RHEUMATOLOGY

Rheumatology 2016;55:1112–1116
doi:10.1093/rheumatology/kew017
Advance Access publication 17 March 2016

Concise report

Serum levels of vascular dysfunction markers reflect disease severity and stage in systemic sclerosis patients

Marta Cossu[1], Romina Andracco[2], Alessandro Santaniello[2], Maurizio Marchini[2], Adriana Severino[2], Monica Caronni[2], Timothy Radstake[1] and Lorenzo Beretta[2],

Abstract

Objective. To improve knowledge of vasculopathy in SSc through the assessment of serum levels of circulating angiogenetic and endothelial dysfunction markers in patients at different stages of the disease.

Methods. Sera from 224 subjects were obtained and concentrations of angiopoietin-2, chemokine (C-X-C motif) ligand (CXCL)-16 (CXCL16), E-selectin, soluble intercellular adhesion molecule-1, IL-8 (CXCL8), soluble vascular adhesion molecule-1 and VEGF were determined by a Luminex assay. Subjects included 43 healthy controls, 47 early SSc patients according to LeRoy and Medsger without other signs and symptoms of evolutive disease, 48 definitive SSc (defSSc) patients according to the 2013 ACR/EULAR criteria without skin or lung fibrosis, 51 lcSSc subjects and 35 dcSSc subjects.

Results. The four groups of patients showed well-distinct clinical and laboratory characteristics, with a linear decreasing trend in forced vital capacity and diffusing capacity for carbon monoxide % predicted values from early SSc to defSSc to lcSSc and to dcSSc, and a linear increasing trend in ESR, and in the prevalence of abnormal CRP, serum gamma globulins and lung fibrosis (all $P < 0.0001$). Highly significant linear trends pointing to an increase in angiopoietin-2 ($P < 0.0001$), CXCL16 ($P < 0.0001$), E-selectin ($P = 0.001$) and soluble intercellular adhesion molecule-1 ($P = 0.002$) in relation to the different disease subsets were observed.

Conclusion. Markers characterizing vascular activation are found to be increased in SSc patients from the earliest stages of disease when clinical and laboratory findings of advanced disease cannot yet be detected. These abnormalities progress with the appraisal of the first sclerodermatous manifestation in defSSc and further increase with the onset of fibrotic manifestations.

Key words: systemic sclerosis, angiogenesis, adhesion molecules, early disease

CLINICAL SCIENCE

Rheumatology key messages

- SSc subsets, including early patients, are well-characterized clinical entities.
- Markers of endothelial dysfunction linearly increase from early to fibrotic SSc.
- Endothelial dysfunction markers are not predictive of organ damage in SSc.

[1]Department of Rheumatology, Clinical Immunology and Laboratory of Translational Immunology, University Medical Center, Utrecht, Netherlands and [2]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Referral Center for Systemic Autoimmune Diseases, Milano, Italy

Submitted 7 May 2015; revised version accepted 27 January 2016

Correspondence to: Lorenzo Beretta, Referral Center for Systemic Autoimmune Diseases, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Via Pace 9, 20122 Milano, Italy.
E-mail: lorberimm@hotmail.com

Introduction

SSc is a complex autoimmune disease characterized by vasculopathy, immune system activation and cellular matrix remodelling leading to fibrosis of the skin and of the internal organs [1]. In the vast majority of patients, the first symptom of the disease is RP, a condition that may antedate by months or years the appraisal of definite sclerodermatous fibrotic features. Subjects with RP and

the occurrence of SSc-specific autoantibodies and/or of typical alterations at nailfold videocapillaroscopy (NVC) are at risk of developing a definite SSc [2, 3] and this condition is thus defined as UCTD at risk for SSc (UCTD/SSc) [4] or early SSc according to LeRoy and Medsger [5].

