hypertension	98 (32.1%)
Diabetes Mellitus	23 (7.5%)
Obesity	6 (2%)
Diagnosis updated after initial treatment strategy (n.)	25 (8.2%)
to PVOD	8 (2.6%)
to group 3 PH	7 (2.3%)
to other categories	10(3.3%)
Pulmonary Function tests	
FVC % (n = 188) Mean ± SD.	95.5 ± 20.1
FEV1 % (n = 161) Mean ± SD.	82.6 ± 19.9
DLCO % (n = 186) Mean ± SD.	41.2 ± 13.4
Early treatment strategy (n = 296)	
Oral monotherapy	169 (55%)
Oral dual Combination therapy	123 (44%)2 (
IV prostanoid therapy	4 (1.3%)

Table 3.1: Baseline characteristics of the studied population.

Continuous variables were expressed as mean ± SD if normally distributed or median and inter-quartile range (IQR) if distribution was skewed. Categorical variables were presented as frequencies and percentages.

2. Baseline and follow-up assessments

Baseline and follow-up of non-invasive and haemodynamic measurements are shown in table 3.2. At diagnosis, the majority of patients (78.7%) were classified as WHO functional class (WHO-FC) III, while the number of patients classified as WHO-FC I-II increased three-fold at follow up (going from 24 to 73 patients). The mean 6MWD at baseline was 252m which increased modestly on follow up by 23m (p=0.001). Median value of NT-proBNP was 854.5 ng/l (IQR 230-3293 ng/l) at baseline and almost halved at follow up to 476 ng/l (IQR 182-1490 ng/l; p=<0.001).

At the first reassessment, 202 patients had repeat haemodynamic assessment at 6 months. At diagnosis, the mean values of mPAP was 41.51 mmHg and PVR was 624.98 dyn·s/cm^5 at baseline; both parameters significantly (p value <0.001) improved on repeat catheterisation (mPAP 38.55 mmHg and PVR 486.64 dyn·s/cm^5). Similarly, all the other haemodynamic parameters, including cardiac output, cardiac index and mixed venous oxygen saturation significantly improved on follow up assessment, with the exception of right atrial pressure where there was only a trend towards a reduction (table3.2).

	N	Baseline	Follow up	p- value
WHO FC n (%)				
I-II		24 (7.8%)	73 (25%)	
III		242(78.7%)	194(66.7%)	
IV		39 (12.8%)	24 (8.2%)	<0.001
6MWD metres	279	252 ± 152	275 ± 154	0.001
NT-pro BNP ng/L	277	854.5 (230-3293)	476 (182-1490)	<0.001
RAP mmHg	201	8.24 ± 4.416	7.62±3.61	0.055
mPAP mmHg	201	41.51±10.84	38.55±11.17	<0.001
PVR $dyn \cdot s/cm^5$	185	624.98 ±26.73	486.64 ±20.56	<0.001
CO l/min	193	4.61 ±1.38	5.03 ±1.37	<0.001
CI	177	2.72 ±0.72	2.95 ±0.72	<0.001
SvO_2 %	191	66.27 ±9.3	67.64 ±8.56	<0.001

Table 3.2: Non-invasive and invasive data at Baseline and at Follow up.

WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; RAP: right atrial pressure; mPAP mean pulmonary artery pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; SvO_2: mixed venous oxygen saturation. N – represents the number where both base line and follow up result was available. Data presented as mean ±SD except NT-proBNP presented as median (IQR).

Changes between baseline and follow up were compared using paired t-test or Wilcoxon signed-rank test where appropriate for numerical data and Chi-square test for categorical data.

3. Survival analysis

The median of the overall survival of our patient population was estimated to be 54 months, with one-year, 3-year and 5-year survival estimated to be 89%, 63.1% and 48.3%, respectively (Figure 2). Annual mortality based on year 3 survival, averaged 14%. Of those that had their initial diagnosis updated during the follow up period, the median survival was estimated 40.6 months, while the remainder of patients with unchanged diagnosis was greater than 60 months, although this trend was not significant (log rank p value = 0.52).

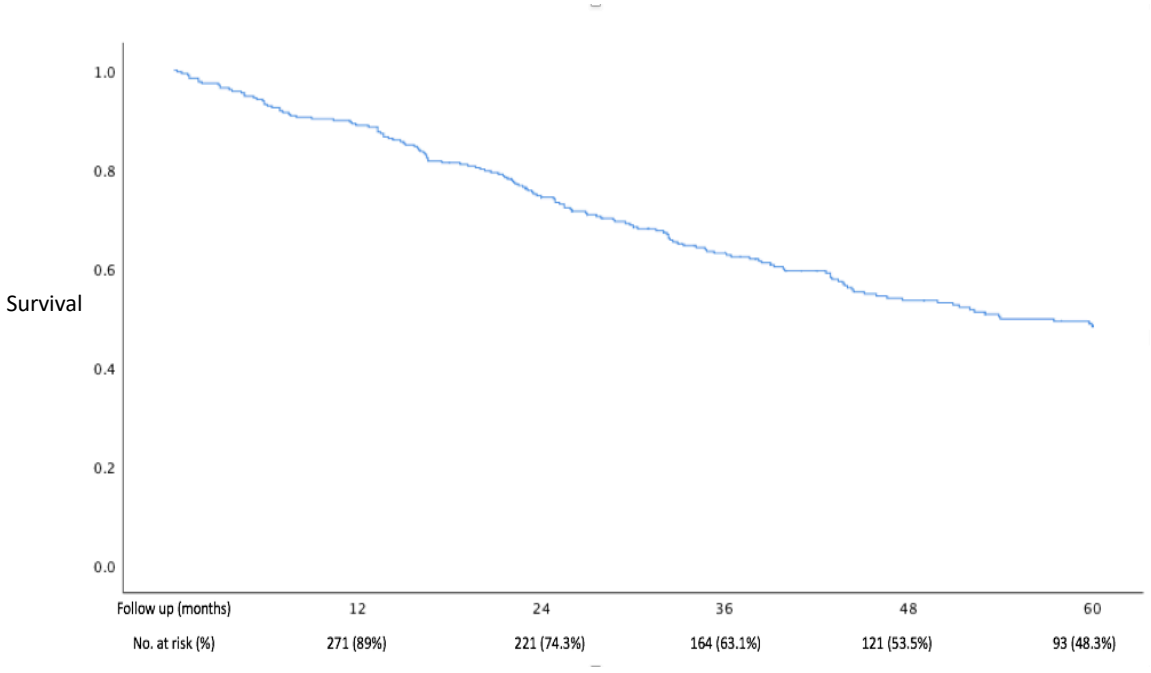


Figure 3.2: Kaplan-Meier survival curve for overall survival of the studied population.

3.3.3.1 Univariable analyses

The univariable associations between baseline characteristics, non-invasive and invasive variables with survival are shown in table 3.3 and figure 3.3. Age and lungs diffusion capacity were found to be significantly associated with mortality (HR 1.031 (95%CI 1.015-1.048) for every year and HR 1.973 (95%CI 1.129-3.449) if DLCO<50%). All the non-invasive (WHO-FC, 6MWD and NT-proBNP) and the invasive (RAP, CI and SvO_2) risk variables were significantly associated with survival both at baseline and at first re-assessment, with the exception of NT-proBNP at follow up.

Baseline Characteristics							
Variable	HR	95% CI	p Value	Variable	HR	95% CI	p Value
Age (per 1 y)	1.031	1.015-1.048	<0.001*	Diabetes Mellitus	0.661	0.324-1.35	0.256
Female	0.671	0.436-1.033	0.07	Obesity	0.047	0.001-3.479	0.164
Diffuse vs Limited SSc	1.083	0.619-1.894	0.781	ILD (any degree)	1.379	0.938-2.027	0.094
No. of comorbidities	0.919	0.768-1.1	0.358	FVC <70%	1.142	0.572-2.282	0.706
Chronic Kidney Disease	1.388	0.706-2.728	0.341	FEV1 <70%	1.311	0.784-2.194	0.302
Coronary Artery Disease	1.017	0.656-1.577	0.94	DLCO <50%	1.973	1.129-3.449	0.011*
Systemic Hypertension	1.129	0.801-1.589	0.488	Diagnosis changed after initial workup	1.115	0.631-1.972	0.708

Baseline measurements				Follow up measurements			
WHO-FC III vs I-II	2.303	1.011-5.246	0.047*	WHO-FC III vs I-II	3.057	1.741-5.369	<0.001*
WHO-FC IV vs I-II	6.864	2.859-16.48	<0.001*	WHO-FC IV vs I-II	8.836	4.189-18.634	<0.001*
6MWD per 10m	0.959	0.948-0.970	<0.001*	6MWD per 10m	0.958	0.946-0.97	<0.001*
NT-proBNP per 300ng/dL	1.02	1.014-1.027	<0.001*	NT-proBNP per 300ng/dL	1	0.977-1.003	0.95
RAP per 1mmHg	1.061	1.028-1.94	<0.001*	RAP per mmHg	1.107	1.041-1.176	0.001*
mPAP per 1mmHg	1.036	1.021-1.051	<0.001*	mPAP per mmHg	1.030	1.012-1.049	<0.001*
CO per L/min	0.749	0.647-0.866	<0.001*	CO per L/min	0.762	0.634-0.915	0.004*
CI L/min/m^2	0.587	0.446-0.772	<0.001*	CI L/min/m^2	0.647	0.465-0.899	0.009*
PVR per 100	1.1581	1.111-1.208	<0.001*	PVR per 100	1.159	1.081-1.242	<0.001*
SvO_2 per 1%	0.948	0.933-0.963	<0.001*	SvO_2 per 1%	0.952	0.934-0.971	<0.001*

Table 3.3: Univariable Cox regression proportional hazard ratios (HR) for prediction of mortality according to the baseline characteristics, non-invasive (WHO-FC, 6MWD and NT-proBNP) and invasive variables (RAP, CI and SvO_2) measured at baseline and follow up.

WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; RAP: right atrial pressure; mPAP mean pulmonary artery pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; SvO_2:mixed venous oxygen saturation.

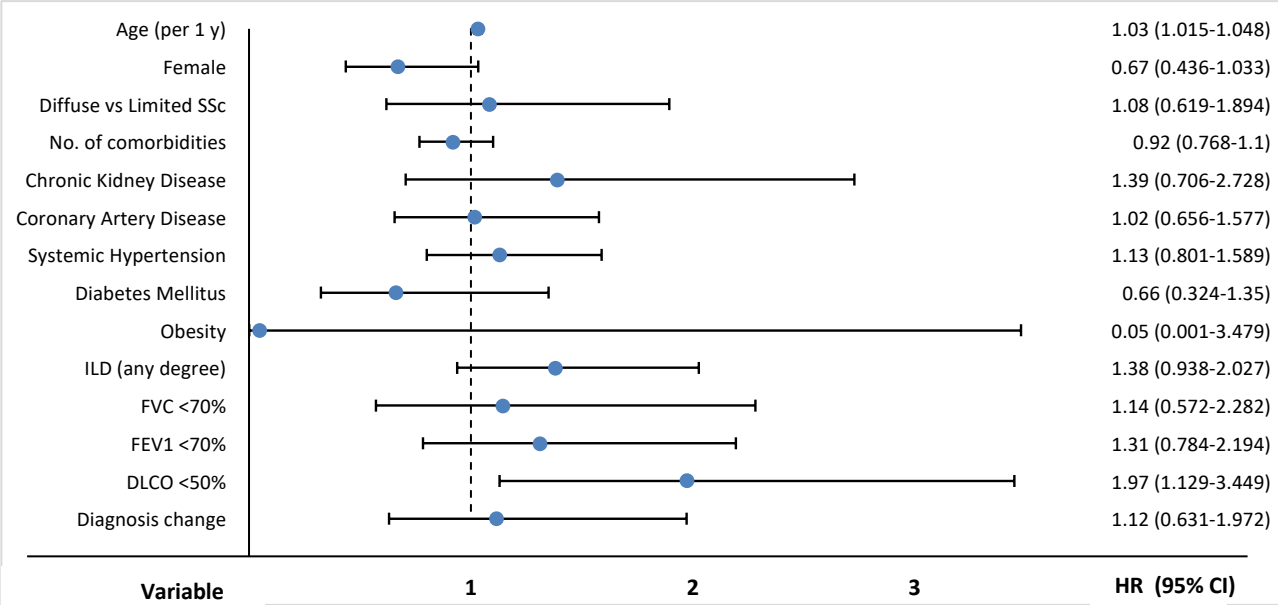


Figure 3.3: Forest plot for uni-variable analysis of the baseline characteristics

HR: hazard ratio; ILD: interstitial lung disease; FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second; DLCO: diffusion lung capacity.

Table 3.4 shows the uni-variable analysis according to the ESC/ERS 2015 guidelines proposed risk categories (table 3.5) for the non-invasive and invasive measurements at baseline and follow up. At baseline, all the variables different risk categories were discriminatory with the exception of RAP and CI intermediate vs low risk categories. Similarly, at follow up, all non-invasive and invasive measurements were significant at discriminating different levels of risk with the exception of RAP intermediate vs low risk categories. Overall, non-invasive variables showed greater discrimination than the invasive variables, both at baseline and follow up. This was most apparent for the two most extreme risk levels (low vs high).

Baseline risk categories				Follow up risk categories			
Variable	HR	95% CI	p Value	Variable	HR	95% CI	p Value
WHO-FC III vs I-II	2.303	1.011-5.246	0.047*	WHO-FC III vs I-II	3.057	1.741-5.369	<0.001*
WHO-FC IV vs I-II	6.864	2.859-16.48	<0.001*	WHO-FC IV vs I-II	8.836	4.189-18.634	<0.001*
6MWD intermediate vs low risk	8.337	2.045-33.984	0.003*	6MWD intermediate vs low risk	3.577	1.443-8.867	0.006*
6MWD high vs low risk	18.852	4.61-77.09	<0.001*	6MWD high vs low risk	8.259	3.246-21.016	<0.001*
NT-proBNP intermediate vs low risk	2.291	1.368-3.839	0.002*	NT-proBNP intermediate vs low risk	1.692	1.033-2.771	0.037*
NT-proBNP high vs low risk	4.487	2.808-7.169	<0.001*	NT-proBNP high vs low risk	4.836	3.023-7.738	<0.001*
RAP intermediate vs low risk	1.212	0.842-1.743	0.3	RAP intermediate vs low risk	1.297	0.838-2.007	0.243
RAP high vs low risk	2.311	1.443-3.701	<0.001*	RAP high vs low risk	3.16	1.132-8.822	0.028*
CI intermediate vs low risk	1.445	0.957-2.183	0.8	CI intermediate vs low risk	2.062	1.284-3.312	0.003*
CI high vs low risk	2.632	1.734-3.995	<0.001*	CI high vs low risk	4.195	1.661-10.6	0.002*
SvO_2 intermediate vs low risk	2.335	1.487-3.669	<0.001*	SvO_2 intermediate vs low risk	2.157	1.311-3.548	0.002*
SvO_2 high vs low risk	2.946	1.996-4.349	<0.001*	SvO_2 high vs low risk	3.553	2.038-6.195	<0.001*

Table 3.4: Univariable Cox regression proportional hazard ratios (HR) for prediction of mortality using baseline and follow up data according non-invasive (WHO-FC, 6MWD and NT-proBNP) and invasive variables (RAP, CI and SvO_2) risk categories as per the ESC/ERS 2015 guidelines measured at baseline and follow up.

WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; RAP: right atrial pressure; mPAP mean pulmonary artery pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; SvO_2:mixed venous oxygen saturation.

ESC/ERS 2015 guidelines risk categories			
Variable	**Low**	**Intermediate**	**High**
WHO FC	I-II	III	IV
6MWD m	>440	165 – 440	<165
NT-proBNP ng/L	<300	300–1400	>1400
RAP mmHg	<8	8–14	>14
CI L/min/m2	$\geq$ 2.5	2.0–2.4	< 2.0
SvO_2 %	>65%	60–65%	<60%

Table 3.5: Selected variables from the ESC/ERS 2015 guidelines for different risk categories.

WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; RAP: right atrial pressure; CI: cardiac index; SvO_2:mixed venous oxygen saturation.

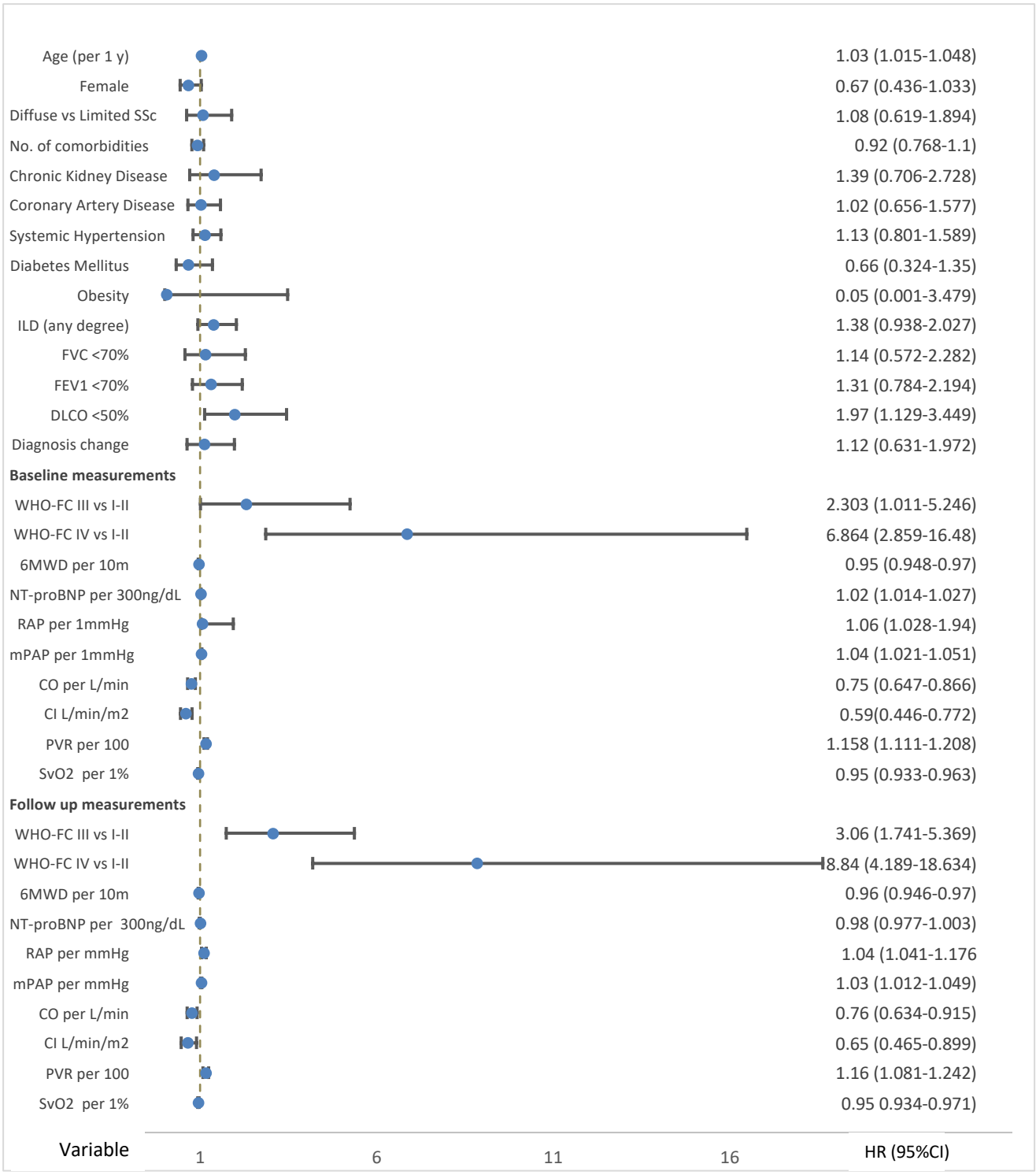


Figure 3.4: Forest plot for uni-variable analysis of baseline and follow up characteristics, non-invasive variables (WHO-FC, 6MWD and NT-proBNP) and invasive variables (RAP, CI and SvO_2)

ILD: interstitial lung disease; FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second; DLCO: diffusion lung capacity WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; RAP: right atrial pressure; mPAP mean pulmonary artery pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; SvO2:mixed venous oxygen saturation; HR: hazard ratio.

Multivariable analysis

Multivariable Cox regression proportional hazards analysis was performed according to the ESC/ERS 2015 risk categories and is shown in table 3.6. Adjusting for other variables as categorical entities with the proposed cut-offs, 6MWD intermediate or high-risk categories (vs. low risk) were the only discriminatory variables at baseline (HR 5.265, 95%CI 1.222-2.691 and p=0.026, HR 6.781, 95%CI 1.515-0.357, p=0.012, respectively). At the time of follow up, high versus low risk level of NT-proBNP was the only significant discriminating factor (HR 2.208, 95%CI 1.167-4.179, p=0.015).

Baseline risk categories				**Follow up measurements**			
Variable	HR	95% CI	p Value	Variable	HR	95% CI	p Value
WHO-FC III vs I-II	0.960	0.371-2.479	0.932	WHO-FC III vs I-II	1.341	0.649-2.771	0.428
WHO-FC IV vs I-II	1.850	0.663-5.160	0.240	WHO-FC IV vs I-II	1.708	0.547-5.334	0.357
6MWD intermediate vs low risk	5.265	1.222-2.691	0.026*	6MWD intermediate vs low risk	2.072	0.594-7.226	0.253
6MWD high vs low risk	6.781	1.515-0.357	0.012*	6MWD high vs low risk	2.590	0.652-10.282	0.176
NT-proBNP intermediate vs low risk	1.660	0.944-2.922	0.079	NT-proBNP intermediate vs low risk	.986	0.532-1.829	0.965
NT-proBNP high vs low risk	1.882	0.997-3.553	0.051	NT-proBNP high vs low risk	2.208	1.167-4.179	0.015*
RAP intermediate vs low risk	0.964	0.648-1.435	0.858	RAP intermediate vs low risk	1.032	0.628-1.697	0.901
RAP high vs low risk	0.803	0.447-1.443	0.463	RAP high vs low risk	.866	0.224-3.343	0.834
CI intermediate vs low risk	1.160	0.748-1.800	0.507	CI intermediate vs low risk	1.577	0.924-2.694	0.095
CI high vs low risk	1.525	0.936-2.486	0.090	CI high vs low risk	2.630	0.889-7.777	0.081
SvO_2 intermediate vs low risk	1.702	0.981-2.954	0.058	SvO_2 intermediate vs low risk	1.110	0.603-2.042	0.738
SvO_2 high vs low risk	1.534	0.885-2.658	0.128	SvO_2 high vs low risk	1.414	0.631-3.166	0.400

Table 3.6: Multivariable Cox regression proportional hazard ratios (HR) for prediction of mortality using baseline and follow up data according ESC/ERS risk categories.

WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; RAP: right atrial pressure; mPAP mean pulmonary artery pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; SvO_2:mixed venous oxygen saturation.

Multi-variable analysis using the score calculated for each variable as per the proposed cut-offs is shown in table 3.7 and figure 3.5. The analysis identified non-invasive variables as significant (WHO-FC, 6MWD and NT-proBNP, HR of 1.79, 1.53 and 1.41, respectively). In contrast, none of the invasive measurements (RAP, CI and SvO_2) were statistically significant at 5-year survival predication at baseline. At follow up, the risk score calculated from NT-proBNP and CI measurements were the only statistically significant predictors of survival (HR 1.51 and 1.57, respectively).