Vasculopathy is pivotal to the development and progression of SSc and endothelial damage can be observed from the earliest stages of SSc. Definite SSc patients present with more severe NVC alterations compared with UCTD/SSc subjects [6] and in UCTD/SSc more severe NVC patterns are associated with shorter times of progression toward a definite SSc [3]. Moreover, adhesion molecules such as soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), chemokine (C-C motif) ligand-2 (CCL2) and chemokine (C-X-C motif) ligand (CXCL)-8 were shown to be increased in UCTD/SSc and definite SSc patients compared with controls and with a gradual increase from UCTD/SSc to fibrotic SSc [7] belonging to the limited or the diffuse cutaneous subsets (lcSSc and dcSSc [8], respectively). Overall, patients with SSc have an increase in vascular damage markers compared with healthy controls [9] and an increased risk of clinical complications in relation to the extent of vascular damage on NVC [10].

Despite these premises, a thorough study of circulating angiogenetic factors and endothelial dysfunction markers in SSc patients that includes the full spectrum of the disease has not been carried out yet. Here we assessed to what extent selected biomarkers of vasculopathy differ among the main subsets of the disease, including UCTD/SSc patients, definite SSc patients according to the 2013 ACR/EULAR criteria [11] and without skin or lung fibrosis (here referred to as defSSc), lcSSc and dcSSc patients.

Methods

Patients and controls

A total of 181 SSc patients referring to the Scleroderma Unit of our institution between January 2012 and September 2014 were considered. This group included 47 UCTD/SSc patients [5]; 48 defSSc patients according to the 2013 ACR/EULAR criteria without skin fibrosis (modified Rodnan skin score = 0) [12] and without any sign of lung fibrosis defined as a typical involvement of the lung parenchyma >5% on high resolution CT [13] accompanied by a reduced forced vital capacity (FVC) or diffusing capacity for carbon monoxide (DLco) <80% of predicted values; 51 lcSSc patients; and 35 dcSSc patients. Forty-three healthy controls matched for sex and age were also included as a comparison group.

Each patient underwent a complete evaluation to correctly allocate the patients to the different study groups. The presence of SSc-specific antibodies, ACA, anti-Topo and anti-RNA polymerase III antibodies, was determined by reviewing the patients' medical records. Clinical features indicative for defSSc [14] were evaluated, including telangiectasia, digital ulcers or pitting scars and puffy fingers; the presence of a gastroesophageal reflux disease was additionally evaluated. Where skin fibrosis was present, patients were categorized as having lcSSc or dcSSc according to LeRoy *et al.* [8]. Pulmonary function tests, with FVC and DLco determinations, were conducted according to published standards [15]. The presence of interstitial lung disease was defined as described above. The right-ventricular systolic pressure was derived by Doppler echocardiography, setting the right atrial pressure to 5 mmHg. The presence of pulmonary arterial hypertension was confirmed by right-heart catheterization. Patients underwent a complete blood test, including the determination of serum haemoglobin, CRP and ESR, and determination of gamma globulin with electrophoresis; limits of local laboratory values were used as reference. NVC was performed in UCTD/SSc patients or in subjects where the total score of nine points necessary to make the diagnosis of defSSc [3] was not otherwise reached, by one operator (M.C.) and scored according to Cutolo and Smith [10]. The study was approved by the local ethics committee (comitato etico Area B) and all the patients and controls gave their consent to participate in the study.

Vascular and angiogenetic factors

A total of seven markers were selected for the analysis: angiopoietin-2 (Ang-2), CXCL16, E-selectin, sICAM-1, CXCL8, sVCAM-1 and VEGF. Peripheral blood was obtained at enrolment by venipuncture; serum was separated by centrifugation at 1500 *g* for 10 min, aliquotted and stored at −80 °C until the analysis via a Luminex 200 instrument (Luminex Corporation, Austin, TX, USA) according to the manufacturer's instructions. All the concentrations are expressed in picograms per millilitre.

Statistical analysis

All the analyses were conducted using IBM SPSS Statistics v 22.0 software (IBM Corp., Armonk NY, USA). Continuous variables are expressed as mean (S.D.). To evaluate differences among groups one-way ANOVA with appropriate Tukey's *post hoc* or Dunnet's T3 comparisons was used if the data were, respectively, normally or non-normally distributed. The ANOVA polynomial linear test was used to evaluate trends for continuous variables; the Somers' D test for trend for categorical variables was used otherwise. Correlations among variables were determined via Pearson's r; comparison of continuous variables between dichotomous groups was performed by means of Student's *t* test. To correct for multiple testings, results were declared significant at the 0.01 threshold.

Results

Demographic data

Baseline demographic data are reported in Table 1. The four studied populations showed well-distinct clinical and laboratory characteristics indicative of worsening trends in most parameters from UCTD/SSc to defSSc to lcSSc to dcSSc. We thus observed a linear decrease in Hb serum

Table 1 Clinical characteristics of study groups

Feature	UCTD/SSc (n = 47)	defSSc (n = 48)	lcSSc (n = 51)	dcSSc (n = 35)
Age, mean (S.D.), years	52.7 (14.2)	62 (13.2)	62.1 (10.4)	54.6 (12.6)
Age Raynaud, mean (S.D.), years	42.5 (12.6	44.7 (14.3)	44.2 (12.6)	40.6 (15.1)
Age disease, mean (S.D.), years	NA	53.2 (13.4)	48.4 (10.3)	42.6 (14.3)
Time-to-evolution, mean (S.D.), years	NA	8.5 (10)	4.2 (6.7)[a]	2.3 (3.6)[b]
Capillaroscopy, n (%)	42 (89.4)	48 (100)	NA	NA
Autoantibody, n (%)				
None	6 (12.8)	1 (2.1)	3 (5.9)	1 (2.9)
ANA (non-specific)	9 (19.1)	1 (2.1)	11 (15.6)	7 (19.9)
RNAP	3 (6.4)	0 (0)	3 (5.9)	3 (8.7)
ACA	22 (46.8)	42 (87.5)	22 (43.1)	1 (2.9)[c]
Topo I	7 (14.9)	4 (8.3)	15 (29.4)	24 (68.6)[c]
Telangectasia, n (%)	0 (0)	29 (60.4)	34 (66.7)	30 (85.7)
Puffy fingers, n (%)	0 (0)	40 (83.3)	NA	NA
Active digital ulcers, n (%)	0 (0)	3 (6.3)	12 (23.5)	7 (20)
Pitting scars, n (%)	0 (0)	7 (14.6)	9 (17.6)	2 (5.7)
Lung fibrosis, n (%)	0 (0)	0 (0)	17 (33.3)	17 (48.6)
Serositis, n (%)	0 (0)	3 (6.3)	6 (11.8)	3 (8.6)
GERD, n (%)	10 (21.3)	37 (77.1)	46 (90.2)	31 (88.6)
FVC[d], % predicted, mean (S.D.)	113.7 (23.1)	109.8 (15.2)	99.3 (23)[e]	86.4 (19.6)[f,g,h]
DLco[d], % predicted, mean (S.D.)	83.9 (21.1)	74.2 (15.9)	63.2 (18.2)[i,j]	58.4 (22.1)[b,k]
RVSP, mean (S.D.), mmHg	26.2 (6.3)	29.3 (10)	31.2 (20.8)	26.1 (7.5)
ESR[d], mean (S.D.), mm/h	13.3 (11)	20.5 (12.6)	23.7 (21.8)[l]	29.5 (28.4)[f]
Haemoglobin[d], mean (S.D.), g/dl	13.5 (1.1)	13.3 (1.1)	12.8 (1.3)[l]	12.4 (1.3)[e]
Increased CRP[m], n (%)	1 (2.1)	8 (16.7)	10 (19.6)	13 (37.1)
Hypergammaglobulinaemia, n (%)	5 (10.6)	7 (14.6)	8 (15.7)	7 (20)
Use of immunosoppressants, n (%)	0 (0)	1 (2.1)	1 (13.7)	12 (34.3)