Baseline risk score				**Follow up risk** score			
Variable	HR	95% CI	p Value	Variable	HR	95% CI	p Value
WHO FC risk score	1.79	1.19 - 2.69	0.005*	WHO FC risk score	1.36	0.8 - 2.31	0.255
6MWD risk score	1.53	1.07 - 2.21	0.02*	6MWD risk score	1.38	0.84 - 2.28	0.206
NT-proBNP risk score	1.41	1.04 - 1.92	0.028*	NT-proBNP risk score	1.51	1.08 - 2.11	0.016*
RAP risk score	0.93	0.71 - 1.21	0.574	RAP risk score	1.03	0.67 - 1.57	0.015
CI risk score	1.2	0.94 - 1.52	0.142	CI risk score	1.57	1.03 - 2.4	0.038*
SvO_2 risk score	1.16	0.89 - 1.5	0.275	SvO_2 risk score	1.16	0.78 – 1.71	0.459

Table 3.7: Multivariable Cox regression proportional hazard ratios (HR) of the baseline and follow-up variables according to risk score of each variable as per the ESC/ERS 2015 guidelines.

WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; RAP: right atrial pressure; mPAP mean pulmonary artery pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; SvO_2:mixed venous oxygen saturation.

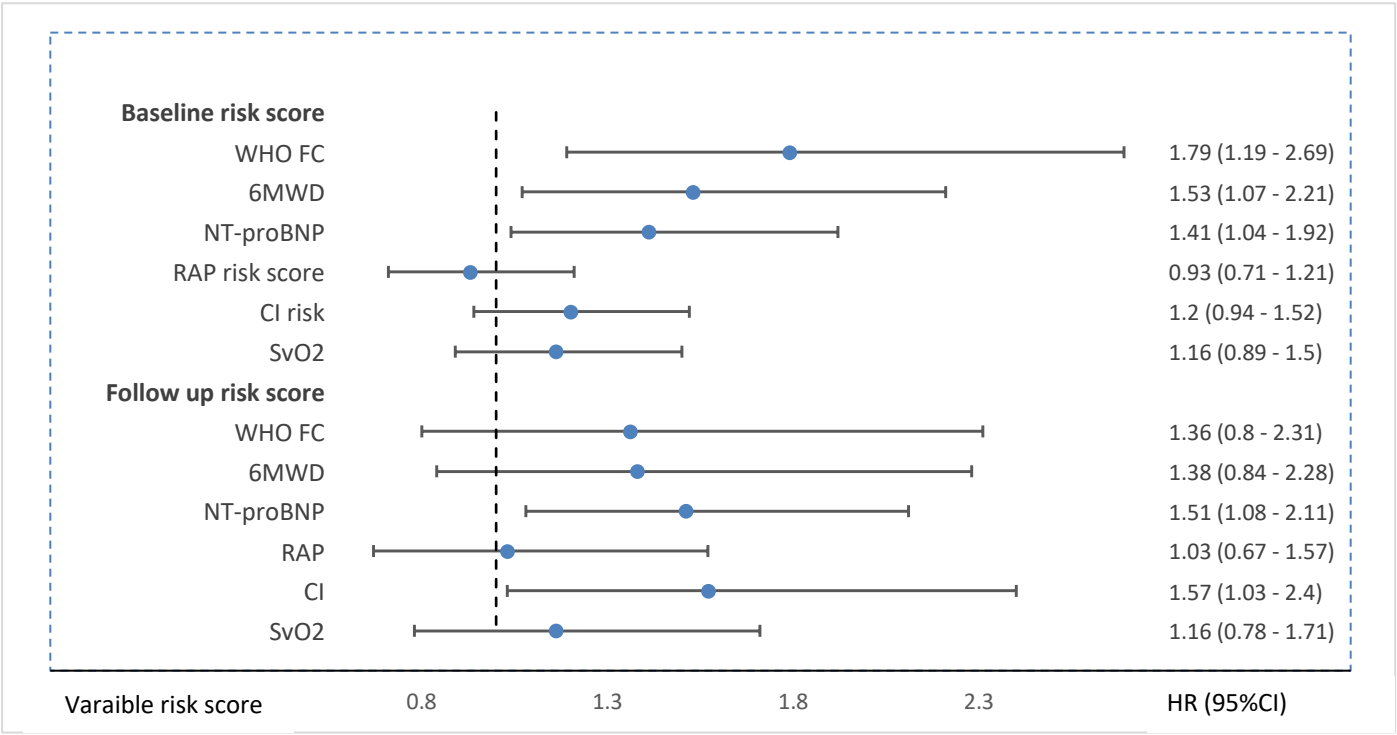


Figure 3.5: Forest plot of the multi-variable analyses of the baseline and follow-up variables according to risk score of each variable as per the ESC/ERS 2015 guidelines.

WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; RAP: right atrial pressure; mPAP mean pulmonary artery pressure; PVR: pulmonary vascular resistance; CO: cardiac output; CI: cardiac index; SvO_2:mixed venous oxygen saturation.

Applying the calculated REVEAL 2.0 risk score (as per table 3.9) in the multi-variable analysis (Table 3.8), yielded similar findings. At baseline only the non-invasive parameters derived score were significantly associated with survival but none of the demographics data (male older than 60 years), comorbidities (CKD or DLCO<40%) or the invasive parameters (RAP or PVR) were. At follow up, only the 6MWD was the only independently significant variable with HR of 1.619 and 95% CI= 1.085-2.415, p value=0.018 .

Baseline parameter risk category				**Follow up parameters risk category**			
Variable risk score	HR	95% CI	p Value	Variable	HR	95% CI	p Value
Male >60yo	1.181	0.791-1.763	0.417	Male >60yo	1.468	0.889-2.424	0.134
CKD	0.687	0.239-1.976	0.486	CKD	0.841	0.289-2.445	0.750
DLCO	1.345	0.823-2.198	0.236	DLCO	1.321	0.712-2.454	0.377
WHO FC	1.909	1.116-3.263	0.018*	WHO FC	1.130	0.578-2.209	0.721
6MWD	1.461	1.075-1.986	0.015*	6MWD	1.619	1.085-2.415	0.018*
NT-proBNP	1.287	1.067-1.553	0.008*	NT-proBNP	1.187	0.984-1.430	0.073
RAP	0.548	0.130-2.309	0.413	RAP	2.080	0.267-16.229	0.485
PVR	1.096	0.595-2.018	0.769	PVR	1.307	0.702-2.432	0.399

Table 3.8: Multivariable Cox regression proportional hazards for prediction of mortality using baseline and follow up data according to REVEAL 2.0 risk calculation.

HR: hazard ratio; CI: confidence interval; CKD: chronic kidney disease; DLCO: diffusion lung capacity; WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; RAP: right atrial pressure; PVR: pulmonary vascular resistance.

Variable	Score		
SSc-PAH	+1		
Male >60yo	+2		
CKD	+1		
DLCO <40%	+1		
WHO FC	FC I = -1	FC III = +1	FC IV = +2
6MWD	>440 m = -2	320 to <440 m = -1	<165 m = +1
NT-proBNP	<300 ng/L = -2	>1100 ng/L = +2	
RAP	>20mmHg = +1		
PVR	< 5 Wood's units = -1		

Table 3.9: REVEAL 2.0 risk category score used in the analysis

SSc-PAH: systemic sclerosis associated pulmonary arterial hypertension; CKD: chronic kidney disease; DLCO: diffusion lung capacity; WHO FC: World Health Organisation functional class; 6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; RAP: right atrial pressure; PVR: pulmonary vascular resistance.

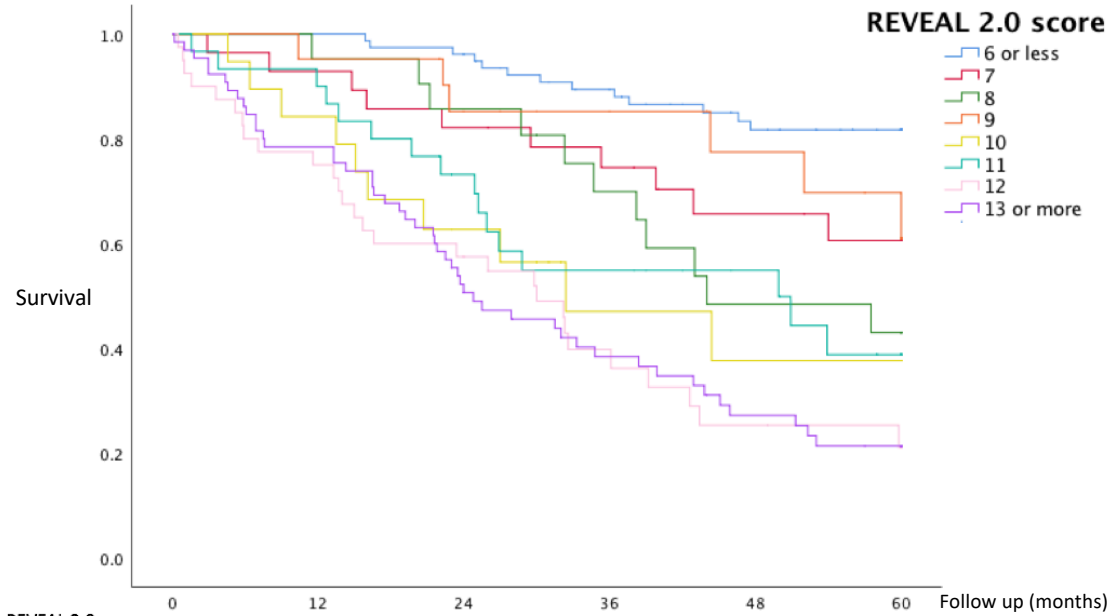
3.3.3.1 Risk assessment models

We have applied several risk assessment models to our cohort at baseline and follow up according to the different risk assessment scoring systems:

REVEAL 2.0 risk score

At baseline, using the exact score [86] resulted in good discrimination between different risk groups (figure 3.6, log rank p value <0.001). Similarly, the simplified three category score (low risk <6, intermediate risk 7 or 8 or high risk >8) discriminates three groups with a 12-month survival of 100%, 94.1% or 82.1%, respectively (log rank p<0.001; Figure 3.7). However, the majority of patients were categorised as high risk (58% of 305 patients).

At follow up (figures 3.8 and 3.9) using either REVEAL 2.0 based scores, both provided good discrimination (log rank p<0.001). Fewer patients were categorised as high-risk categories using the actual REVEAL 2.0 score on follow up. On the simplified score this was demonstrated by more even distribution of the population between the three levels of risk.



REVEAL 2.0 score						
6 or less	78	78 (100%)	75 (96.2%)	63 (89.4%)	50 (81.7%)	40 (81.7%)
7	28	26 (92.9%)	23 (82.1%)	19 (74.5%)	14 (65.7%)	12 (60.6%)
8	21	20 (95.2%)	17 (85.7%)	13 (69.9%)	9 (48.4%)	7 (43%)
9	21	20 (95.2%)	16 (85.2%)	13 (85.2%)	10 (77.5%)	7 (61%)
10	19	16 (84.2%)	10 (62.7%)	5 (47%)	4 (37.6%)	3 (37.6%)
11	30	27 (90%)	20 (73.2%)	15 (54.9%)	11 (54.9%)	4 (38.8%)
12	40	30 (75%)	23 (57.5%)	12 (39.8%)	7 (25.3%)	4 (21.1%)
13 or more	65	51 (78.5%)	32 (50.6%)	21 (38.4%)	14(27.2%)	9 (21.4%)

Figure 3.6: Kaplan-Meier survival curves for REVEAL 2.0 score measured at baseline

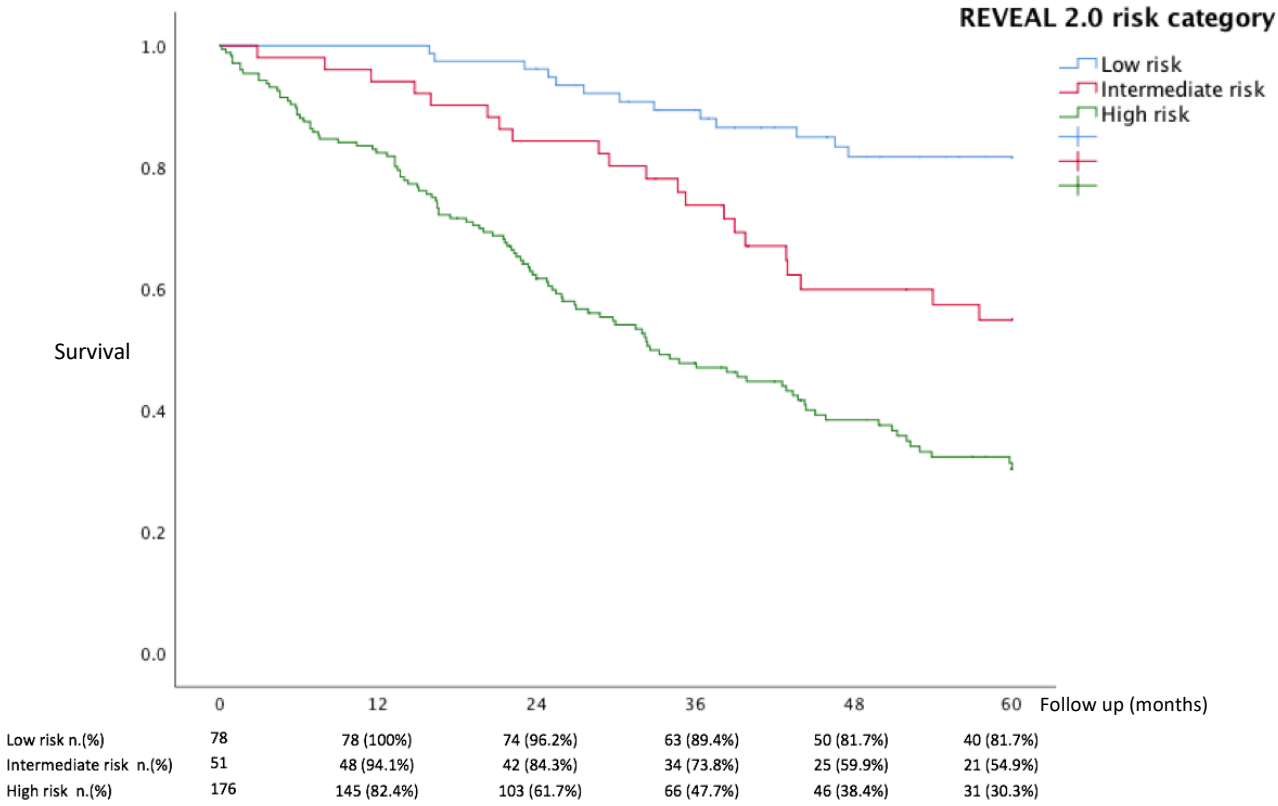
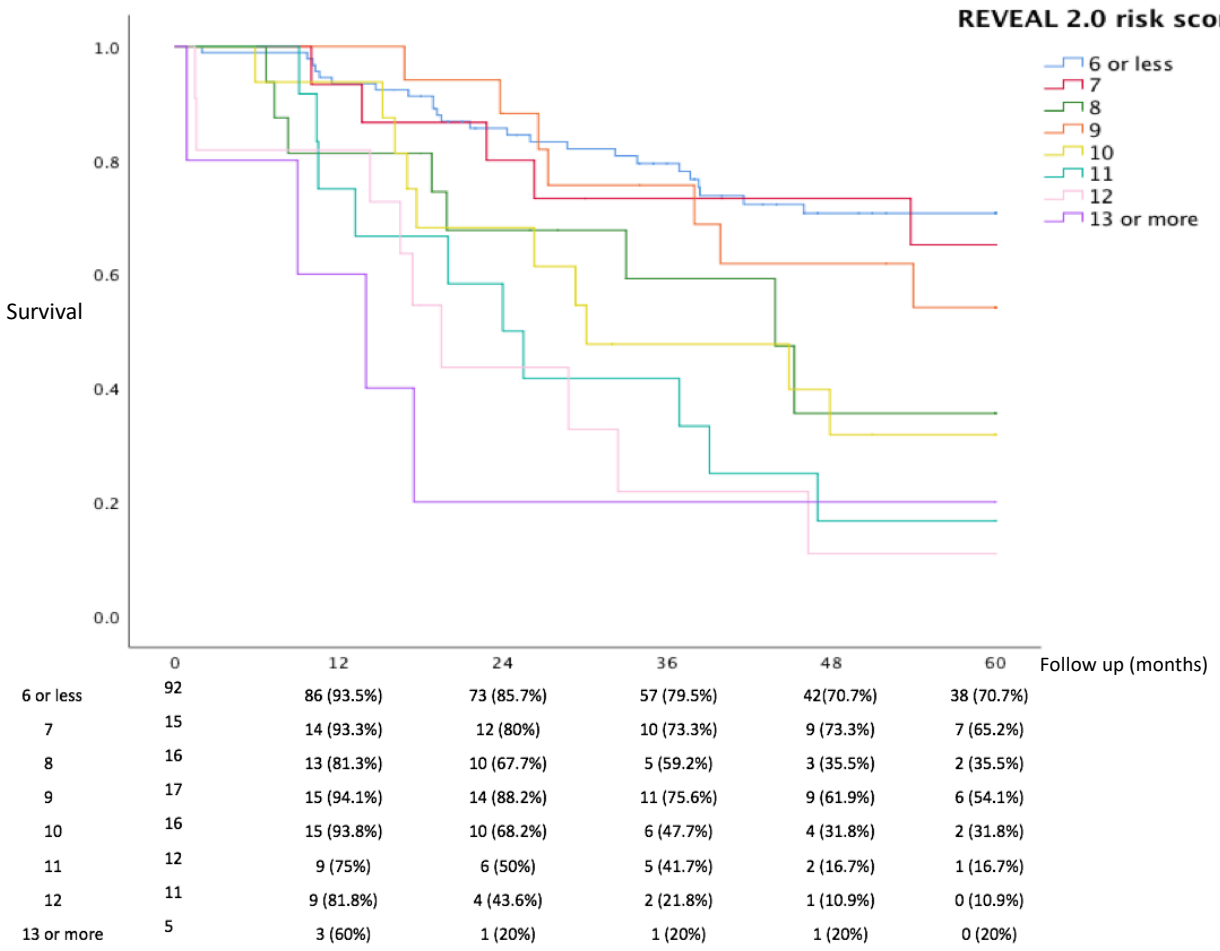


Figure 3.7: Kaplan-Meier survival curves for simplified REVEAL 2.0 risk score measured at baseline (low risk <6, intermediate risk 7or 8 and high risk >8)



6 or less	92	86 (93.5%)	73 (85.7%)	57 (79.5%)	42(70.7%)	38 (70.7%)
7	15	14 (93.3%)	12 (80%)	10 (73.3%)	9 (73.3%)	7 (65.2%)
8	16	13 (81.3%)	10 (67.7%)	5 (59.2%)	3 (35.5%)	2 (35.5%)
9	17	15 (94.1%)	14 (88.2%)	11 (75.6%)	9 (61.9%)	6 (54.1%)
10	16	15 (93.8%)	10 (68.2%)	6 (47.7%)	4 (31.8%)	2 (31.8%)
11	12	9 (75%)	6 (50%)	5 (41.7%)	2 (16.7%)	1 (16.7%)
12	11	9 (81.8%)	4 (43.6%)	2 (21.8%)	1 (10.9%)	0 (10.9%)
13 or more	5	3 (60%)	1 (20%)	1 (20%)	1 (20%)	0 (20%)

Figure 3.8: Kaplan-Meier survival curves for REVEAL 2.0 score measured at follow up

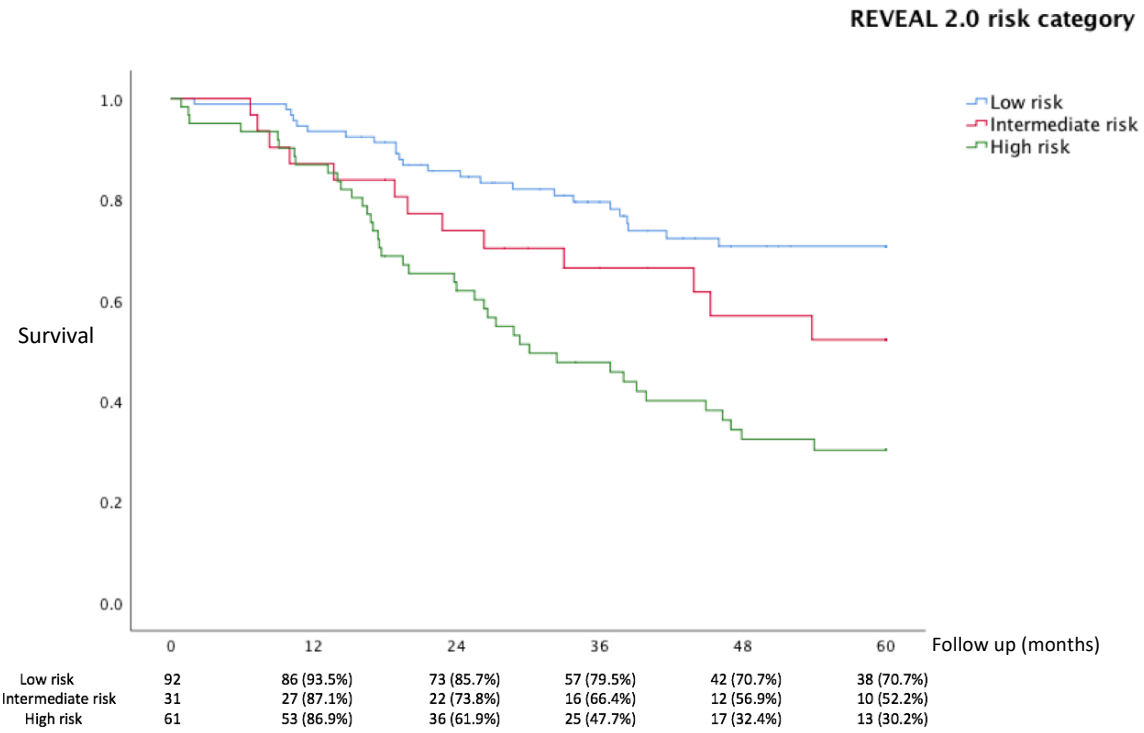


Figure 3.9: Kaplan-Meier survival curves for simplified REVEAL 2.0 risk category measured at follow-up (low risk <6, intermediate risk 7or 8 and high risk >8)

The French model

A group of French clinicians suggested a model based on the number of the low risk criteria [55], having none, 1, 2, 3 or all of a selected 4 variables (2 non-invasive WHO FC, 6MWD and 2 invasive variables RAP and CI) from the ESC/ERS 2015 low-risk criteria (table 3.5). The scores based on this approach did significantly discriminate between the different risk levels both at baseline and follow up (log rank p value <0.001) but yielded slightly less accurate discrimination (figures 3.10 and 3.11). However, similar to the simplified REVEAL 2.0 score, the majority of patients were categorised in the higher risk categories at baseline (172 of 274 patients with none or only one low risk criterion). Of note, only very few patients (5 at baseline and 10 at follow up) had all the low risk criteria.