UCDT/SSc, undifferentiated connective tissue disease at risk for SSc according to [5] (early SSc according to [2]); defSSc according to [3] without lung or skin fibrosis. Raynaud age is age at the onset of RP. Disease age is age at the onset of the first non-Raynaud's manifestation. ANA without ACA or anti-Topo I or RNA-Polymerase III antibody positivity. [a]One patient with double ACA/Topo I positivity. [b]$P < 0.01$ vs defSSc. *Post hoc* comparisons on ANOVA (Tukey's test). [c]$P < 0.05$ vs defSSc. [d]One-way ANOVA polynomial test for trend $P < 0.0001$. [e]$P < 0.005$ vs UCDT/SSc. [f]$P < 0.0001$ vs UCDT/SSc. [g]$P < 0.0001$ vs defSSc. [h]$P < 0.05$ vs lcSSc. [i]$P < 0.0001$ vs UCDT/SSc. [j]$P < 0.005$ vs defSSc. [k]$P < 0.005$ vs defSSc. [l]$P < 0.05$ vs UCDT/SSc. [m]Somers' D test for ordinal trend, $P < 0.0001$. defSSc: definitive SSc; DLco: diffusing capacity for carbon monoxide; FVC: forced vital capacity; GERD: gastro-oesophageal reflux disease; n: number; NA: not available; RNAP, RNA polymerase III; RVSP: right ventricular systolic pressure.

levels and in FVC and DLco % predicted values, an increase in ESR serum values (one-way ANOVA polynomial test for trend $P < 0.0001$) and in the prevalence of abnormal CRP values, hypergammaglobulinaemia or lung fibrosis (Somers' D test for ordinal trend, $P < 0.0001$). dcSSc patients had an increased prevalence of anti-Topo I antibodies compared with lcSSc and defSSc patients, where ACA was the most common antibody. The mean interval from the onset of RP to the appraisal of the first SSc-related manifestation was longer for defSSc [8.5 (10) years] compared with both lcSSc [4.2 (6.7) years] and dcSSc patients [2.3 (3.6) years] (*post hoc* comparisons on ANOVA, $P < 0.05$ and $P < 0.01$, respectively), and for ACA^+ vs ACA^- subjects [7.5 (9) vs 3.2 (5.6) years, $P = 0.01$]; the results were confirmed in multivariate models (supplementary Table S1, available at *Rheumatology* Online).

Circulating markers

The mean (S.D.) concentrations of circulating markers in the study groups are reported in Table 2. A number of pairwise comparisons were significant when comparing SSc subsets with healthy controls and a highly significant linear trend across the different study groups was observed for Ang-2, CXCL16, E-selectin and sICAM-1 (also depicted in supplementary Fig. S1, available at *Rheumatology* Online). The descriptive statistics, as well as pairwise comparisons among groups for fibrotic SSc vs non-fibrotic SSc, are reported in supplementary Table S2, available at *Rheumatology* Online.

Correlation analysis did not show any meaningful association (assuming a Pearson's $r > 0.3$ or < -0.3 irrespective of the P-values) between vascular markers and clinical variables (age at Raynaud's onset, age at blood drawn, FVC or DLco % of predicted values, right-ventricular systolic pressure, ESR, haemoglobin). The concentrations of vascular markers did not differ in patients with or without arthritis, serositis, increased CRP, active digital ulcers and/or pitting scars and telangiectasia (supplementary Tables S3–S8, available at *Rheumatology* Online). CXCL16 was higher in patients with pulmonary fibrosis