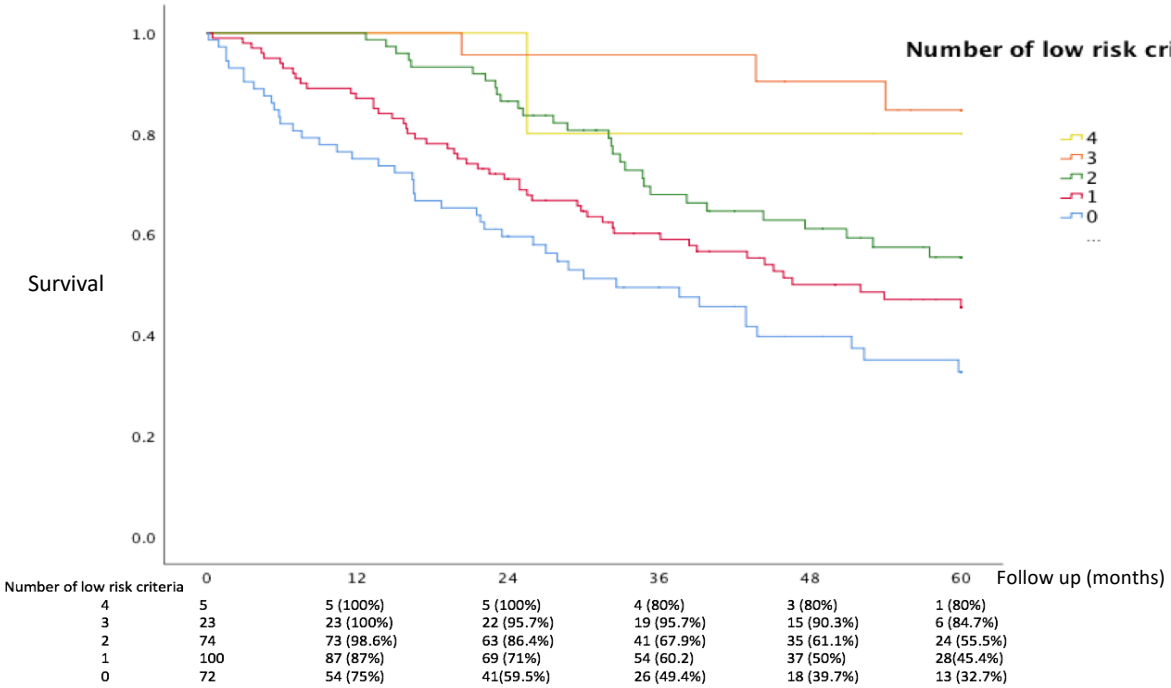
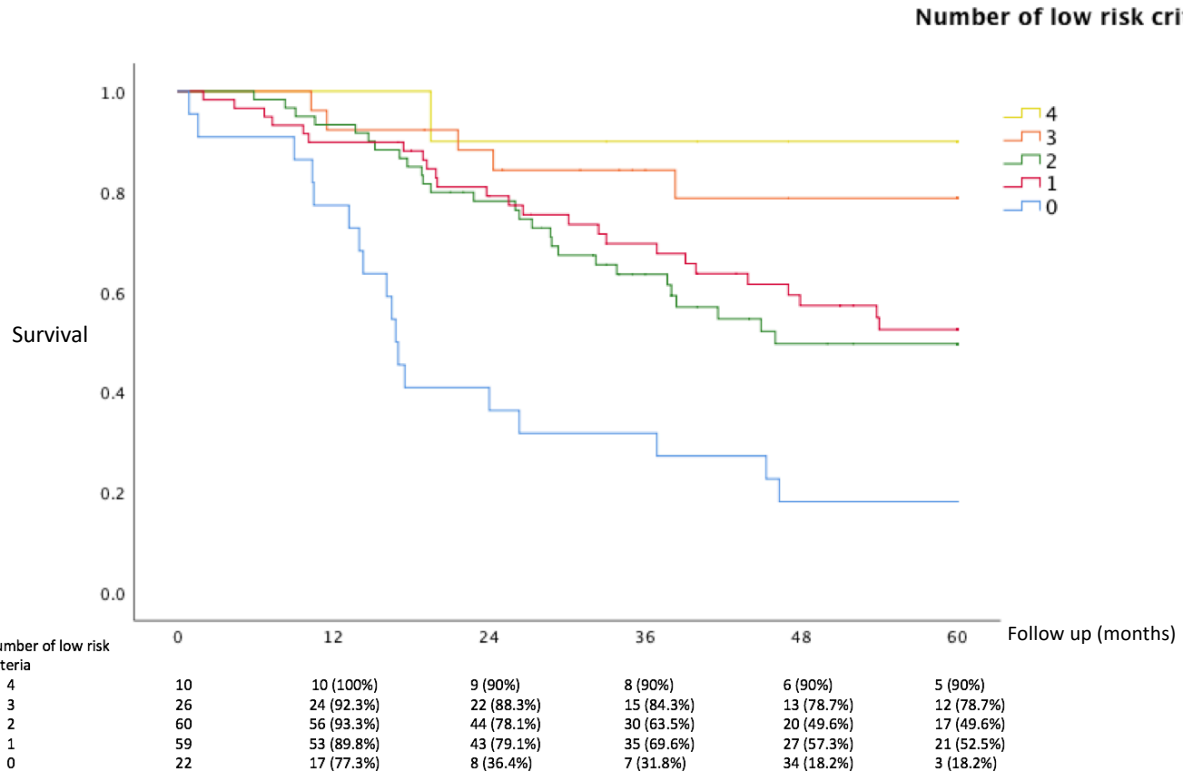


Figure 3.10: Kaplan-Meier survival curve for number of low risk criteria (WHO FC, 6MWD, RAP and CI) at baseline.

6MWD: 6-minute walking distance; CI: cardiac index; RAP: right atrial pressure; WHO FC: World Health Organisation functional class.



Number of low risk criteria						
4	10	10 (100%)	9 (90%)	8 (90%)	6 (90%)	5 (90%)
3	26	24 (92.3%)	22 (88.3%)	15 (84.3%)	13 (78.7%)	12 (78.7%)
2	60	56 (93.3%)	44 (78.1%)	30 (63.5%)	20 (49.6%)	17 (49.6%)
1	59	53 (89.8%)	43 (79.1%)	35 (69.6%)	27 (57.3%)	21 (52.5%)
0	22	17 (77.3%)	8 (36.4%)	7 (31.8%)	34 (18.2%)	3 (18.2%)

Figure 3.11: Kaplan-Meier survival curve for number of low risk criteria at follow up (WHO FC, 6MWD, RAP and CI)

6MWD: 6-minute walking distance; CI: cardiac index; RAP: right atrial pressure; WHO FC: World Health Organisation functional class.

The French non-invasive model

The same group also proposed a non-invasive model, with four levels of risk according to having either none, 1, 2 or all 3 of the low-risk criteria for WHO-FC, 6MWD and NT-Pro BNP [55]. Applying this score to the study population yielded moderately good overall discrimination (log rank p value <0.001) among the different risk categories at baseline and follow up (figures 3.12 and 3.13). Like the above model, the vast majority of patients were in the higher risk groups having none or only one low risk criteria at baseline (231 of 263 patients) and follow up (195 of 249 patients).

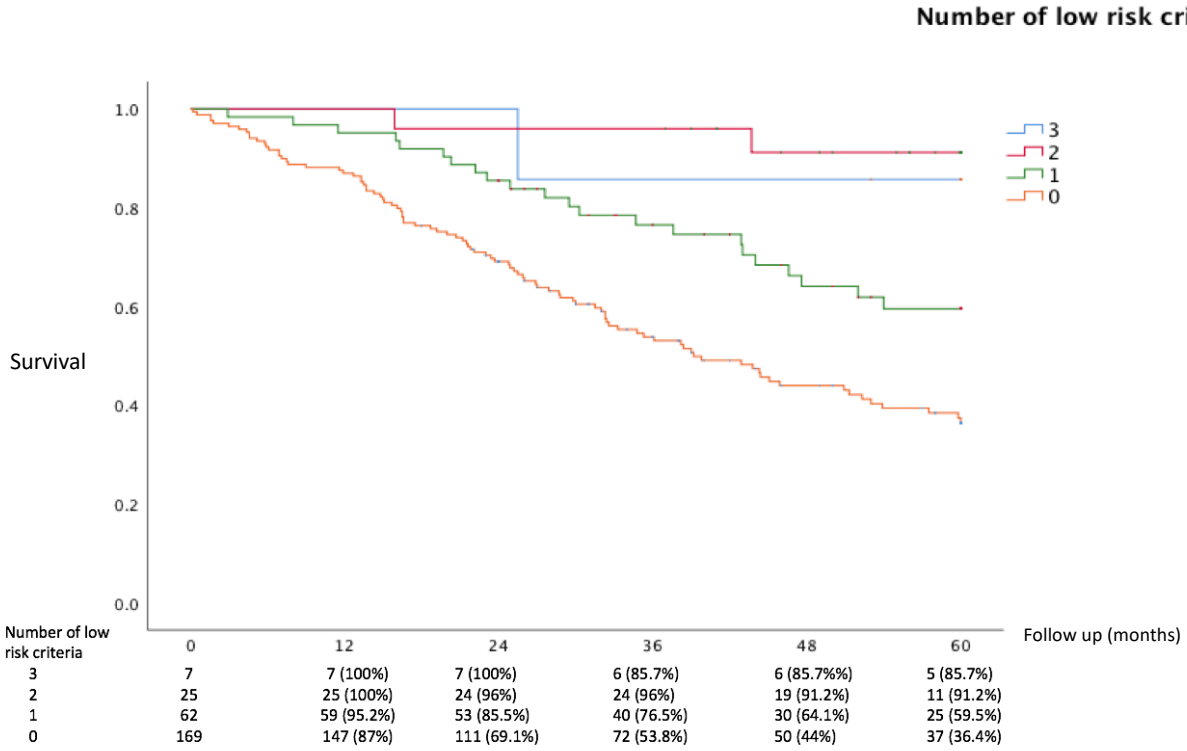
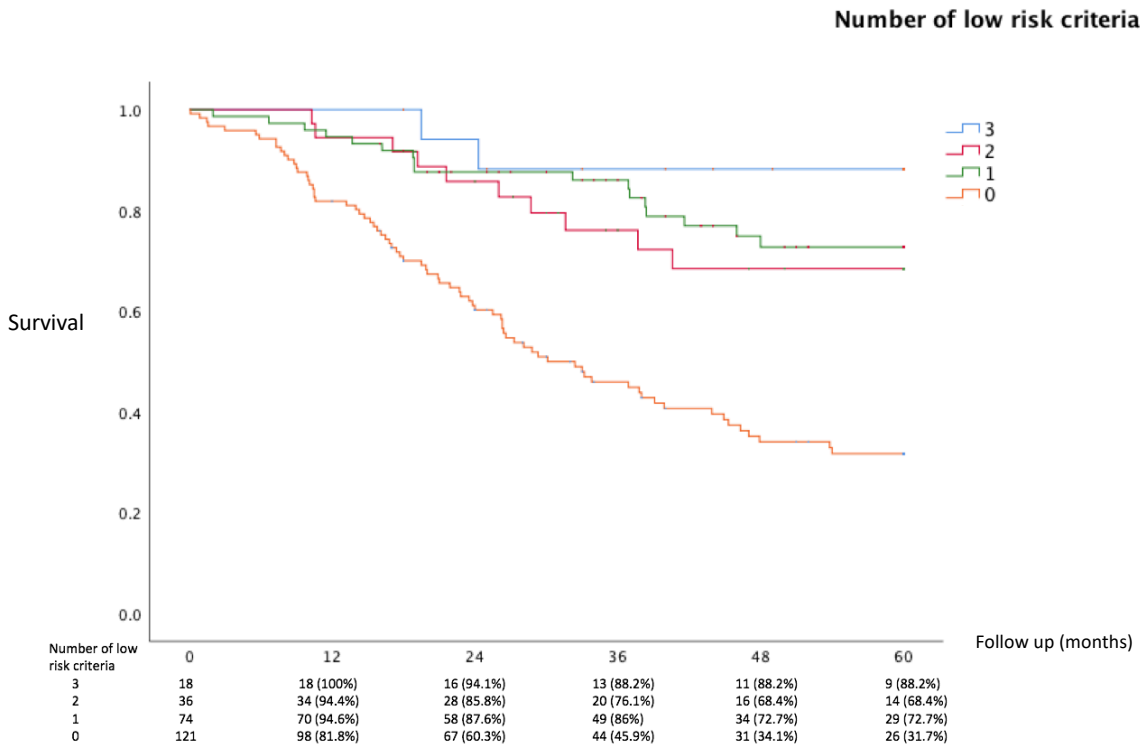


Figure 3.12: Kaplan-Meier survival curve for number of non-invasive low risk criteria at baseline (WHO FC, 6MWD and NT-proBNP)

6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; WHO FC: World Health Organisation functional class.



Number of low risk criteria	0	12	24	36	48	60
3	18	18 (100%)	16 (94.1%)	13 (88.2%)	11 (88.2%)	9 (88.2%)
2	36	34 (94.4%)	28 (85.8%)	20 (76.1%)	16 (68.4%)	14 (68.4%)
1	74	70 (94.6%)	58 (87.6%)	49 (86%)	34 (72.7%)	29 (72.7%)
0	121	98 (81.8%)	67 (60.3%)	44 (45.9%)	31 (34.1%)	26 (31.7%)

Figure 3.13: Kaplan-Meier survival curve for number of non-invasive low risk criteria at follow up (WHO-FC, 6MWD and NT-proBNP)

6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; WHO FC: World Health Organisation functional class.

Swedish/Compera model

A third method also based on the ESC/ERS 2015 guidelines was suggested by the Swedish group [53] and also applied on the COMPERA registry (that includes data from specialized centres from several European countries) [54]. Patients were categorized as low, Intermediate, or high risk according to the risk assessment instrument from the guidelines documented in table 3.5. After grading each of the available variables (WHO FC, 6MWD, NT-proBNP, RAP, CI, and SvO_2) and averaging the sum obtained, the result was rounded to the nearest integer and used to define each of the patient's risk group.

At baseline, this model resulted in good discrimination (log rank p <0.001) and the majority (about two thirds) were in the intermediate risk category. Those patients with a high risk calculated score at baseline had significantly worse outcome, with only 68% surviving the first year, while 100% and 94% survived in the low and intermediate risk groups, respectively.

On follow up, the distribution of patients across the three risk groups remained broadly similar, although there was an increased proportion of patients in the low risk category (19% to 27%). Survival one year after the first follow up assessment was 97% among those in the low risk group, 87.8% in the intermediate risk group and 57.7% in the high-risk group survival.

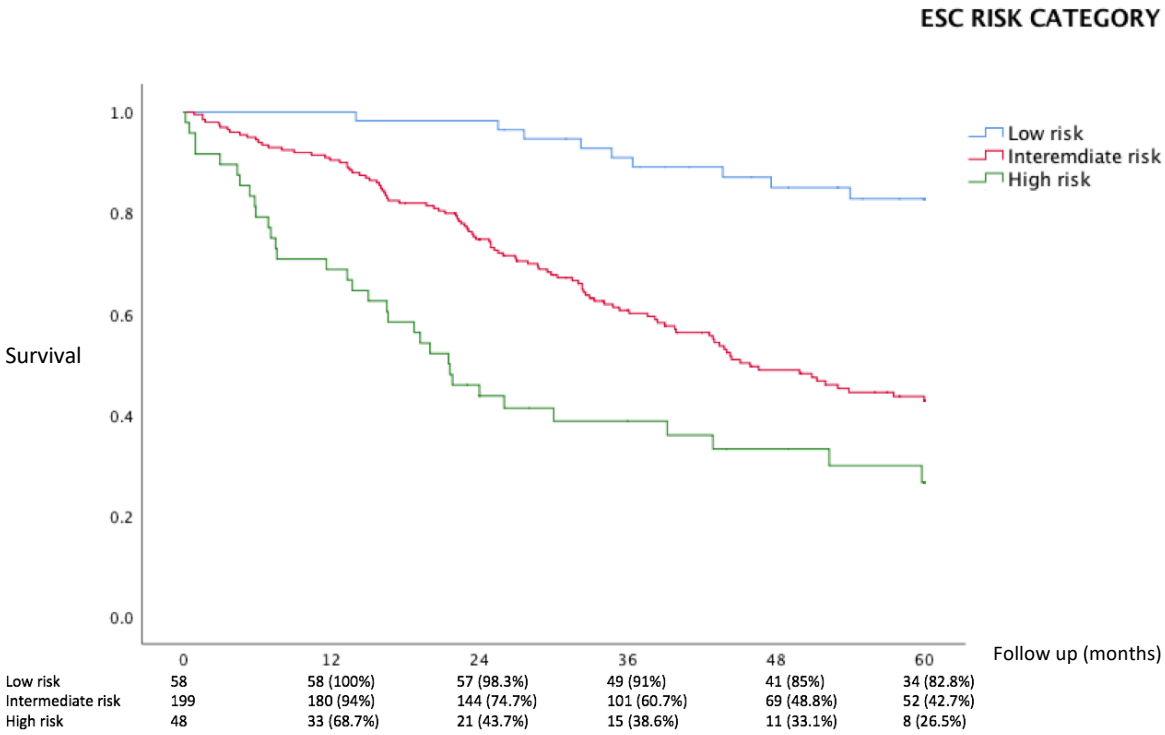
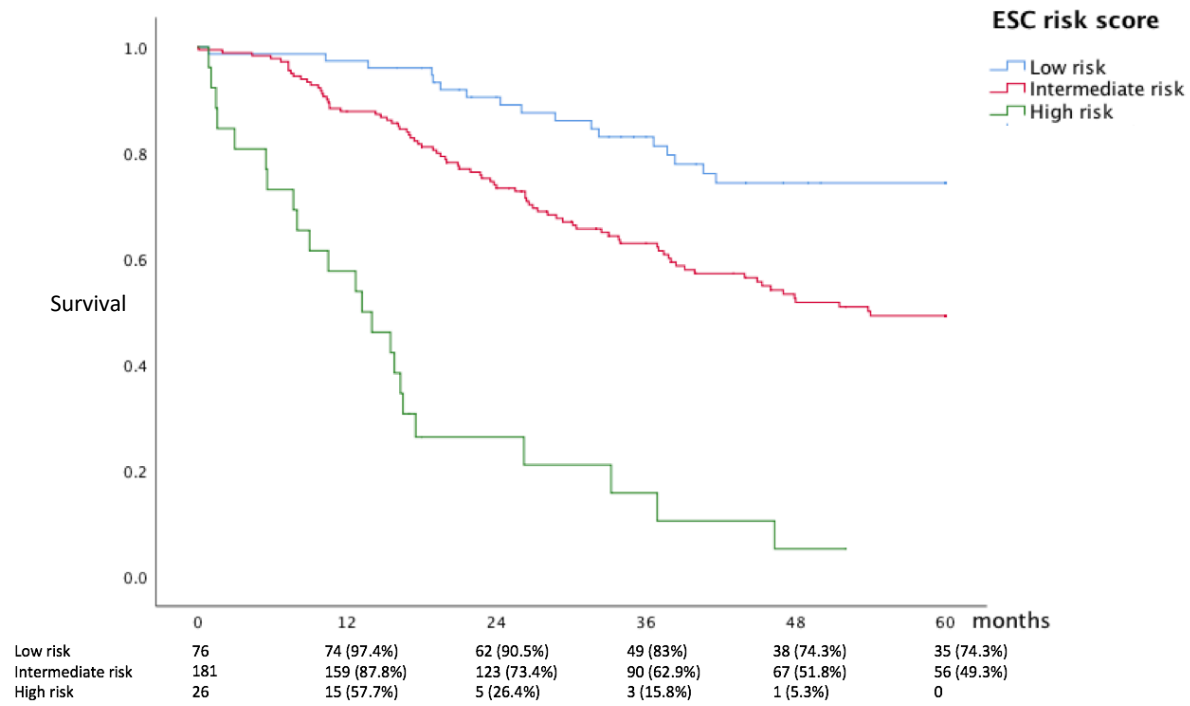


Figure 3.14: Kaplan-Meier survival curve as per the ESC risk score at baseline



Low risk	76	74 (97.4%)	62 (90.5%)	49 (83%)	38 (74.3%)	35 (74.3%)
Intermediate risk	181	159 (87.8%)	123 (73.4%)	90 (62.9%)	67 (51.8%)	56 (49.3%)
High risk	26	15 (57.7%)	5 (26.4%)	3 (15.8%)	1 (5.3%)	0

Figure 3.15: Kaplan-Meier survival curve as per the ESC risk score at follow up.

A proposed non-invasive model

As the multivariable analysis identified non-invasive parameters as independent predictors of outcome, we explored the value of averaging the non-invasive variables (WHO FC, 6MWD, NT-proBNP) using the ESC/ERS 2015 guideline cut-offs (table 5), similar to the method employed in the Swedish & COMPERA models. This approach also resulted in good discrimination (log rank p value <0.001) with a similar distribution to the previous method that also included the invasive parameters (i.e. the majority of patients were in the intermediate risk group both at baseline and follow up). Likewise, the high-risk group had a significantly worse survival rate where the 1 year, 3 year and 5-year survival rates were 71.4%, 34.9% and 25%, respectively at baseline and 68.2%, 21% and 12% when calculated at the time of follow up.

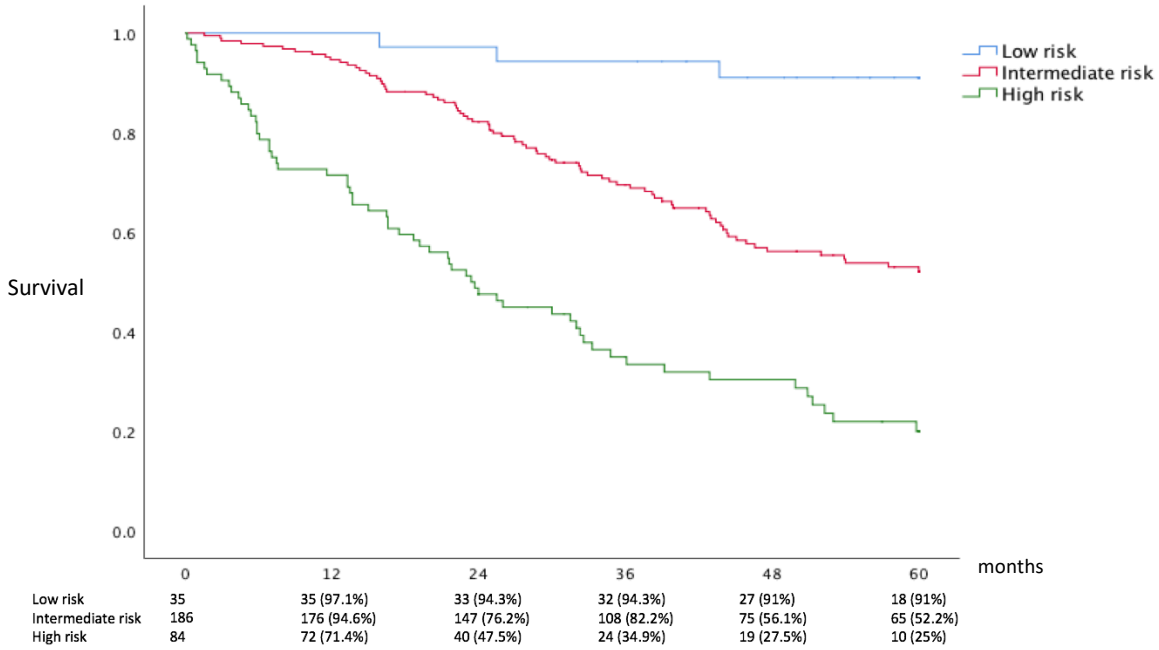


Figure 3.16: Kaplan-Meier survival curve as per the ESC risk score calculated using only the non-invasive parameters (WHO-FC, 6MWD and NT-proBNP) measured at baseline.

6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; WHO FC: World Health Organisation functional class.

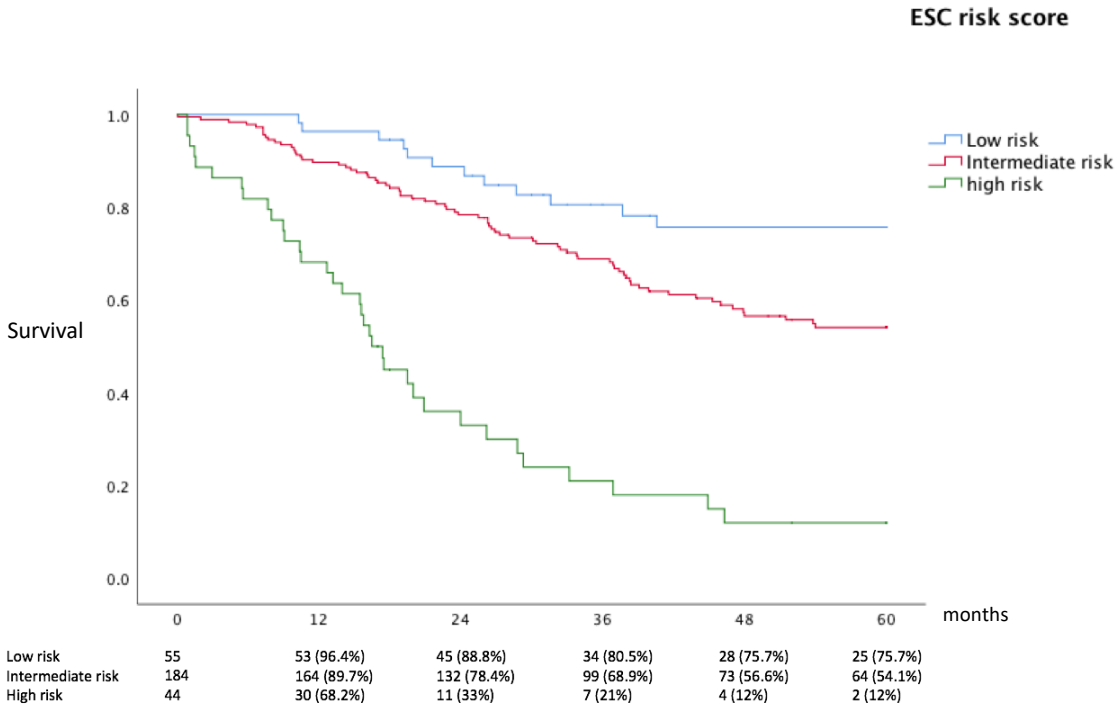


Figure 3.17: Kaplan-Meier survival curve as per the ESC risk score calculated using only the non-invasive parameters (WHO-FC, 6MWD and NT-proBNP) measured at follow up.

6MWD: 6-minute walking distance; NT-Pro BNP: N-terminal pro beta natriuretic peptide; WHO FC: World Health Organisation functional class.

4. Comparison of the different risk prediction models at baseline

Using C-statistic area under the curve (AUC) both sets of French scoring systems had the lowest AUC, being 0.65 and 0.64 for the non-invasive and full models, respectively (figure 20 and table 8). The full REVEAL 2.0 score had the highest AUC at 0.72, with the simplified REVEAL 2.0 yielding an AUC of 0.68. The simplified non-invasive ESC model improved the AUC (0.69) compared to the ESC score using all the variables (AUC 0.65).

The lowest Bayesian information criterion (BIC) was for the ESC non-invasive score at baseline indicated that it was best fit as a survival prediction model (table 3.11), while the highest BIC was for the REVEAL 2.0 simplified and full scoring systems respectively.

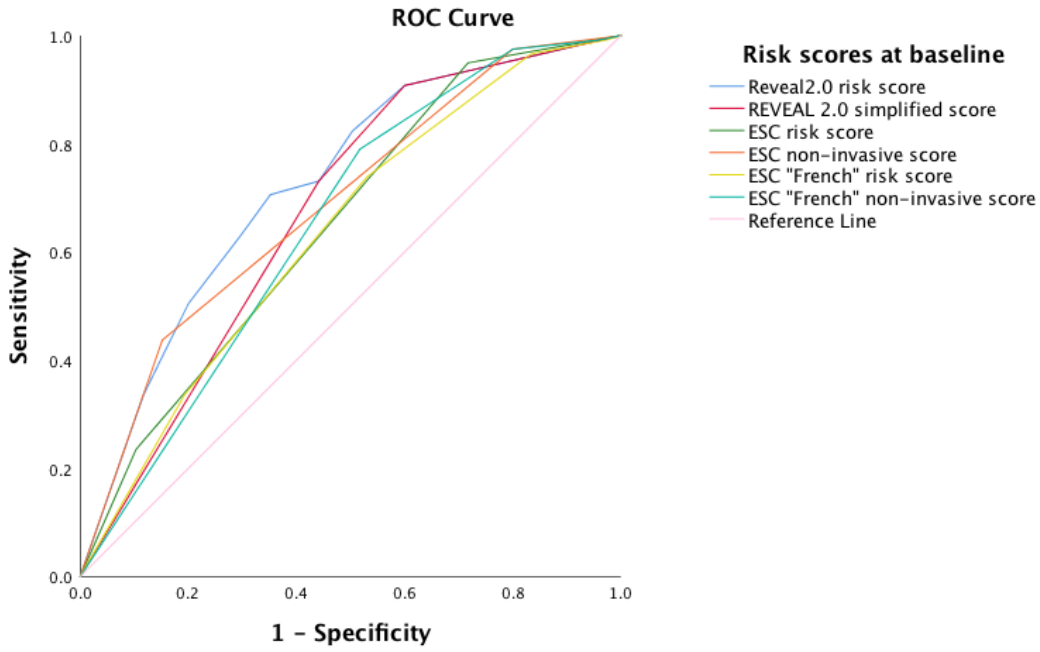


Figure 3.18: Receiver operating characteristic (ROC) analysis of the different risk assessment models

Risk prediction score	AUC	95% CI
REVEAL 2.0 score	0.72	0.66 - 0.79
ESC non-invasive score at baseline	**0.69**	**0.63 - 0.75**
REVEAL 2.0 simplified score	0.67	0.61 - 0.74
Swedish risk score	0.65	0.59 - 0.72
French non-invasive risk score	0.65	0.59 - 0.7
French risk score	0.64	0.57 - 0.71

Table 3.10: Receiver operating characteristic (ROC) calculated area under the curve (AUC) for different scores calculated at baseline (higher is preferred)

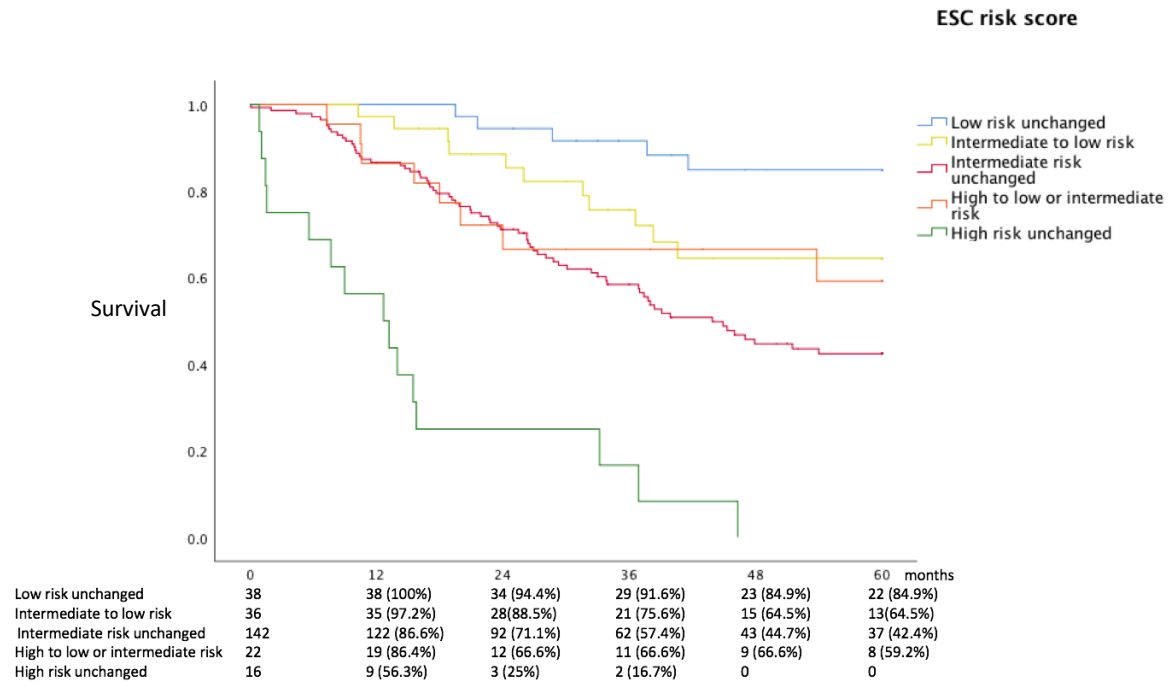
Risk prediction score	**BIC**
ESC non-invasive score	**1205.01**
French non-Invasive at baseline	1215.73
French invasive at baseline	1232.43
Swedish risk score	1234.7
REVEAL 2.0 score	1236.79
REVEAL 2 simplified score	1243.55

Table 3.11: Bayesian information criterion (BIC) for different risk models at baseline (lower figure means a better fit)

5. Effect of ESC risk score modification on outcome

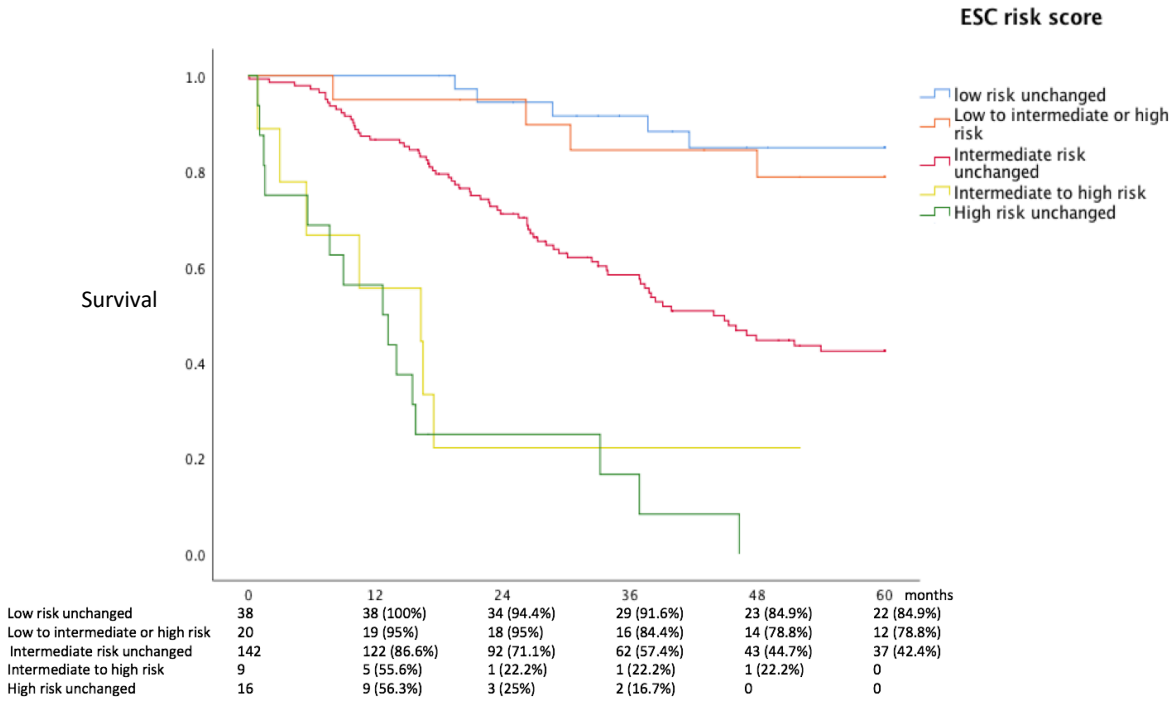
An assessment of the effect of improving or worsening ESC risk profile (measured by averaging the risk parameters measured as per the Swedish model) was undertaken at the time of follow up. Altering the risk score was associated with a significantly different one-year outcome (figures 3.19 and 3.20). Patients with a high risk status at baseline that improved to low or intermediate risk profile at the time of follow up had a one-year survival of 86.4%, which was considerably better than those who remained high risk at the time of follow up (one-year survival of 56.3%). Among patients that had worsening profile at the time of follow up, one-year survival was 55.6% for those whose risk profile deteriorated from intermediate to high risk compared to 86.6% for those whose risk level remained intermediate. Interestingly, although only a small number of patients that had been low risk at baseline worsened at the time follow up (to intermediate or high risk), there was no obvious impact on one-year survival (95% at one year).

Confining analysis to the non-invasive ESC scoring system, improvement from high to intermediate risk was associated with improved survival. In contrast, a worsening from intermediate to high risk had much worse survival. However other risk group changes were not associated with significant changes in outcome (Figures 3.21 and 3.22).



Low risk unchanged	38	38 (100%)	34 (94.4%)	29 (91.6%)	23 (84.9%)	22 (84.9%)
Intermediate to low risk	36	35 (97.2%)	28(88.5%)	21 (75.6%)	15 (64.5%)	13(64.5%)
Intermediate risk unchanged	142	122 (86.6%)	92 (71.1%)	62 (57.4%)	43 (44.7%)	37 (42.4%)
High to low or intermediate risk	22	19 (86.4%)	12 (66.6%)	11 (66.6%)	9 (66.6%)	8 (59.2%)
High risk unchanged	16	9 (56.3%)	3 (25%)	2 (16.7%)	0	0

Figure 3.19: Kaplan-Meier survival curve as for those who had their ESC risk category improved against those whose risk was unchanged at follow up.



Low risk unchanged	38	38 (100%)	34 (94.4%)	29 (91.6%)	23 (84.9%)	22 (84.9%)
Low to intermediate or high risk	20	19 (95%)	18 (95%)	16 (84.4%)	14 (78.8%)	12 (78.8%)
Intermediate risk unchanged	142	122 (86.6%)	92 (71.1%)	62 (57.4%)	43 (44.7%)	37 (42.4%)
Intermediate to high risk	9	5 (55.6%)	1 (22.2%)	1 (22.2%)	1 (22.2%)	0
High risk unchanged	16	9 (56.3%)	3 (25%)	2 (16.7%)	0	0

Figure 3.20: Kaplan-Meier survival curve as for those who had their ESC risk category worsened against those whose risk was unchanged at follow up.

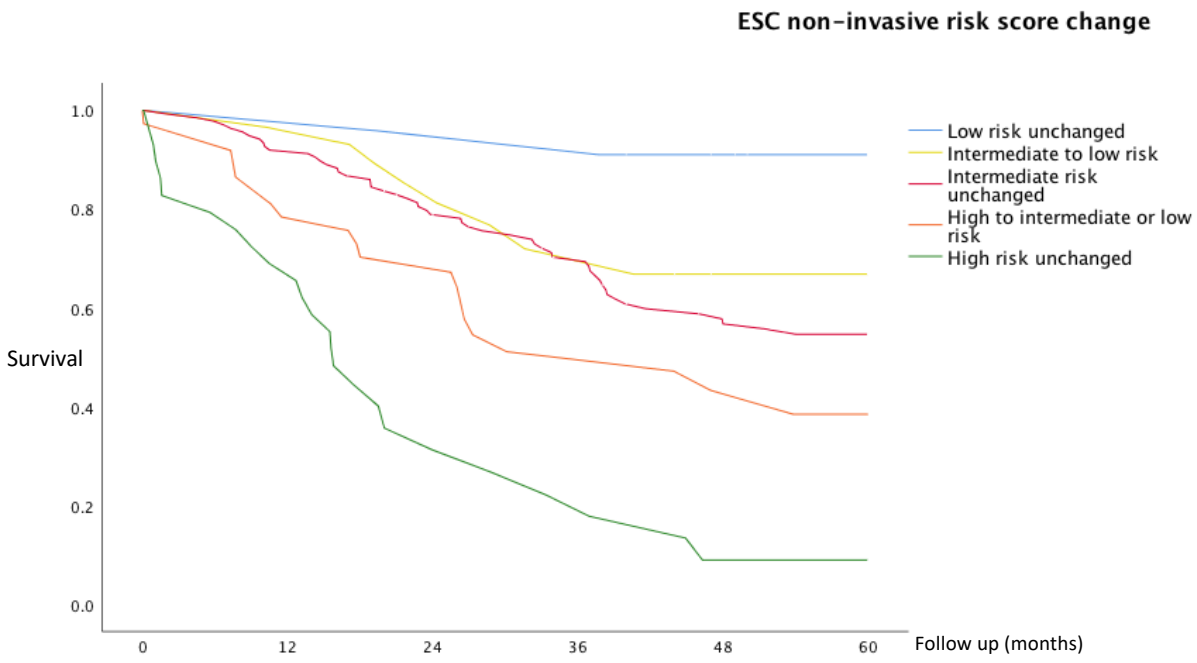


Figure 3.21: Kaplan-Meier survival curve as for those who had their ESC non-invasive risk category improved against those whose risk was unchanged at follow up.

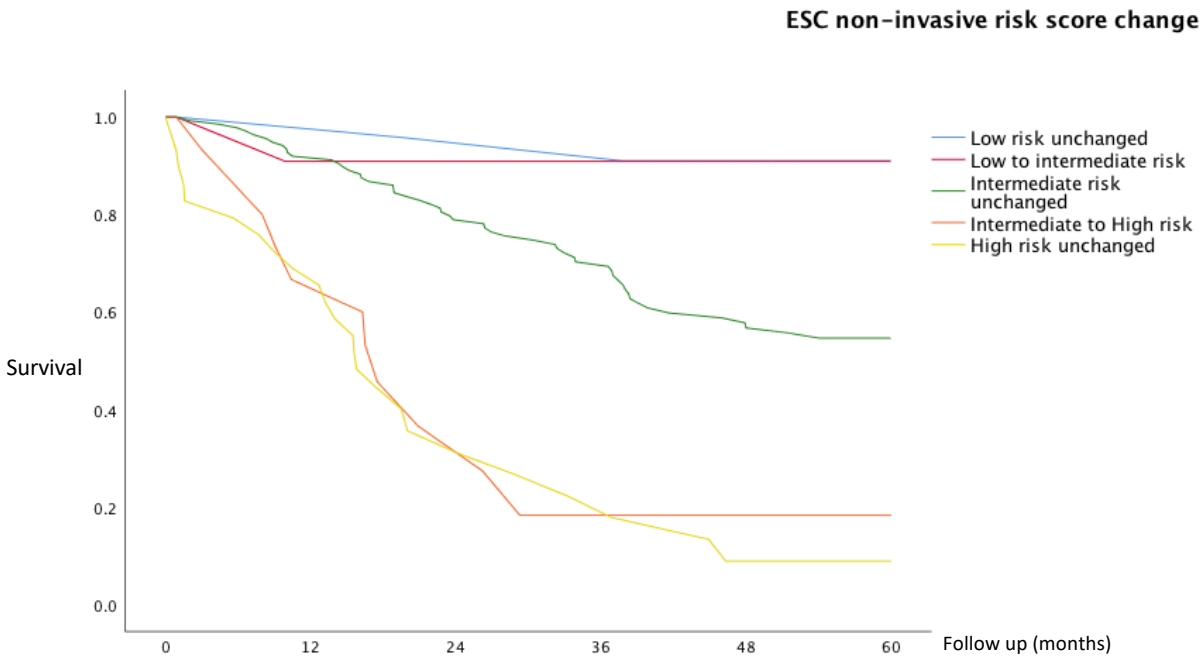


Figure 3.22: Kaplan-Meier survival curve as for those who had their ESC non-invasive risk category worsened against those whose risk was unchanged at follow up.

3.4 Discussion

The main finding of the current study is that in a large group of SSc-PAH patients, using thresholds derived predominantly from IPAH population, the proposed risk models could all discriminate between lower and higher risk. The discriminatory value was also clear at both baseline and on first follow up. Comparative analysis shows that the REVEAL 2.0 score outperforms the Swedish and the French non-invasive and invasive models in its prognostic ability. Of note, some of these scores were limited in the ability to discriminate those with the poorest prognosis among SSc PAH patients.

The French proposed methods of calculating risk are the simplest to calculate and rely on a limited number of parameters (4 or 3) which can be readily incorporated into standard clinical practice. It also identifies a truly very low risk group with very low annual mortality. However, a major limitation applying these scores on a SSc population (especially the non-invasive score), is that the bulk of patients have either no or only one low risk feature. The upshot of this is the inability to discriminate those with the poorest survival. A similar conclusion was reached by the French group when they applied their model on a SSC-PAH population, which resulted in only a modest discrimination at baseline [87]. This seems to be an inevitable consequence of disregarding high-risk data and data derived from other variables.

The full REVEAL 2.0 risk score was the most accurate method in prognostication particularly at baseline as demonstrated by the highest AUC. While this method is well validated, it is nonetheless more complex to calculate. The simplified REVEAL 2.0 version performs less well as it does not identify those patients with the highest risk.

Another limitation of REVEAL 2.0 is the incorporation of non-modifiable risk factors (age, sex, aetiology of PH and comorbidities) in its calculation. While this seems to increase its prognostic validity (as evidenced by higher AUC on the C-statistic), this limits its value when identifying those whose worse prognosis is driven by modifiable risk factors, which would be of particular importance in determining those appropriate for escalated intervention.

The average score of the ESC/ERS 2015 guideline risk categories that have been used in the Swedish and the COMPERA registries performs nearly as well as the REVEAL 2, the difference being that it only includes modifiable parameters. This method can be calculated easily even with some missing data. This risk profiling system identifies three risk groups that have a very different calculated prognosis. However, the allocated risk are different in SSc when compared to that reported in IPAH – with an annual mortality of 3%, 15% and 27% in the low, intermediate and high risk at baseline, and 6% and 14% in low and intermediate risk from follow up (the annual mortality of the high risk from follow up was likely very high but not calculated due to small numbers at first follow up). This indicates that this score identifies a truly high-risk group that should be considered a target for very early aggressive therapy from the outset and the immediate consideration of referral to transplant. We further observed that at the time of first follow up a change in risk identified patients whose prognostic risk had either improved or worsened.

An abbreviated ESC/ERS risk score using only the non-invasive variables (WHO-FC, 6MWD and NT-proBNP) also provides discriminatory value and may be particularly appropriate for the SSc population, since there is a well-recognised disconnect

between haemodynamic severity and outcome. This method is very easy to calculate so potentially user friendly for regular follow up and serial assessments and appears to identify low and intermediate risk populations very clearly at baseline. Using this model, the risk associated with the low and intermediate risk category at first follow up is nearly twice the risk of the same category at baseline. Suggesting that achieving a lower risk profile on therapy is of less benefit than presenting at the same profile before treatment.

Finally, by comparing our results to the respective groups validation work on PAH patients in general, one can conclude that though most of the mortality difference between SSc-PAH and IPAH can be attributed to a greater burden of modifiable risk factors at baseline and follow up. The resulting annual mortality remains consistently higher for each risk group than reported in IPAH populations – confirming that SSc-PAH is associated with a worse outcome than IPAH for equivalent risk category.

The main role of a powerful risk assessment tool is to discriminate different levels of risk, to accurately predict prognosis and to dynamically reflect change in predicted outcome with the change in risk profile. Each of the discussed models have shown different strengths and weaknesses and clinicians should be mindful of these points when applying these models.

Our reported data analysis has several advantages. It reports on a real-life group of phenotypically similar patients (SSc-PAH) and describes their real-life course of management (e.g. if diagnosis changed after initial assessment) within a large national pulmonary hypertension service. This data is of particular value as SSc-PAH seem to have a very distinct phenotype with a unique natural history. This has been

previously explained by pulmonary, cardiac and musculoskeletal involvement, with systemic manifestations and more comorbidities [88]. Additionally, pulmonary vascular disease is not restricted to the precapillary vessels but does extend to pulmonary capillaries and/or veno-occlusive disease [89]. These features have been proposed as explanations for the blunted response to therapy and relatively poorer survival. We have reported on available non-invasive and haemodynamic profile with limited missing diagnostic data at baseline and follow-up except for invasive haemodynamic data at follow up, which was done as per the treating physician's discretion. Our group of patients is also a contemporaneous group of patients and this was reflected by the choice of the initial therapeutic strategy. Most importantly we have demonstrated that adjusting the risk and improving (or worsening) risk profile was associated with different survival. In particular the very poor prognosis of patients that are high risk at baseline and more so for those who remain at high risk at the time follow up, emphasises the need for aggressive therapy (IV prostanoid therapy and consideration of transplantation) from the onset [2]. On the other hand, improved survival was seen in those patients with an improved low risk profile at the time of follow-up. This would support the current treatment guidelines that advocates aiming to achieve low risk profile on serial risk assessments.

Our study is a consecutive observational analysis from a single centre experience of a unique population. As this was an observational study, the findings may be subject to confounding factors for which we have been unable to control. Our data set includes all major clinical variables known to affect outcome, which would strengthen the validity of our results. However, we have not included several parameters that

were part of the REVEAL 2.0 score (history of hospitalisations, vital signs and echo data) or the ESC/ERS 2015 guidelines risk stratification tool (clinical history and imaging data in particular). Again, these may have had an influence on our findings.

3.5 Conclusion

All proposed risk scoring systems for PAH have were effective in discriminating between different risk levels in SScPAH. The Reveal 2.0 and Swedish models outperform the French models in this population of patients. The simplified Reveal 2.0 model given consistent prognostic accuracy both at baseline and first reassessment at 6 months, recognising that most of our patients were in the higher risk group at baseline. Inclusion of non-modifiable factors does, however, appear to limit its value for serial risk assessments. The ESC based models not only provide a similar discriminatory value, but also provides feedback on the impact of response to therapeutic intervention and may be the most useful in clinical practice.

Chapter 4 The role of microvesicles in the screening and risk assessment of systemic sclerosis associated pulmonary arterial hypertension

4.1 Introduction

Pulmonary hypertension (PH) is a progressively debilitating and ultimately fatal condition [90]. The Pathobiology of this disease involves phenotypic changes in the pulmonary arterial endothelium, smooth muscle cells and fibroblasts, inflammatory cells infiltration leading to extensive remodelling of the lung with in situ thrombosis [91, 92]. Patients are usually asymptomatic until the disease is fairly advanced, and the clinical profile can overlap with many other clinical conditions, rendering early diagnosis difficult.

Pulmonary arterial hypertension (PAH) is a common complication of systemic sclerosis (SSc) with an estimated prevalence of 10-15% [17 75 76] and is a leading cause of death in this population of patients [77 78 79]. Moreover, it has been reported that up to 20% of SSc patients can be asymptomatic at the time of PAH diagnosis [18 19]. A meta-analysis reported pooled 1-,2- and 3-year survival to be 81%, 64% and 52%, respectively in incident SSc PH [20]. The high prevalence rate, non-specific symptoms and high morbidity and mortality from PAH, as well the potential benefit from early intervention with more widely available therapeutic options, provide a strong rationale for active screening programs.

Several screening algorithms have been suggested that combine clinical, pulmonary function tests (PFTs) and chemical biomarkers in addition to echocardiography. The ESC/ERS 2009 guidelines has suggested an algorithm which was updated in the latest 2015 version. DETECT (Early, Simple and Reliable Detection of Pulmonary Arterial Hypertension in Systemic Sclerosis) algorithm was developed in 2013 following a large prospective and multicentre study in SSc patients with higher risk of PAH (>3 years' duration from first non-Raynaud's symptom and DLCO <60% predicted) [9].

Cardiac catheterisation remains the gold standard for establishing the diagnosis and for the assessment of haemodynamics on follow up in patients with suspected PH. This is as an invasive procedure that is not without risk, limiting its use as a screening tool. Currently, NT-proBNP is the only widely used serum biomarker in clinical trials and registries and is able to provide valuable prognostic data [93]. However, it is a non-specific marker of ventricular stress and thus a marker of advanced disease.

Extracellular vesicles (EVs) are membrane-contained vesicles released in an evolutionally conserved manner by cells ranging from organisms such as prokaryotes to higher eukaryotes and plants [94]. EVs were first described as platelet-derived particles in normal plasma in 1946 by Chargaff and West [95]. Another early report in 1967 that is often quoted is by Wolf when it was also described as "platelet dust" [96]. More recently, with the discovery that EVs contain RNA, including microRNA, EVs acquired substantially renewed interest as mediators of intercellular communication [97].

EVs can be broadly classified into 3 main classes [94]: (a) Microvesicles (also known as microparticles or ectosomes) that are produced by outward budding and fission of the plasma membrane; (b) Exosomes that are formed within the endosomal network and released upon fusion of multi-vesicular bodies with the plasma membrane; and (c) Apoptotic bodies are released as blebs of cells undergoing apoptosis.

Microvesicles (MVs) are widely regarded to measure between 100 to 1000nm and are released by different cell types into the circulation when they become activated or undergo apoptosis [68, 69]. While original interest has focused mainly on MVs as markers of disease, the understanding of their role in intercellular communication has been evolving rapidly [98]. Principally, 2 main mechanisms by which MVs can mediate. First, MVs may act as circulating signalling modules affecting cellular properties and responses by activation of receptors on the target cell via presentation of membrane-associated bioactive molecules. Second, MVs may mediate signalling by directly transferring part of their content or components including proteins, bioactive lipids or RNA to the recipient cell, potentially resulting in cell activation, phenotypic modification and reprogramming of cell function [98].

As MVs are formed via the direct outward blebbing and pinching of the plasma membrane of activated cells, they have membrane receptors and proteins that are related to the parent cell from which they were derived. They richly expose phosphatidylserine (PS) on their membranes, and the calcium-dependent phospholipid, Annexin V+, which has high affinity for PS [68]. By using flow cytometry targeting cell specific antigens or combination of antigens, it is possible to identify their cellular origins with a high degree of certainty.

The role of MVs as a potential biomarker in PH has been previously proposed by Amabile and colleagues who found that levels of endothelial and leukocyte MVs were elevated in patients with precapillary PH compared to control subjects [70]. In addition, levels of PECAM(+) and VE-cadherin(+), but not E-selectin(+), endothelial MVs predicted hemodynamic severity of the disease. Further work by the same group also showed that there was an association with adverse outcomes of another endothelial subpopulation of MVs, namely those staining with CD62 [71]. In another study reported by Bakouboula and colleagues, elevated levels of endoglin+ (CD105+) MVs were observed in PAH patients compared with control subjects [72]. They also found higher levels in patients with more advanced disease (functional class III or IV and 6MWD <380m). More recently, it was found that patients with SSc-PAH, had elevated levels of CD144+ endothelial MVs in comparison to SSc patients with no PH or healthy controls [73]. Thus, MVs may exhibit the properties of being potential biomarkers, possibly being associated with the inflammatory status, the degree of vascular remodelling and/or tissue damage, important pathophysiological features of PAH. That endothelial dysfunction is an early feature of PAH, may point to MVs being of clinical value as a screening tool in SSc patients.

In work underpinning this research proposal, Professor Clapp's team [74] has characterised in the venous blood of patients with severe PAH subpopulations of MVs staining with markers for endothelial cells (PECAM-1+/CD41-/PDGFRβ-), smooth muscle cells (PDGFRβ+/NG2+/PECAM1-), platelets (CD42a+) and leukocytes (e.g. CD16) by fluorescence-activated cell sorting (FACs) [99]. Thus, MVs are a potential *in vivo* surrogate of vascular remodelling.

The objective of this work was to determine if the total or levels of different MV populations would be useful in screening for SSc patients who might go on to develop or who have PH. This work also aimed to elucidate if MV levels would be a predictor of risk profile of these patients.

4.2 Patients and methods

All adult patients with SSc that were ⩾18 years that were seen in the Pulmonary Hypertension National Service of the Royal Free Hospital (from May 2018 till May 2019 and referred for cardiac catheterisation) were invited to partake in the study and then enrolled after consent. SSc was diagnosed based on the contemporary American College of Rheumatology/European League Against Rheumatism criteria [80].

The Scleroderma Cohort study (SMART), IRAS ID 159715 provides ethics approval for the use of clinical data collected on patients with scleroderma attending the Royal Free Hospital. All patients included are consented prior to data and sample collection.

Baseline characteristics included demographics, scleroderma profile and other organ involvement diagnosed at time of enrolment. Non-invasive assessments (WHO FC, 6MWD and NT-Pro BNP) and full haemodynamic profile (right atrial pressure, mean pulmonary artery pressure, cardiac output, cardiac index, pulmonary vascular resistance and pulmonary artery oxygen saturation) were recorded.

Precapillary PH was identified following right heart catheterisation (RHC) and was defined as mean pulmonary artery pressure (mPAP) of ⩾25, PCWP ⩽15mmHg and pulmonary vascular resistance (PVR) ⩾3 Wood units. Significant lung disease was excluded using both lung function testing (FVC >60%, FEV1 >50%, FEV1/FVC >70%) and CT chest assessment for ILD (<20% parenchymal involvement), emphysema (< 5%) or ⩾ 2 features compatible with pulmonary venous occlusive disease (PVOD). Left heart disease was excluded on echocardiography (LVEF <50%, significant valvular disease, IVSD >1.2cm, left atrial area >20cm^2). Patients with disproportionate precapillary pulmonary hypertension (mPAP >35mmHg, PVR >5WU), were also considered to have PAH if identified lung disease had been demonstrated as stable for more than 2 years on high resolution CT chest (HRCT) and lung function testing, The presence of modest diastolic abnormalities on echocardiography (e.g. isolated left atrial enlargement), was permissible if all other data supported a precapillary cause. Chronic thromboembolic disease (CTEPH) was excluded using V/Q scanning or by pulmonary angiography.

Patients with left heart disease were classified as WHO pulmonary hypertension (PH) group 2, while patients with significant lung disease on high resolution CT chest (HRCT) or on pulmonary function test (PFTs) were classified as group 3 and patients with significant thromboembolic disease on angiography were classified as group 4.

Samples collection and MVs identification process

For every patient undergoing the clinically indicated cardiac catheterisation, 4 ml blood samples were obtained from 4 different sites (peripheral vein, pulmonary artery, pulmonary wedged "capillary" and peripheral artery) into sodium citrate vacutainer tubes (BD, Oxford, United Kingdom). Bloods were processed in Professor Lucie Clapp's laboratory at UCL's Institute of Cardiovascular Science (Rayne building, London UK).

Preparation of platelet poor plasma (PPP)

In order to remove different cell types in the blood including erythrocytes, leukocytes, lymphocytes and platelets, a double centrifugation was applied to obtain platelet poor plasma (PPP) using an adapted protocol [100]. First, the blood was centrifuged at 5,000g for 15 minutes to obtain plasma, which was then stored in 1.5 ml Eppendorf tubes at -80^{o}C until further use. For batch analysis, the plasma was rapidly thawed in a 37^{o}C water bath and centrifuged a second time at 5,000g for 5 minutes to remove most platelets. This would ensure that the PPP is depleted from the majority of platelets, enabling for clearer analysis via flow cytometry. Aliquots of PPP (100µl) were transferred to new 1.5ml Eppendorf tubes and centrifuged at 17,000g for 60 minutes at 4^{o}C. Most of the supernatant was decanted leaving an MV pellet of approximately 15µl in volume. The MV pellet was then reconstituted in 175µl of Annexin V binding buffer (BD Pharmingen, Wokingham, UK), divided into 35µl aliquots and plated into a 96 well U-bottomed polypropylene plate (Greiner, Sigma Aldrich, Gillingham, UK).

Identification of MVs from PPP

Annexin V conjugated to fluorescein isothiocyanate (FITC) was diluted in Annexin V binding buffer (BD Pharmingen, Wokingham, UK) at a 1:10 dilution and 5µl of that was added to appropriate wells of a 96-well U-bottom multiplate to assess total MV counts. Monoclonal mouse anti-human antibodies against cell surface markers were used to characterise the cell of origin from which the MVs are derived, details of these antibodies are outlined in table 4.1. Endothelial MVs were identified by being positive to anti-human platelet endothelial cell adhesion molecule (PECAM1)/CD31 and CD42a negative. Smooth muscle MVs were identified as being negative to PECAM1 and positive to any of the following antibodies: anti-human platelet-derived growth factor receptor beta (PDGFRβ), anti-human endoglin/CD105, anti-human neural glial antigen 2 (NG2) or anti-human intracellular cell adhesion molecule (ICAM)/CD54. For identifying platelet MVs in PPP, samples were incubated with mouse anti-human glycoprotein IX (GP9)/CD42a.

Antibody	Conjugated fluorophore	Company (Catalogue number)	Dilution
Annexin V	FITC (fluorescein isothiocyanate)	BD Pharmingen (556419)	1:100
PDGFRβ (platelet-derived growth factor receptor beta)	PerCP-Cy5.5 (peridinin-chlorophyll-protein - Cyanine7)	BD Pharmingen (558774)	1:50
NG2 (neural glial antigen 2)	PE (phycoerythrin)	R&D Systems (MA5-28549)	1:50
Endoglin (CD105)	PE/Cy7 (phycoerythrin/Cyanine7)	Biolegend (120409)	1:50
ICAM1 (intracellular cell adhesion molecule)	BV510 (Brilliant Violet 510)	BD Pharmingen (740170)	1:50
PECAM1 (human platelet endothelial cell adhesion molecule)	APC-Cy7 (allophocyanin- Cyanine7)	BD Pharmingen (563653)	1:50
CD42a / glycoprotein IX (GP9)	BV421 (Brilliant Violet 421)	BD Pharmingen (565444)	1:50

Table 4.1: Fluorochrome conjugated antibodies and reagents used for flow cytometry analysis of microvesicles. As annexin V is a protein and not an antibody, FITC-annexin V does not have a clonal origin, unlike the antibodies used to label receptors and their respective isotype control antibodies.

Relevant isotype control antibodies were also used on all samples to distinguish non-specific staining (false positive). Single colour control staining by annexin V-FITC, PE-annexin V, and PECAM1-APC-Cy7 were used to compensate digitally during analysis on the FlowJo software (Tree Star Inc., Oregon, USA) after sample acquisition. This method of compensation is essential during multi-colour flow cytometric analysis, as it corrects for spill over, which happens when the fluorescent emission of a fluorochrome is registered by a detector designed to measure the signal of another fluorochrome. All antibodies were diluted in phosphate buffer saline (PBS) containing 0.01% foetal bovine serum (FBS) and used at final dilutions (1:50 or 1:100), figure 4.1. After adding the antibodies (test and isotype), annexin V and samples, the 96 well plate was covered with aluminium foil to shield from light and incubated at room

temperature on a gentle plate shaker for 15 minutes. After that 200µl of Annexin V binding buffer was added to each well to dilute the samples and terminate the staining.

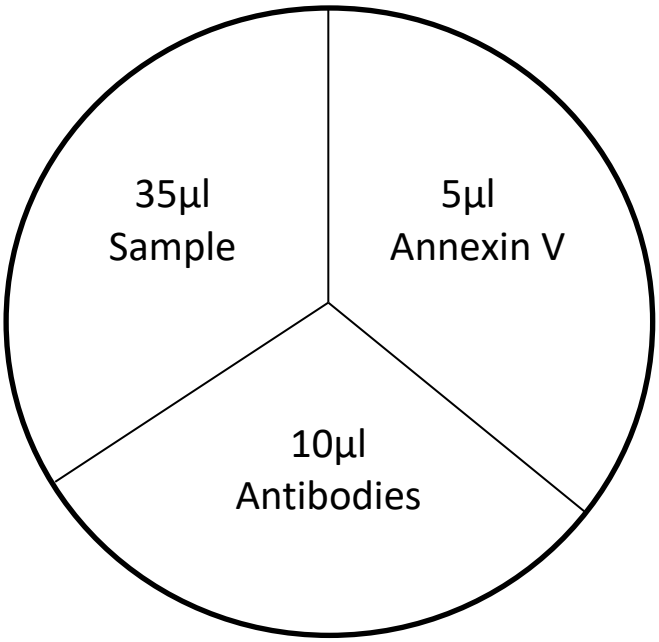


Figure 4.1: The constitution of each well of the analysed sample plated into a 96 well U-bottomed polypropylene plate (Greiner, Sigma Aldrich, Gillingham, UK). 5µl Annexin V (1:10 dilution) and 10µl of antibodies (1µl of each of PECAM1, PDGFRβ, Endoglin, ICAM1, NG2 and CD42 added to 4µl of PBS/FBS) were added to 35µl of the sample.

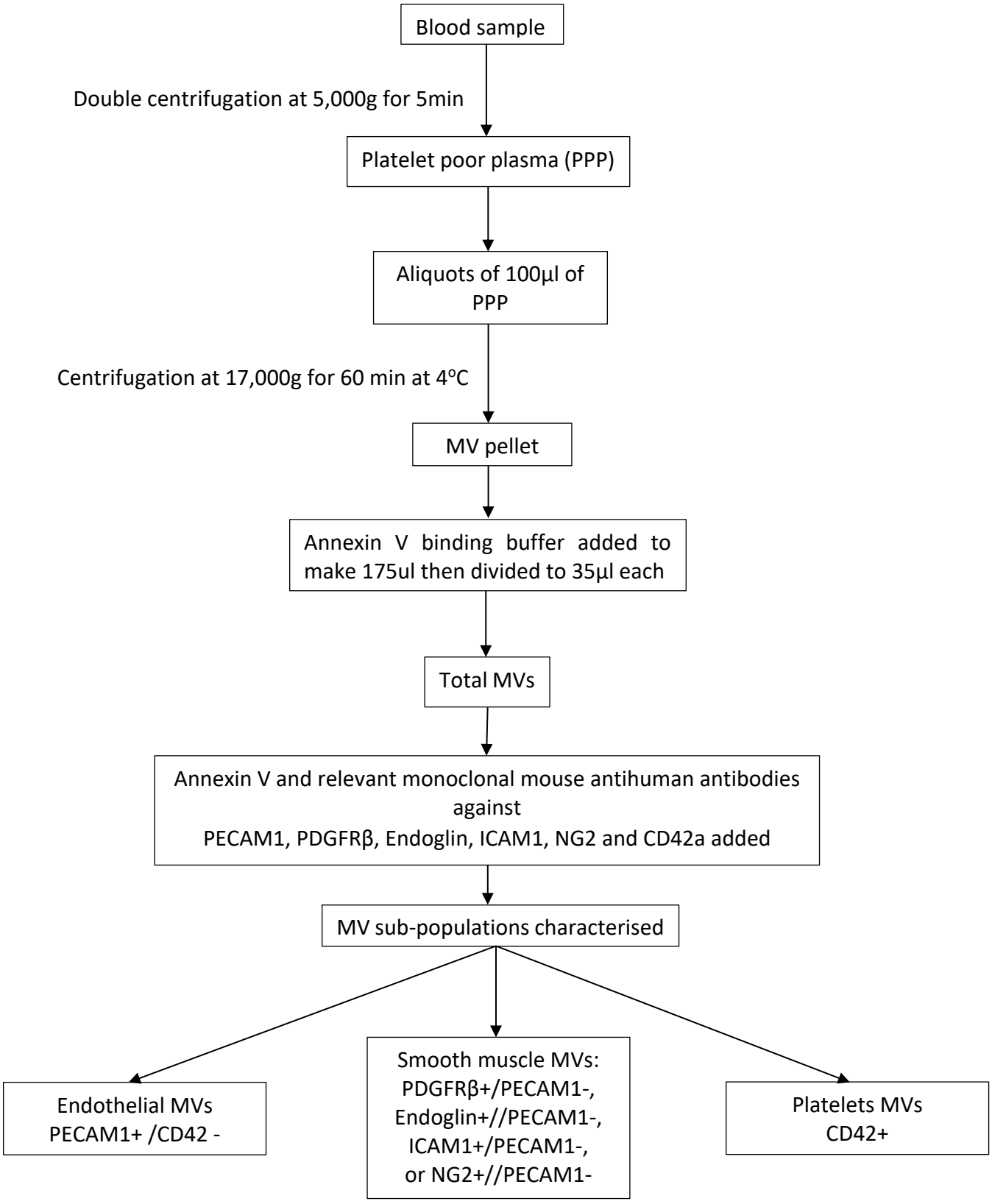


Figure 4.2: Flow chart of the preparation of platelet poor plasma and identification of micro vesicles (MVs).

Flow cytometric analysis of MVs

Analysis of MVs was performed on the FACSVerse^{TM} cytometer (BD Biosciences, Wokingham, UK). To optimise the forward (FSC) and side scatter (SSC) settings, 1.1µm latex beads (Sigma-Aldridge, Poole, Dorset, UK) were run and logarithmic FSC and SSC plots obtained. The gates were set to obtain particles smaller than approximately 1.1µm in diameter. When MVs were run through the cytometer, 12µl from each well was run at a medium flow rate of 2µl/sec. As Annexin V is a constitutive marker for MVs, total MV were defined as particles <1.1µm in size and Annexin V+.

Data was collected and analysed with FlowJo computer programme. Optimal compensation was set for the appropriate channels detecting FITC, PE, and APC-Cy7 using single stained controls acquired on the analysis software as the data was collected digitally. A known concentration of 3µm latex beads (Sigma-Aldridge, Poole, Dorset, UK) were also run as an internal standard for enumeration.

Determination of MV number per ml of plasma.

As the number of MV events analysed via flow cytometry changes depending on variables such as the amount of plasma analysed, forward and side scatter parameters, and the type of cytometer used, it is important to enumerate the MV count in a standardised fashion. The use of latex beads as an internal standard was first introduced by Combes and colleagues [101] where the number of MVs per ml of plasma was determined by using the proportion of known concentration of 3µm beads counted and the volume of plasma from which the MVs were analysed. A predetermined number (200,000) of the 3µm latex beads (Sigma-Aldridge, Poole,

Dorset, UK) was calculated according to the manufacturer's recommendations and added to a well in the 96-well plate. This count was achieved approximately by diluting 6µl of 3µm latex beads was in 2ml of deionised water (dH_2O) that had first been filtered through a 0.22µl syringe filter. Of that, 10µl was added to 240µl filtered dH_2O and run through the flow cytometer at the exact same settings as the MV analysis. A gate was drawn around the main cloud of 3µm beads, and the total events count was recorded and used to calculate the number of MVs per ml of plasma.

The following equation (Figure 4.3) was adapted from Brogan et al. [100], which converts flow cytometer events to an estimated count of MVs per ml of plasma.

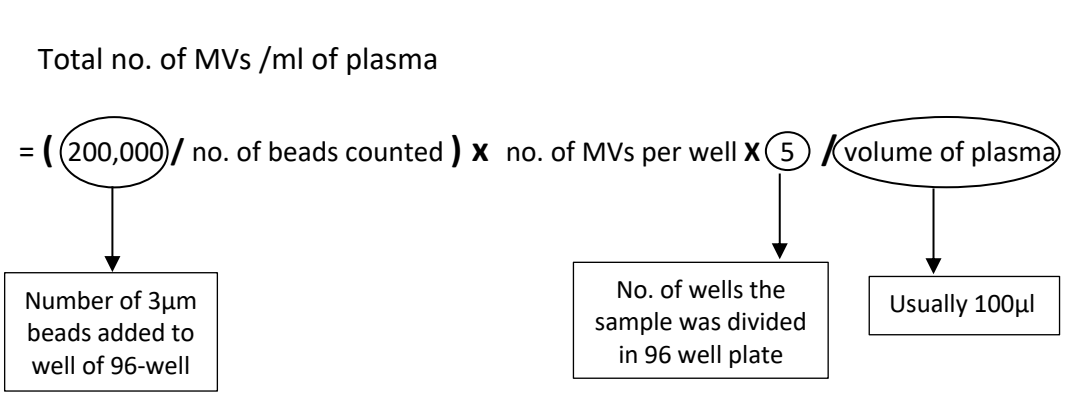


Figure 4.3: Conversion equation for MV number per ml of plasma calculated from flow cytometer event counts

The enumeration of microparticles from raw flow cytometry events count required a fixed number (200,000) of 3µm latex beads per well of the 96 well plate used, number of beads counted as raw events after acquisition, number of ml of plasma (100µl) used per platelet poor plasma to obtain the MVs, and the number of wells the sample was divided into during plating in the 96 well plate.

Statistical analysis

Data were analysed using SPSS software (version 26, IBM, New York USA) and Prism software (version 9, GraphPad, California USA). Each variable's data was plotted on histogram and Kolmogorov–Smirnov test was used to test for normal distribution. Continuous variables were expressed as mean ± SD if normally distributed or median ± quartile range if distribution was skewed. Categorical variables were presented as number and percentage. Log transformation was used if the data was significantly skewed. Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test for numerical data and Fisher's test for categorical data. Correlation between different variables was done using Spearman's rank correlation coefficient and odds ratios (OR). ROC curve was reported as area under the curve (AUC) and 95% confidence intervals (CI) to measure how well a parameter could distinguish between diagnostic groups. Statistical significance was regarded when P values were less than 0.05 (two sided).

4.3 Results

Fifty-nine patients were recruited in this study. Of these, 33 patients had SSc PH (mean PAP of 37 mm Hg) and 26 had PH ruled out on cardiac catheterisation. With further investigations, SSc PAH was confirmed in 30 patients. Two patients had their diagnosis updated to SSc PH secondary to left heart disease (group 2 disease due to worsening breathlessness on ERA (endothelin receptor antagonist) therapy and confirmed by a high wedge pressure on repeat catheter. One patient was classified as group 3 after confirming extensive lung disease not initially identified on pre-procedure HRCT scanning.

Table 4.2 presents the baseline characteristics of SSc patients with and without PH. The mean age of the PH group was not statistically different (60.42 and 61.77 years for no PH versus PH, respectively). The majority of patients with SSc were female, and although there were fewer females In the SSc PH group (78.8% versus and 92.3%) this was not statistically different.

Diffuse SSc was significantly more common in the non-PH group, with almost half of the patients (45.8%) being diagnosed with this form of SSc while only 3 patients had diffuse disease in those who had PH confirmed ($p<0.01$).

Comorbidities distribution was significantly different, with both systemic hypertension and interstitial lung disease being more common in the group without PH. The distribution of the other comorbidities was not statistically different between the two groups.

	SSc no PH (n=26)	**SSc PH (n=33)**	p Value
Age (years ±SD)	60.42 ±7.98	61.76 ±9.33	0.564
Females n. (%)	24(92.3%)	26 (78.8%)	0.152
SSc subtype			
Limited	12 (50%)	26 (78.8%)	
Diffuse	11 (45.8%)	3 (9.1%)	
Mixed connective tissue disease	1 (4.2%)	4 (12.1%)	0.006*
Comorbidities			
Hypertension	7 (26.9%)	2 (6.1%)	0.032*
Diabetes Mellitus	0	2 (6.1%)	0.202
Hypothyroidism	4 (15.4%)	8 (24.2%)	0.401
Chronic Kidney disease	0	3 (9.1%)	0.115
Interstitial lung disease	12 (46%)	5 (15.2%)	0.01*
Coronary artery dis.	3 (11.5%)	3 (9.1%)	0.757
WHO functional class			
I	6 (24%)	0	
II	8 (32%)	6 (18.8%)	
III	11 (44%)	23 (71.9%)	0.005*
IV	0	3 (9.4%)	
6MWD m.	350.5 (166.5-425)	224 (176-425)	0.104
NT-proBNP ng/L	177 (100-348)	470 (142.5-969.5)	0.001*
Right atrial pressure mmHg	4.5 (2-6.5)	7 (4.75-9.5)	0.007*
Mean pulmonary arterial pressure mmHg	20 (14.75-22.25)	37(27.75-37)	<0.001*
Cardiac Output L/min	4.85 (4.13-5.53)	4.6 (3.8-5.48)	0.734
Cardiac Index	2.75 (2.5-3.3)	2.7 (2.25-3.35)	0.548
Pulmonary Vascular Resistance	184 (124.25-222.25)	357 (253.5-612)	<0.001*
Mixed venous Oxygen sat. %	72.5 (71-75.25)	66.5 (60.75-73.25)	<0.001*
Arterial oxygen sat. %	96 (94-98)	94 (92-96)	0.021*
ESC risk category			
Low risk	19 (73.1%)	10 (30.3%)	
Intermediate risk	7 (26.9%)	21 (63.6%)	
High risk	0	2 (6.1%)	0.004*

Table 4.2: Baseline characteristics of the study population who had scleroderma in the absence or presence of pulmonary hypertension. Continuous variables were expressed as mean ± SD if normally distributed or median ± quartile range if distribution was skewed. Categorical variables were presented as number and percentage. Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test for numerical data and Fisher's test for categorical data.

As expected, patients with SSc PH were more symptomatic, with 82% classified as being in WHO functional class (FC) III or IV versus 44% classified as having FC III in the non -PH group, with none classified as having FC IV). The 6-minute walking distance was lower in the PH group (median=350.5m versus 224m) but this was not found to be statistically significant. NT pro-BNP was significantly higher in the PH group with a median of 470 ng/dl (IQR 142.5-969.5 ng/dl) about three times the median measurement of the no-PH group 177 ng/dl (IQR 100-348ng/dl) (p=0.001).

1. Haemodynamic data and ESC/ERS risk category

As expected, the mean pulmonary artery pressure, right atrial pressure and pulmonary vascular resistance measurements were significantly higher at the PH group when compared to those without PH (median of 37mmHg vs. 20 mmHg, median of 7mmHg vs. median of 4.5mmHg, and a median of 357 dyn·s/cm^5 vs. a median of 184 dyn·s/cm^5, respectively. Similarly, systemic arterial and pulmonary artery oxygen saturations were significantly less in the PH group (median 96% vs. median 94% and median 66.5%, vs. median 72.5% , respectively). However, cardiac output and cardiac index were higher in the non-PH group though this did not reach statistical significance.

Using the ESC/ERS 2015 risk assessment tool on our acquired data, the majority of the PH group were in the intermediate risk category whilst majority of patients were in the low risk category in the no PH group. Of the two patients that were considered high risk, both were confirmed to have PH.

2. Microvesicles levels in SSc patients with and without Pulmonary Hypertension

Comparison of different MVs populations in samples collected from the peripheral vein in SSc PH patients and those with SSc without PH is demonstrated in figures (4.4, 4.5, 4.6 and 4.7). There was no statistical difference in either the total Annexin+ MVs, or the different MVs subpopulations except ICAM+/PECAM1- MVs which reached borderline statistical significance, being higher in the no PH group (p value=0.0479).

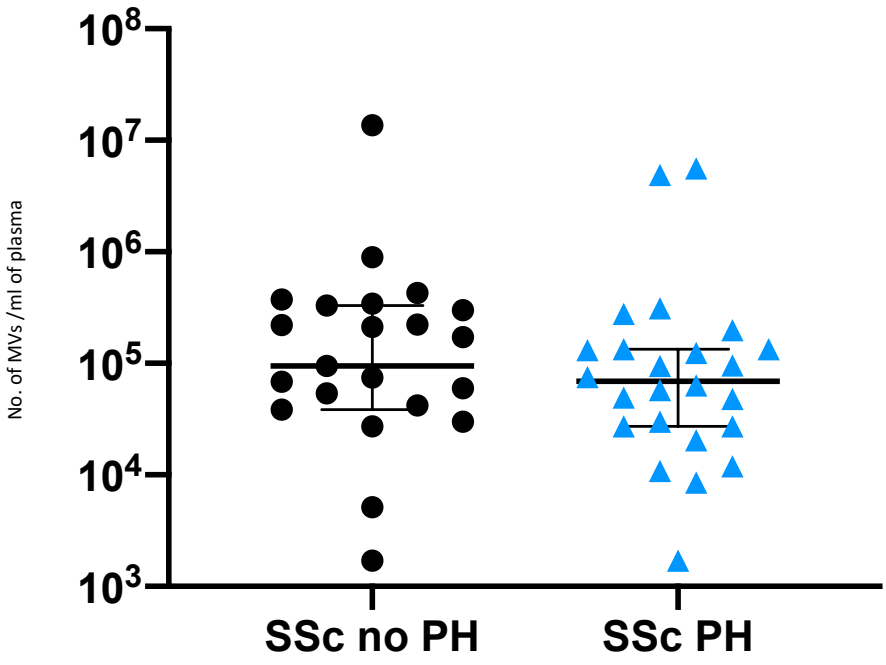


Figure (4.4) Femoral vein samples of total Annexin V+ MVs count displayed on a log 10 scale in scleroderma patients without pulmonary hypertension (SSc no PH) and patients with scleroderma pulmonary hypertension (SSc PH). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test, p value= 0.377.

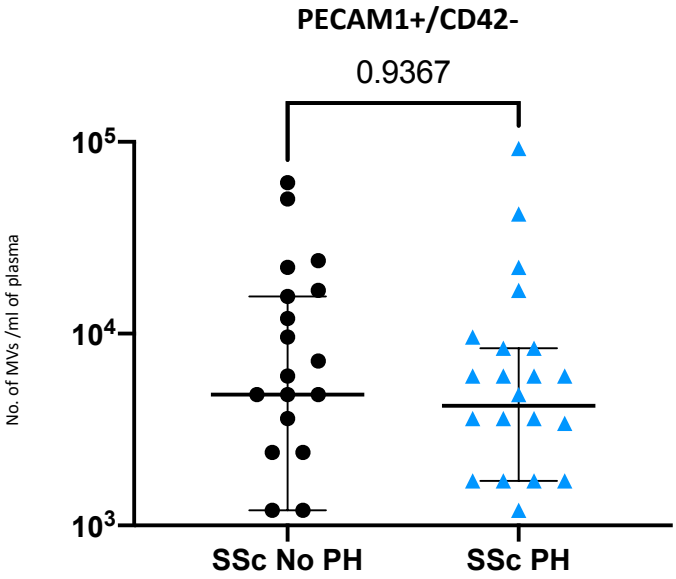


Figure (4.5) Annexin positive MVs stained with the endothelial marker, PECAM1, but not the platelet marker, CD42, in a femoral vein sample. Data points are displayed on a log 10 scale in scleroderma patients without pulmonary hypertension (SSc no PH) and patients with scleroderma pulmonary hypertension (SSc PH). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test, p value=0.937.

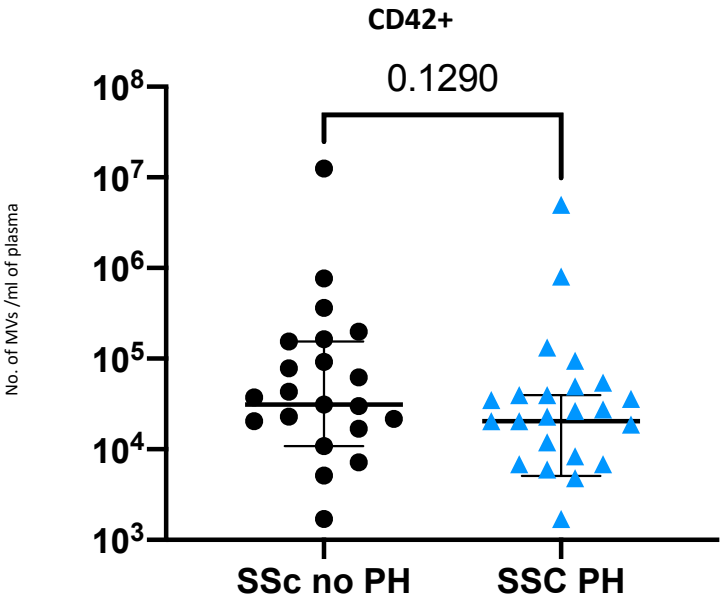


Figure (4.6) MVs count in femoral vein where vesicles have been stained positive with annexin V and the platelet marker, CD42+. Data are displayed on a log 10 scale in scleroderma patients without pulmonary hypertension (SSc no PH) and patients with scleroderma pulmonary hypertension (SSc PH). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test, p value=0.129.

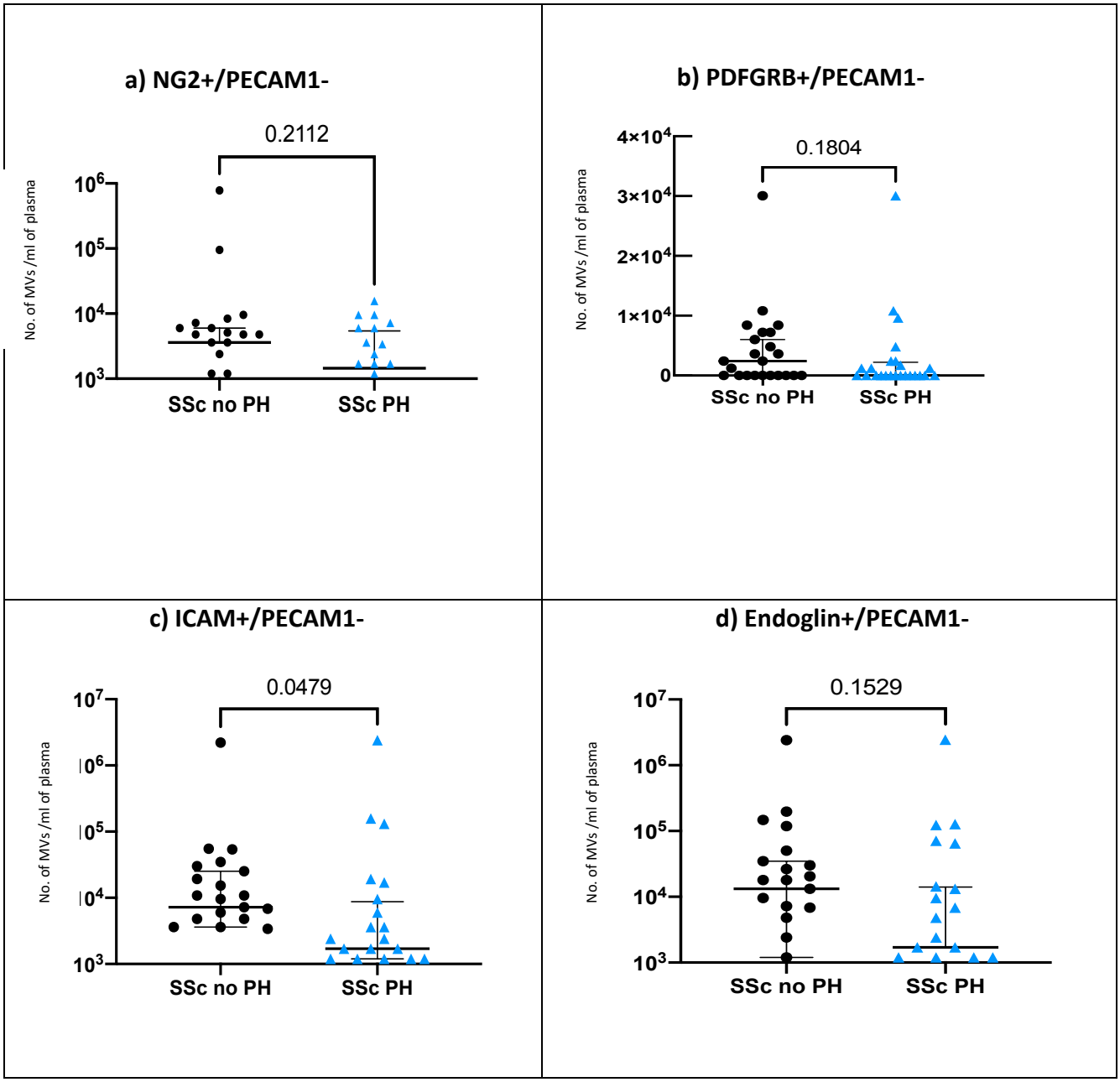


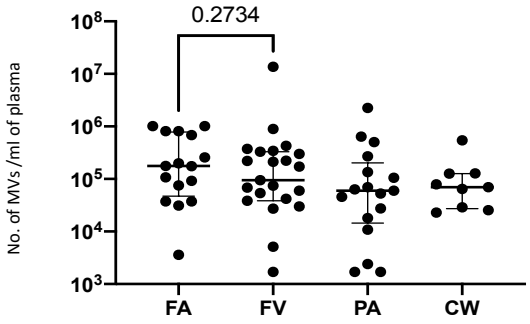
Figure (4.7)

MVs count in femoral vein where vesicles have been stained positive with annexin V and different putative smooth muscle cell surface markers (NG2, PDFGRB, ICAM, endoglin) and negative for the endothelial marker, PECAM1. Data are displayed on a log 10 scale (a, c, d) and linear scale (b). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test.

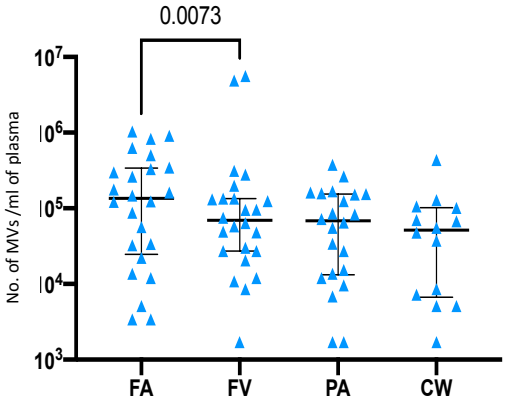
3. Comparison of microvesicles levels in SSc patients with and without pulmonary hypertension using multiple sampling sites.

In order to establish whether the site of sampling was superior in terms of sensitivity or selectively of the MV population, we collected blood from femoral artery, femoral vein, pulmonary artery and a pulmonary capillary (wedged) sample. Figures (4.8 and 4.9) demonstrate different MVs counts from samples collected across the 4 different sites during cardiac catheterisation of SSc patients.

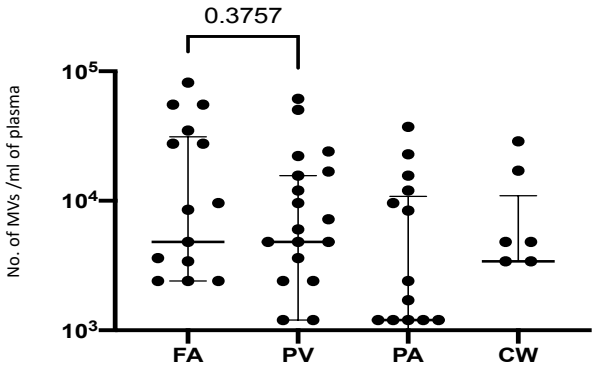
Interestingly, with some exceptions, there was an overall trend that the femoral artery sample total and sub-population of MV counts were higher than in the femoral vein, pulmonary artery and pulmonary capillary (wedged) samples. This only reached statistical significance in the SSc patient population with pulmonary hypertension for total Annexin V+, endothelial but not platelets MVs. In the smooth muscle MVs, this trend was also noted and reached statistical significance in the ICAM+/PECAM1- sub-population of MVs but not others (NG2+/PECAM1-, Endoglin+/PECAM1- and PDGFRB+/PECAM1-). Total and the individual MVs sub-populations, were indistinguishable in the femoral vein, pulmonary artery and the pulmonary capillary wedge samples.



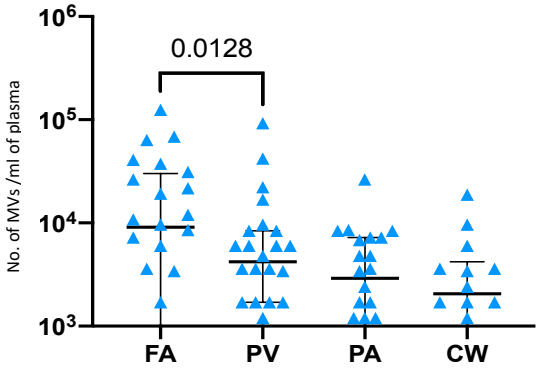
Total MVs count in SSc no PH



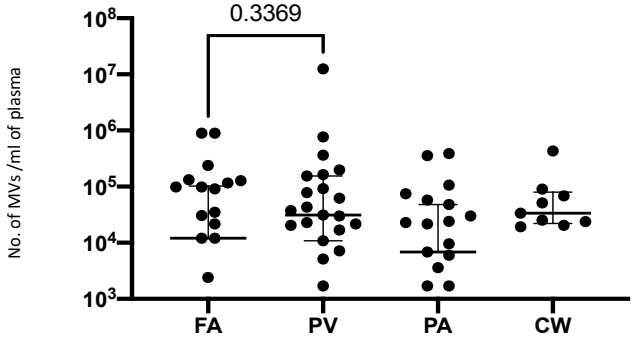
Total MVs count in SSc PH



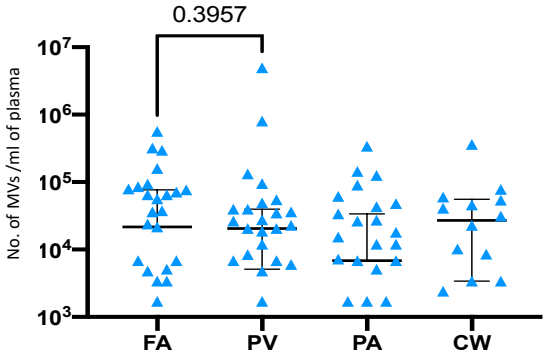
Endothelial (PECAM1+/CD42-) MVs in SSc no PH



Endothelial (PECAM1+/CD42-) MVs in SSc PH



Platelets (CD42+) MVs count in SSc no PH



Platelets (CD42+) MVs count in SSc PH

Figure (4.8) Different MVs count on log 10 scale across different sample sites (FA= femoral artery, FV=femoral vein, PA= pulmonary artery and CW= pulmonary capillary (wedged) sample. Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test.

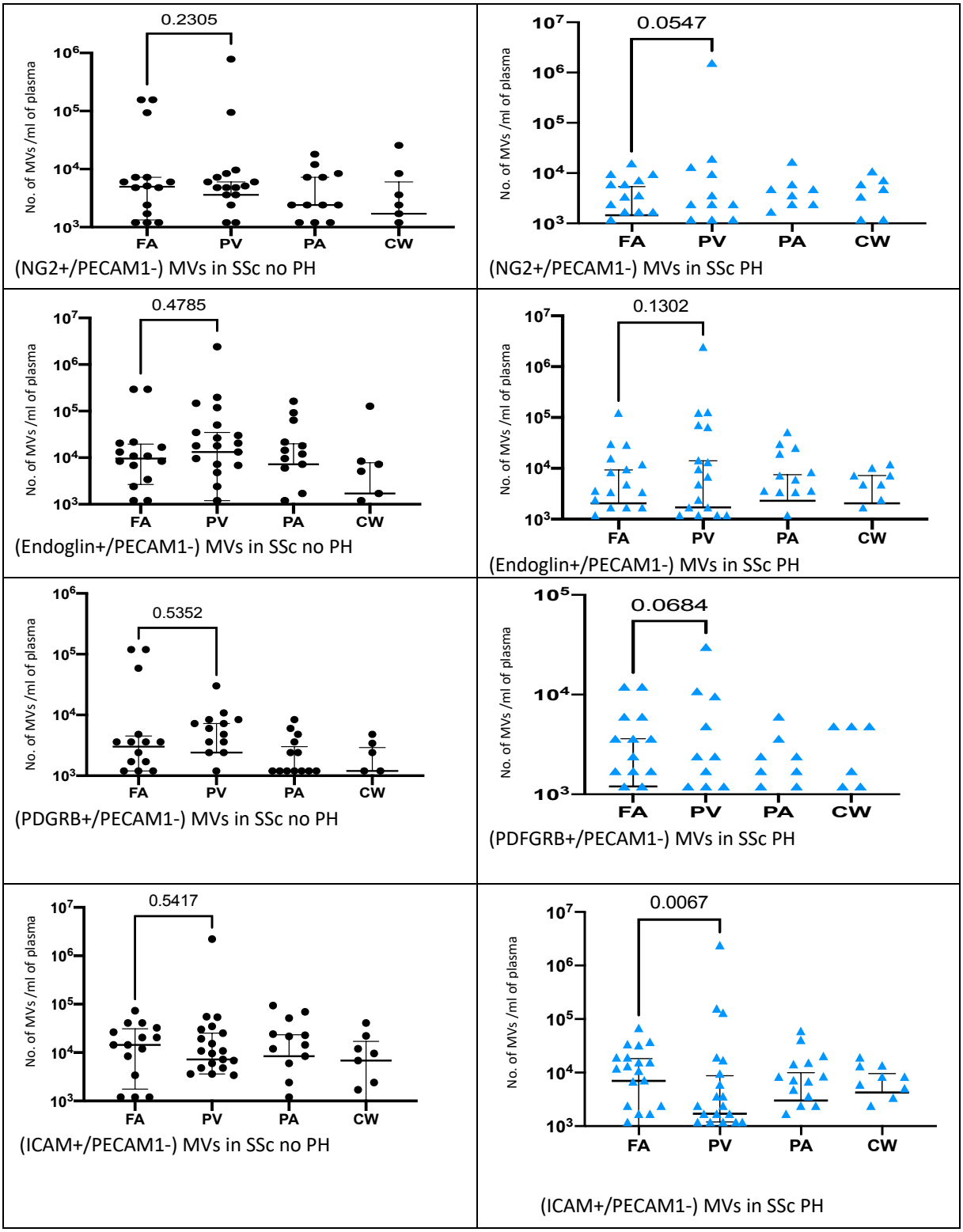


Figure (4.9) Different smooth muscles MVs counts on log 10 scale across different sample sites (FA= femoral artery, FV or PV =femoral vein, PA= pulmonary artery and CW= pulmonary capillary (wedged) sample. Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test.

lasma

4. Femoral artery microvesicles levels in SSc patients with and without Pulmonary Hypertension

As the femoral arterial samples appeared to differentiate between other sites of collection, further analysis was undertaken to determine if MV's from this site could differentiate between PH and non-PH patients (figures 4.10, 4.11, 4.12 and 4.13). Such analysis showed that there was no statistically significant difference between the two groups for total Annexin V+, endothelial, platelets and smooth muscles MVs except (NG2+/PECAM1-) and (Endoglin+/PECAM1-) which were statistically higher in the no PH group (p values were 0.021 and 0.033, respectively).

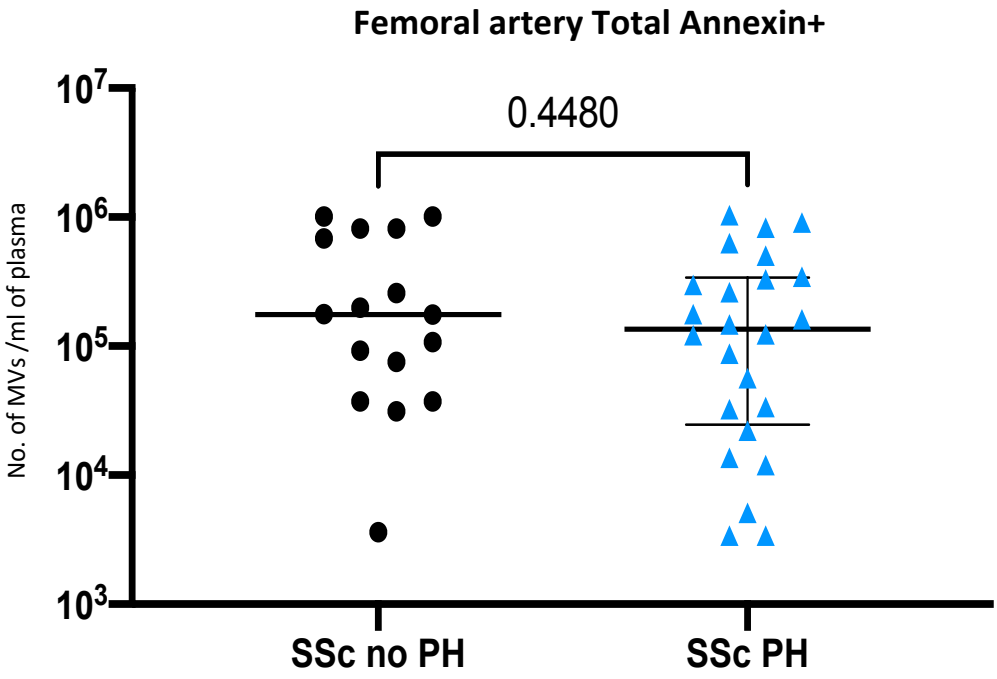


Figure (4.10) Total Annexin V+ MV count in platelet poor plasma derived from femoral artery samples in scleroderma patients without (SSc no PH) and with pulmonary hypertension (SSc PH). Data are presented on a log scale as median with min and max. The p value= 0.488. Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test.

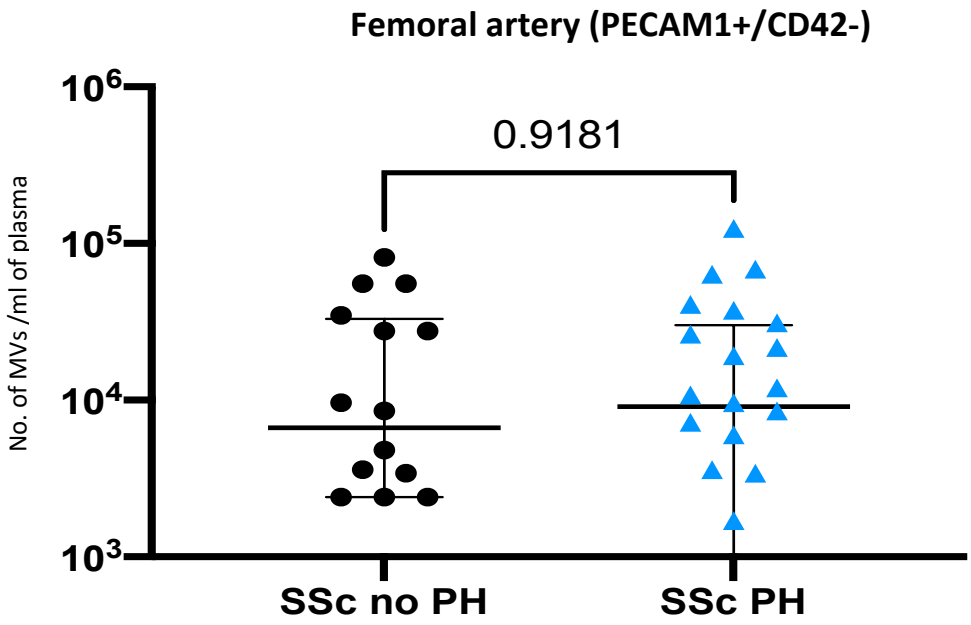


Figure (4.11): Femoral artery sample endothelial (PECAM1+/CD42-) MVs count on log 10 scale of scleroderma patients without pulmonary hypertension (SSc no PH) and patients with scleroderma pulmonary hypertension (SSc PH). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test, p value=0.918.

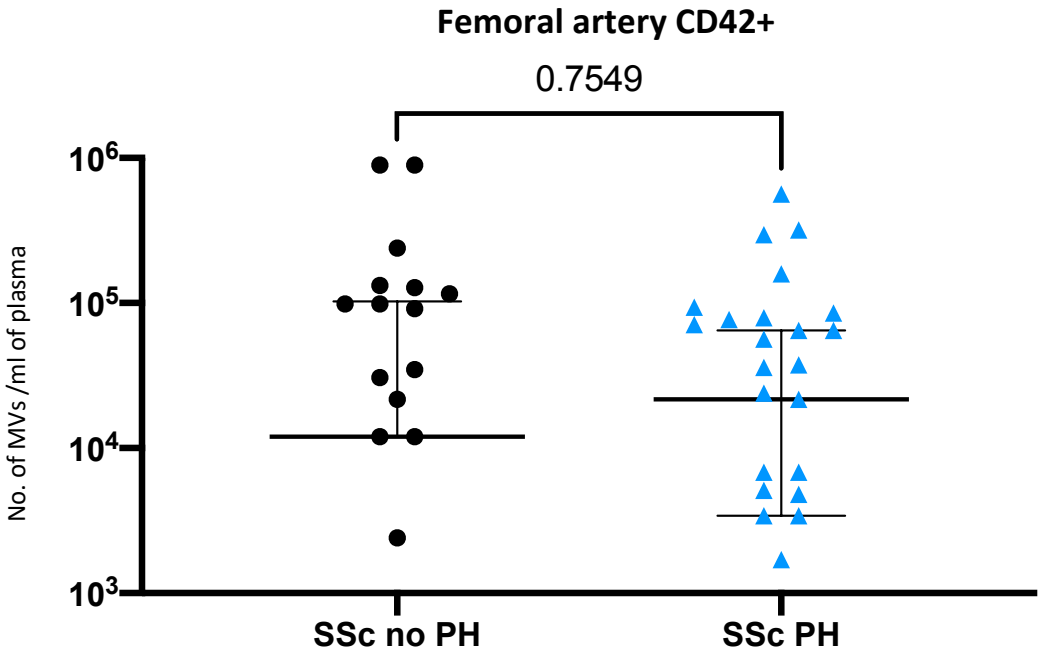


Figure (4.12): Femoral artery sample platelets (CD42+) MVs count on log 10 scale in scleroderma patients without pulmonary hypertension (SSc no PH) and patients with scleroderma pulmonary hypertension (SSc PH). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test, p value=0.755.

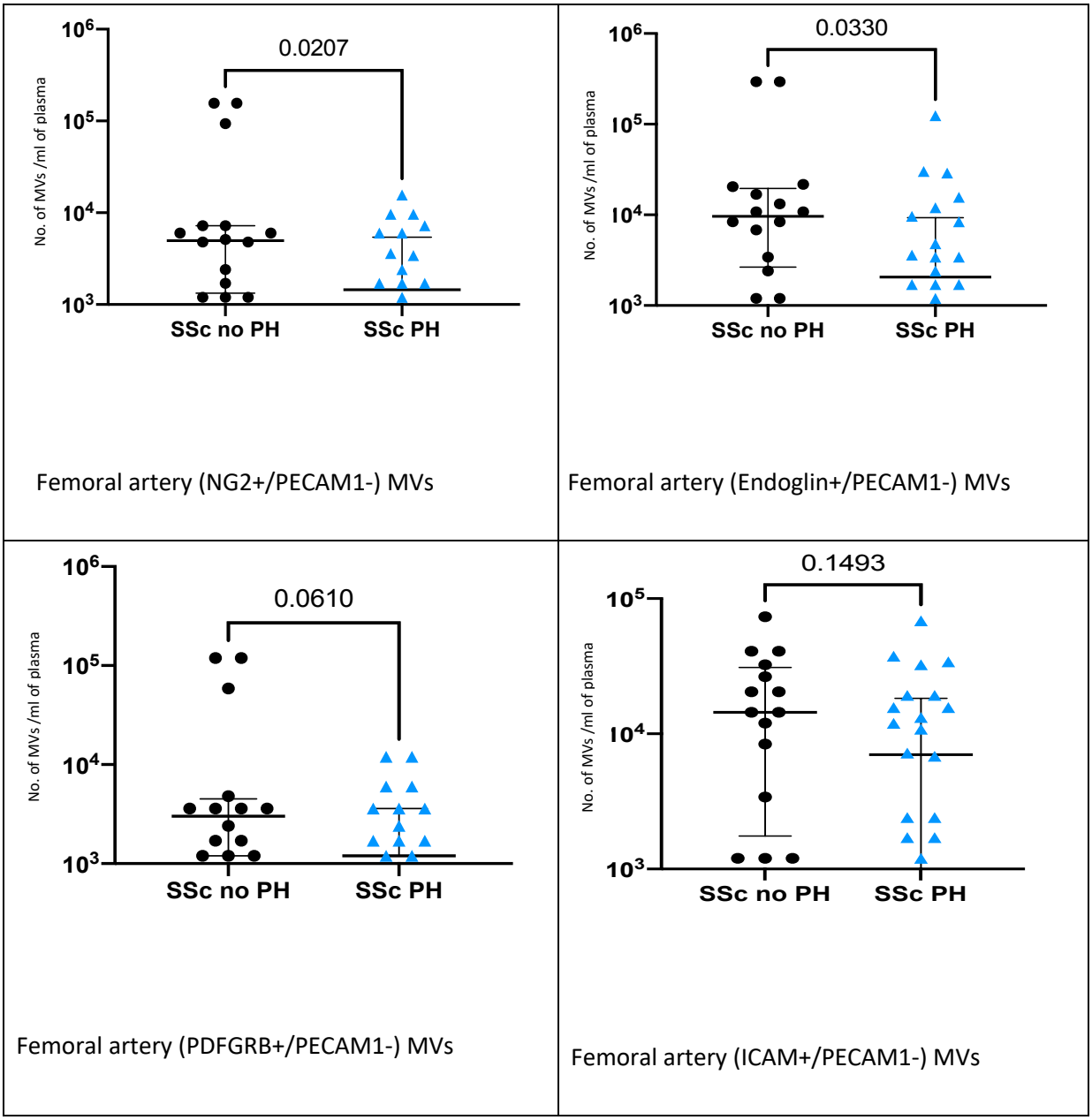


Figure (4.13): Femoral artery sample different smooth muscles MVs counts on log scale (NG2+/PECAM1-, PDGRB+/PECAM1-, ICAM+/PECAM1- and Endoglin+/PECAM1-) in scleroderma patients without pulmonary hypertension (SSc no PH) and patients with scleroderma pulmonary hypertension (SSc PH). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test.

4.3.4.1 Characterisation of MVs from patients with limited SSc

PAH is well known to be more common in limited than diffuse SSc. In our population, 45.8% of the no-PH group had diffuse SSc while only 9.1 % of the SSc PH group which could have affected the MVs counts (table 4.2). In order to try an adjust for this uneven distribution of the different SSc phenotypes which may have affected the results (diffuse SSc being associated with a greater degree of inflammation), we undertook a post-Hoc analysis to re-test our hypothesis in the limited SSc patient study population only.

Table (4.3) demonstrates the baseline characteristics of the PH and no PH in limited SSc patients only. As in whole study population there was no significant difference in the demographics (age and sex) between the two groups. The no PH group had more comorbidities with 50% had interstitial lung disease (vs. 7.7% in the limited SSc PH patients, p value =0.007) and 41.7% had systemic hypertension (vs 7.7% in the limited SSc PH patients, p value =0.022). The non-invasive and the invasive baseline measurements differences between the two groups were similar to the whole study population.

Limited SSc	**Limited SSc no PH (n=12)**	**Limited SSc PH (n=26)**	p Value
Age (years ±SD)	62.17 ±6.9	63.08 ±9.19	0.762
Females n. (%)	11(91.7%)	21 (80.8%)	0.643
Comorbidities			
Hypertension	5 (41.7%)	2 (7.7%)	0.022*
Diabetes Mellitus	0	2 (7.7%)	0.462
Hypothyroidism	3 (25%)	7 (26.9%)	0.615
Chronic Kidney disease	0	3 (11.5%)	0.538
Interstitial lung disease	6 (50%)	2 (7.7%)	0.007*
Coronary artery dis.	1 (8.3%)	3 (11.5%)	0.625
WHO functional class			
I	1 (9.1%)	0	
II	4 (36.4%)	4 (16%)	
III	6 (54.5%)	18 (72%)	0.147
IV	0	3 (12%)	
6MWD m.	308.5 (198.5-438)	207.5 (81.5-567)	0.225
NT-proBNP ng/L	200 (12.75-518.5)	567 (167-969.5)	0.045*
Right atrial pressure mmHg	3.5 (3-6)	7 (4.75-9.5)	0.002*
Mean pulmonary a. pressure mmHg	19 (16.75-21)	33 (27.75-42.25)	0.001*
Cardiac Output L/min	4.8 (3.975-5.025)	5.1 (4.45-5.73)	0.724
Cardiac Index	2.7 (2.48-3.03)	2.9 (2.5-3.33)	0.720
Pulmonary Vascular Resistance	187.5 (160.25-233.5)	354 (274.5-506)	<0.001*
Mixed venous Oxygen sat. %	71.9 ((±3.14)	64.45 ((±8.43)	0.001*
Arterial oxygen sat. %	95.2 (±2.65)	91.7 (±5.84)	0.102
ESC risk category			
Low risk	8 (66.7%)	7 (26.9%)	
Intermediate risk	4 (33.3%)	17 (65.4%)	
High risk	0	2 (7.7%)	0.057

Table (4.3) Baseline characteristics of the limited SSc patients without (limited SSc no PH, n=12) and with pulmonary hypertension (limited SSc PH, n=26).

Continuous variables were expressed as mean ± SD if normally distributed or median ± quartile range if distribution was skewed. Categorical variables were presented as number and percentage. Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test for numerical data and Fisher's test for categorical data.

Microvesicles levels in limited SSc patients with and without Pulmonary Hypertension

None of the MVs total or specific populations measured in different sites were significantly different between the limited SSc-PH patients and those with limited SSc without PH (figures 4.14, 4.15, 4.16 and 4.17).

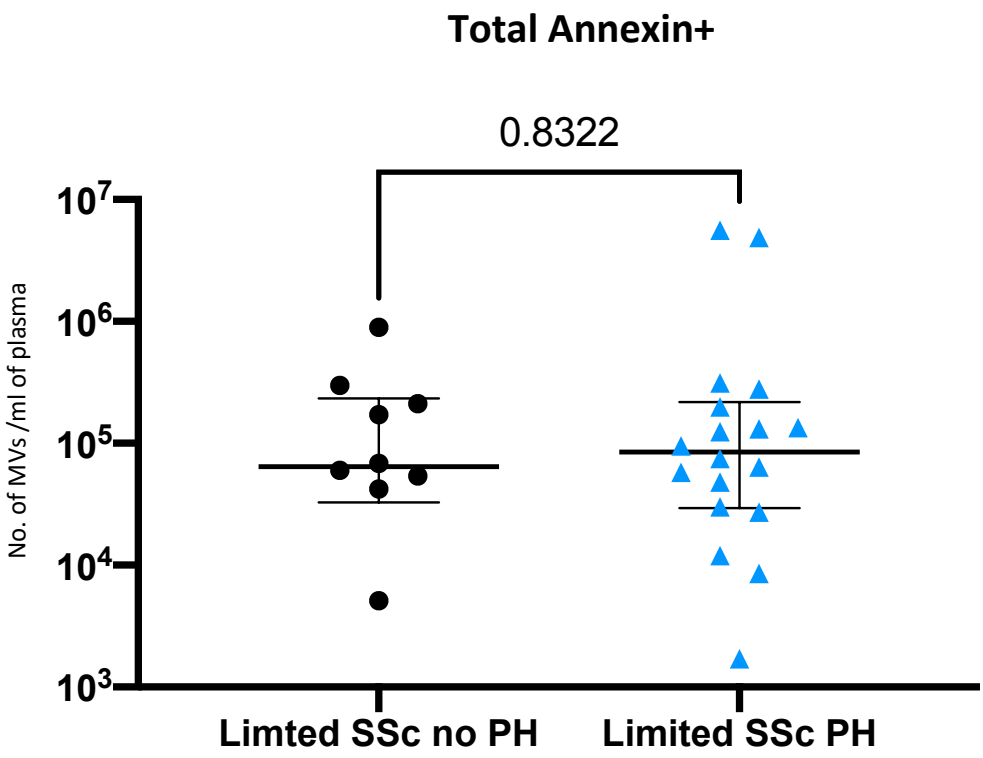


Figure (4.14): Total Annexin V+ MVs count in a femoral vein sample from patients with limited scleroderma diagnosed without (limited SSc no PH) or with pulmonary hypertension (limited SSc PH). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test, P value= 0.8322.

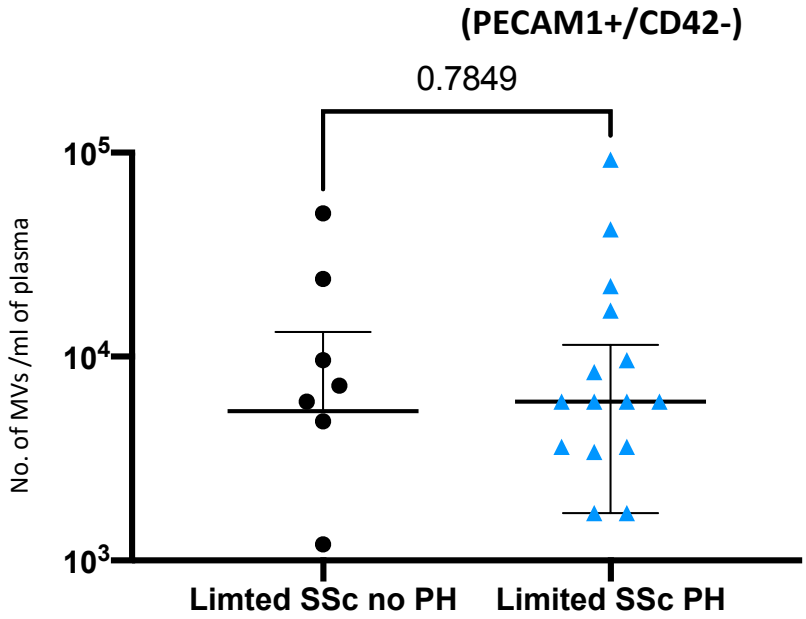


Figure (4.15): MV count in femoral vein samples from patients with limited scleroderma without (limited SSc no PH) and with pulmonary hypertension (limited SSc PH). Annexin V+ MV count were taken as those that stained positive for the endothelial cell surface marker, PECAM1 and negative for the platelet marker CD42, and were plotted as the log of total count per ml in platelet poor plasma. Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test, P value=0.785.

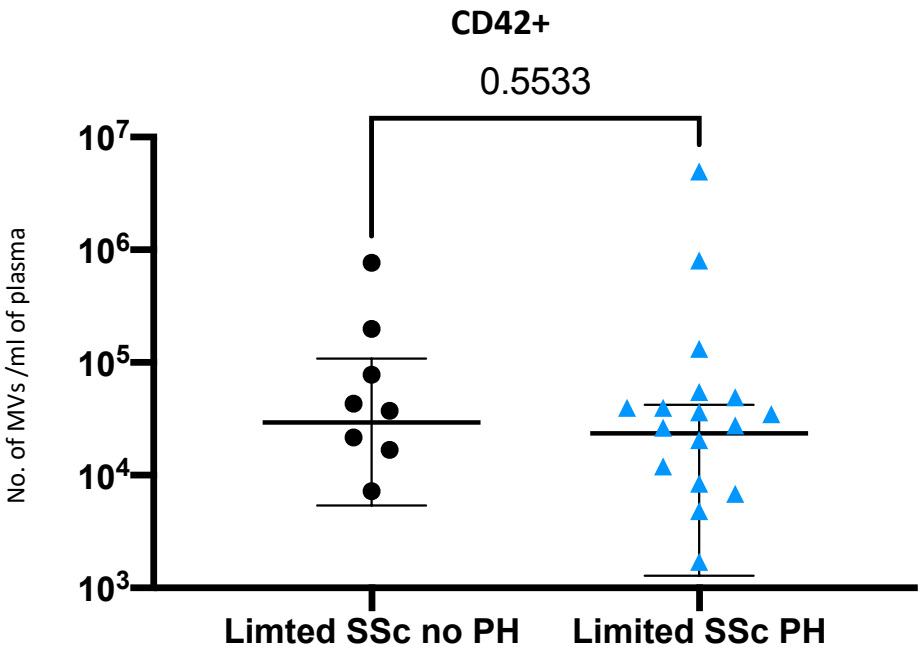


Figure (4.16) Femoral vein sample platelets (CD42+) MVs count on log 10 scale in limited scleroderma patients without pulmonary hypertension (limited SSc no PH) and with pulmonary hypertension (limited SSc PH). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test, p value=0.553.

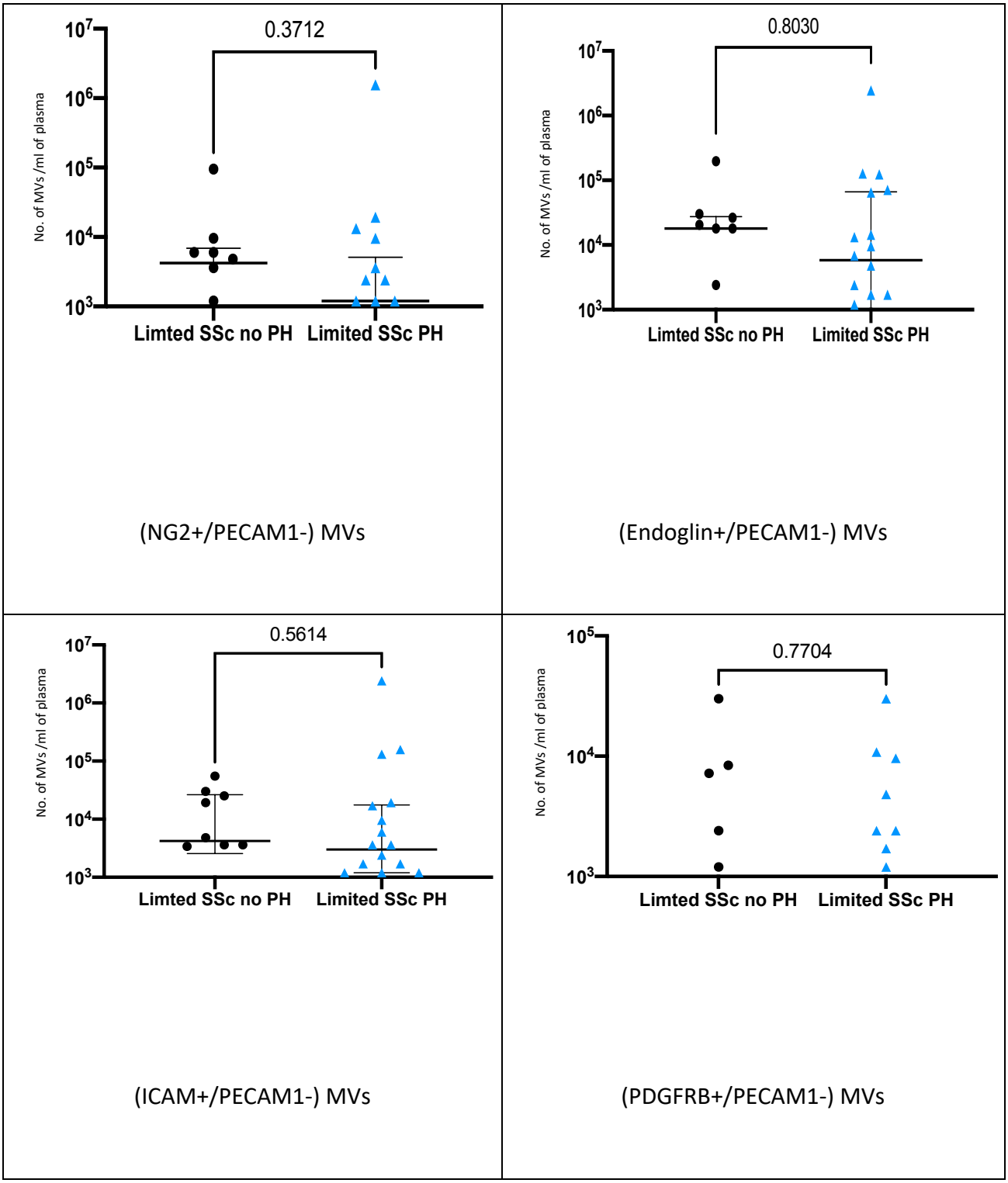
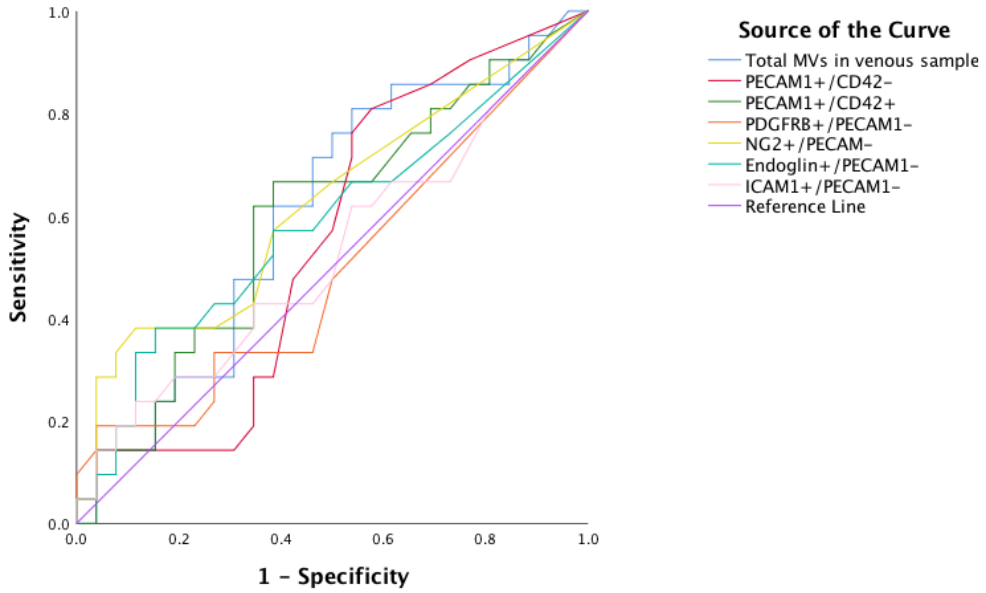


Figure (4.17) Femoral vein sample different smooth muscles MVs (NG2+/PECAM1-, PDGRB+/PECAM1-, ICAM+/PECAM1- and Endoglin+/PECAM1-) count on log 10 scale in limited scleroderma patients without pulmonary hypertension (limited SSc no PH) and with pulmonary hypertension (limited SSc PH). Comparison between groups was done using non-parametric Mann-Whitney-Pearson U test.

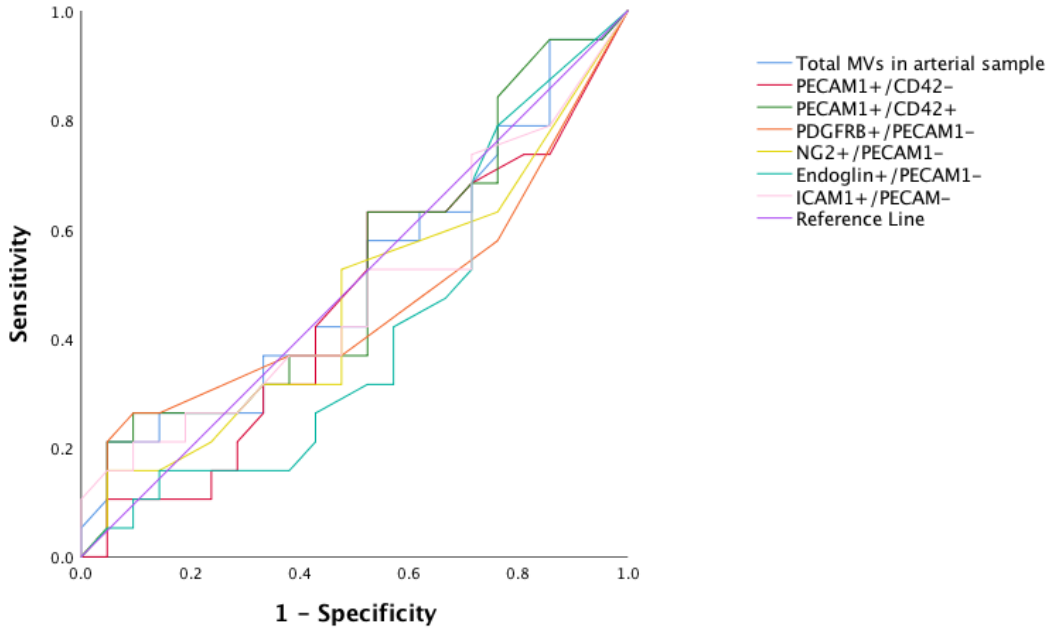
5. MVs counts and risk prediction in SSc PH

Figures (4.18-19) demonstrate the ROC analysis of the different measured MVs populations from different sites and predictability of higher ESC risk status. None of the measured total or specific sub-population MV count were of significant diagnostic value to a higher risk status.



Test Result Variable(s)	Area	P Value	95% CI
Total Annexin V+ MVs	0.607	0.211	0.443-0.771
PECAM1+/CD42-	0.551	0.549	0.383-0.719
PECAM1+/CD42+	0.595	0.266	0.430-0.761
PDGFRB+/PECAM1-	0.498	0.983	0.328-0.668
NG2+/PECAM1-	0.624	0.149	0.459-0.788
Endoglin+/PECAM1-	0.586	0.315	0.418-0.754
ICAM1+/PECAM1-	0.526	0.765	0.356-0.696

Figure 4.18: ROC analysis of the different measured MVs populations from venous sample and predictability of higher ESC risk status.



Arterial Sample	Area	P Value	95% CI
Total Annexin V+ MVs	0.510	0.914	0.326-0.694
PECAM1+/CD42-	0.462	0.685	0.280-0.644
PECAM1+/CD42+	0.519	0.839	0.335-0.703
PDGFRB+/PECAM1-	0.459	0.655	0.271-0.647
NG2+/PECAM1-	0.464	0.695	0.281-0.647
Endoglin+/PECAM1-	0.412	0.343	0.231-0.593
ICAM1+/PECAM-	0.481	0.839	0.297-0.666

Figure 4.19: ROC analysis of the different measured MVs populations from arterial sample and predictability of higher ESC risk status.

4.4 Discussion

The main finding of this study is that in the SSc patient population, MVs were not shown to be useful biomarkers for the early detection of PAH. Total MV and the characterised MV subpopulations from endothelial, smooth muscle or platelet origin failed to differentiate PH from non-PH in the studied population, irrespective of the site of sampling.

Compared to previous reports and our historical registry data, the patient population characteristics included in this study is a typical representation of SSc-PAH at the time of diagnosis [102]. However, differences between the PH and No-PH groups may have contributed to the negative findings. While the PH group majority had limited subtype as expected, the control non-PH group had a significant proportion of patients with diffuse subtype (45.5% in the non-PH group and 9.1% of the PH group). In addition, there was a significantly higher proportion of the non-PH group that had ILD (46% in non-PH group compared with 15.2% in the PH group). Finally, while the PH group were clearly more symptomatic (as per the WHO-FC), the non-PH group also had significant symptoms, with 76% of patients diagnosed as being in WHO functional class II or III. The objective functional assessment of 6MWD mean of 309m (±140.83) in the non-PH group was not statistically significant than the PH group either. Thus, we have a no PH group that were not a true 'screening' population. These baseline characteristics are important to consider when interpreting the MVs data.

While there was no statistically significant difference in the total annexin V+ MVs count between the SSc PH and those without PH in the studied patients. Unexpectedly, the smooth muscles MVs (NG2+/ PECAM1-) and (Endoglin+/PECAM1-) were also found to be significantly lower in the PH patients' group in the femoral artery samples and the (ICAM1+/PECAM1-) in the peripheral venous samples. However, on reviewing the crude data the difference was driven by some outliers in the no PH group. Interestingly, this difference was abolished when the analysis was repeated after excluding the diffuse SSc patients from both groups.

ROC analysis further supports the lack of value of different MVs counts measured in different sites (peripheral vein, pulmonary artery, pulmonary capillary and peripheral artery) in the prediction of PH in the studied group of SSc patients.

An interesting finding in this study, however, was the significant gradient between the peripheral arterial and peripheral venous counts of some of the MVs (the total, PECAM1+/CD42- and ICAM1+/PECAM1-) in the SSc-PH patients. To our knowledge, this is the first report of such a finding. This may indicate the role of the lung vasculature as an important producer of such particles and also the role of peripheral vasculature as a filter to these particles. The pathobiological and the clinical role of this observation is not entirely clear, and the studied population number was small.

The diagnostic criteria for diagnosis of PH are likely to change as per the most recent World Symposium in Pulmonary Hypertension [4]. Applying a lower cut-off of 20mmHg as per the newer recommendations, the different MVs counts measured in the 4 sites

during cardiac catheterisation also did not successfully predict the mPAP of more than 20mmHg.

Diffuse SSc is a more aggressive inflammatory condition in comparison to the more indolent limited subtype , especially early in the course of the disease [103]. MVs data could have been affected by the diffuse subtype, hence we did a sub analysis of the limited SSc group of patients with and without PH. However, this also showed no statistical difference in the measured counts across the different sites in PH and non-PH patients.

The role of MVs in risk prediction was also limited in the studied PH population. ROC analysis showed poor differentiation of the different measured MVs counts in the differentiation of low versus higher risk group of patients as per the ESC/ERS risk score. This was further supported by the poor MV counts correlation with the different non-invasive and haemodynamic parameters obtained at the time of sampling.

While the results of this analysis are negative, caution has to be taken before dismissing the role MVs in screening and risk prediction as the lack of difference may be explained by several factors related to the population studied and the MVs markers measured.

The average age of our non-PH group of patients has been significantly higher than several previous screening studies for PH in patients with SSc. For example, the DETECT study mean age was 54.7 years [9], while a Belgian study group reported a

mean age of 54 years for their SSc population at the time of screening for PH [104]. Moreover, in the DETECT study, 82% of the non-PH SSc patients included had functional class I or II [9] and the Belgian group reported 91.1% with functional class I or II [104]. Our "control" group was significantly older and were more symptomatic at the time of investigation.

Further, the pre-identified MVs populations were based on the work undertaken by Gurung and colleagues for the different MV populations in a heterogenous PAH group of patients and were compared against a group of age and sex matched healthy volunteers [74]. Whilst in that study there was significant difference in almost all the studied MVs (total and specific populations), the inability to reproduce similar results could be explained by the SSc pathogenic mechanisms which may well produce similar level of MVs even in the absence of PH.

The hallmark of SSc pathogenesis is extensive fibrosis. Fibrosis is characterised by the deposition of mainly collagen and fibronectin in the extracellular matrix, but as fibrosis becomes chronically active, it leads to permanent tissue remodelling and organ damage. The progressive fibrosis in SSc is usually preceded by microvascular injury and endothelial dysfunction plays a key role in this process. This endothelial injury contributes to leukocyte and platelets activation, production of pro-inflammatory and pro-fibrotic cytokines that can lead to defective angiogenesis and vasculogenesis resulting in severe vasculopathy and progressive fibrosis [100].

The measurement of MVs in SSc have documented conflicting results. Iversen and colleagues reported that total, platelets MVs, leukocytes MVs, and endothelial MVs

were 22−42% lower in SSc patients than in healthy controls [105]. However, another study found that circulating endothelial MVs were significantly elevated in SSc patients compared to controls and reflected the extent of capillaroscopic alterations [106]. In addition higher platelets MPs but not endothelial MPs were reported in SSc patients compared to healthy controls [107]. The same group also demonstrated the significant correlation between the measured endothelial and platelets MVs and objective assessment vascular function by cold challenge and reperfusion test.

A recent study has suggested more positive findings than we have found, Lammi et al. have found that PECAM1+ (CD31+) were similar on both SSc-PAH and SSc no PH patients [73]. However, another endothelial MV marker, VE-Cadherin+ (CD144+) was found to be significantly elevated in the SSc PAH group versus SSc with no PH. This was a small study with only 10 patients with SScPAH and another 10 with SSc with no PH. The average age was also younger than our group of patients (57years in the PH group and 54 years in the non-PH group) and patients had a better 6MWD (379m and 383m respectively). However, the distribution of the SSc disease subtype (PH and non-PH were 90% and 40% with diffuse subtype respectively), and functional class (50% and 40% were WHO FC III respectively) were not dis-similar.

4.5 Conclusion

We have not successfully established a role of measuring total or different MVs populations as a biomarker for the screening or risk assessment of pulmonary hypertension in patients with systemic sclerosis. This study may be limited by the relatively more advanced SSc disease in comparison to the typical population in other screening studies. Also, the distribution of the scleroderma subtypes and comorbidities in the “control” group may have played a role in these findings.

Chapter 5 Conclusions and future considerations

1. Impact of routine screening on detection, severity and outcome of pulmonary arterial hypertension in systemic sclerosis.

It was demonstrated in the second chapter that there has been no significant change in the demographics and severity of SSc-PAH in the contemporary era vs. the historical one. While it could be easy to conclude that active screening programs has not been able to translate into truly earlier diagnosis in the disease process, I feel that a more likely explanation is probably the underwhelming penetration of such screening programs. In the landmark DETECT paper there was 62% referral for invasive RHC in asymptomatic patients [9]. This approach has significantly improved sensitivity but in the same time one can understand that this significant proportion requiring an invasive procedure that is not without a risk, may have limited the pick-up of this rigorous approach by both asymptomatic SSc patients and arguably by the SSc physicians. In order to support this argument, future consideration should include a survey among these physicians to discuss the true adoption of this (or any other) screening program and if there were any common reservations.

These findings would support an the need for a non-invasive biomarker that make the referral for invasive RHC less needed without compromising the negative predictive value of the current approach.

5.2 Risk Assessment in Scleroderma Associated Pulmonary Arterial Hypertension. Validation and comparison of different risk assessment models.

On comparison between all the current assessment models it was clear that in our population, SSc PAH was more likely to be in the higher risk categories at baseline and at the time of follow up. Each of the scoring systems used had advantages and disadvantages. The REVEAL 2.0 score had the best prognostic ability, but the inclusion of non-modifiable factors limited its usefulness at the time of follow up to track response to therapeutic interventions if any, an aspect that the ESC based models excelled at. The ESC based models not only provide a similar discriminatory value at baseline and at follow up, but also provides feedback on the impact of response to therapeutic intervention and may be therefore the most useful in clinical practice.

However, these findings demonstrated that there remains a need for a biomarker to further risk stratify SSc PAH at the time of diagnosis of those that may benefit from an escalated intervention at the outset. A marker that can not only improve the discriminatory ability of current models but also reflects the change of the risk category with therapeutic interventions.

5.3 The role of Microvesicles in the screening and risk assessment of systemic sclerosis associated pulmonary arterial hypertension

The main finding of this study is that in the studied population, MVs were not shown to be useful biomarkers for the early detection of PAH or those with higher risk profile. Total MVs count and the characterised MV subpopulations from endothelial, smooth muscle or platelet origin failed to differentiate PH from non-PH in the studied population, irrespective of the site of sampling. While the results of this analysis are negative, caution has to be taken before dismissing the role MVs in screening and risk prediction as the lack of difference may be explained by several factors related to the population studied and the MVs markers measured. These limitations include the older age in our non-PH group, SSc phenotype and the significant symptoms profile in comparison to historical control groups in standard screening studies.

An interesting finding in this study, was the significant gradient between the peripheral arterial and peripheral venous counts of some of the MVs (the total, PECAM1+/CD42- and ICAM1+/PECAM1-) in the SSc-PH patients. To our knowledge, this is the first report of such a finding. This may indicate the role of the lung vasculature as an important producer of such particles and also the role of peripheral vasculature as a filter to these particles. The pathobiological role and the clinical effect of this observation is not entirely clear, and the studied population number was small.

In order to truly test the role of the MVs in screening and risk prediction, we suggest that the current study would further extend the numbers in the SSc-PAH group but

also extends into another group that truly reflects a screening population (asymptomatic with lower risk profile). On the other hand, further work may need to be undertaken to further characterise MVs and different subpopulations in symptomatic patients with advanced SSc.

COVID-19

In early 2020 COVID-19 has evolved to a pandemic on a scale that mankind hasn't faced for several generations. The consequences of that has unfortunately not spared this research work. With lockdown restrictions in particular to high risk groups, further recruitment of patients became impossible. As a frontline worker myself, redeployment to more urgent clinical duties was a major detriment for conduction of research. Lab analysis of the samples has been significantly delayed due to government-imposed restrictions on non-essential work.

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