TABLE 2 Serum concentrations of analytes

Analyte	Controls (n = 43)	UCTD/SSc (n = 47)	defSSc (n = 48)	lcSSc (n = 51)	dcSSc (n = 35)	Test for trend
Angiopoietin-2, mean (S.D.)	1949.76 (700.92)	2876.13 (1780.83)*	**3137.61 (1135.95)*** **	**3677.18 (1873.73)*** **	**3025.91 (1550.97)*** **	**F = 15.923, P < 0.0001**
CXCL16, mean (S.D.)	1075 (233.98)	1225.14 (307.96)	1248.41 (392.85)	**1330.44 (375.48)*** **	**1427.49 (378.41)*** **	**F = 21.86, P < 0.0001**
E-selectin, mean (S.D.)	22 550.69 (8968.17)	23 671.39 (9113.41)	26 526.22 (10654.6)*	27 731.69 (11 316.37)*	29 320.8 (9945.2)*	**F = 12.22, P = 0.001**
sICAM-1, mean (S.D.)	349.17 (222.75)	424.15 (307.06)	530.74 (329.99)	546.95 (407.02)*	552.4 (408.91)	**F = 9.44, P = 0.002**
IL-8/CXCL8, mean (S.D.)	146.53 (405.38)	289.23 (1019.19)	266.87 (1119.88)	215.12 (598.43)	218.81 (692.2)	F = 0.02, P = NS
sVCAM-1, mean (S.D.)	617.95 (207.18)	533.02 (219.21)	632.07 (237.85)	648.23 (274.66)	699.64 (192.15)	F = 5.859, P = 0.016
VEGF, mean (S.D.)	59.22 (32.99)	74.56 (41.08)	75.42 (38.05)	84.6 (50.2)	76.54 (48.04)	F = 4.397, P = 0.032

*P < 0.05; **P = 0.001; ***P < 0.001 vs control. Data shown in bold are significant at the P = 0.01 threshold. F: one-way ANOVA polynomial test for trend statistics.

compared with patients without lung involvement [1476.15 (358.69) vs 1258.57 (360.38) pg/ml, P = 0.002, supplementary Fig. S2, available at *Rheumatology* Online); Ang-2 was increased in subjects with hypergammaglobulinaemia [3055.8 (1540.32) vs 3984.09 (1914.24) pg/ml, P = 0.006].

Discussion

To improve our knowledge of Ssc-related vasculopathy, we studied a unique and comprehensive array of SSc subsets that includes the prototypical fibrotic cutaneous form of the disease (i.e. lcSSc and dcSSc) [8], UCTD/SSc patients [5] and the particular subset of defSSc patients that do not display lung- or skin-SSc related fibrotic manifestations.

From a clinical point of view, it is clear that the four disease subsets are distinct and characterized by different degrees of severity ranging from UCTD/SSc (the less severe) to dcSSc (the most severe) with defSSc representing an intermediate stage of disease severity between early and fibrotic SSc. This gradient of severity is reflected by a statistically significant linear trend in several clinical and laboratory parameters as clearly depicted in Table1.

From the biological point of view, the presence of four clinical entities characterized by different severity is reflected by the serum levels of most of the vascular markers we analysed. Striking linear trends for Ang-2, CXCL16, E-selectin and sICAM-1 serum levels can be observed in relation to the different disease subsets (Table 2, supplementary Fig. S1, available at *Rheumatology* Online). While a formal statistical difference between healthy controls and UCTD/SSc could not be detected, our findings clearly point out the presence of an initial state of vasculopathy in preclinical SSc that worsens as the disease gets more severe.

According to our results, several aspect of vasculopathy are involved in disease severity and stage, including a defective angiogenesis (Ang-2, VEGF, chemokines), an activation of endothelial cells (E-selectin, sICAM-1) and the regulation of endothelial function and modulation of tissue microenvironment (chemokines). Even though the different SSc subsets have strikingly different clinical and laboratory characteristics (Table 1) and vascular markers are in tight relation with these subsets (Table 2), we did not find any meaningful correlation with any specific organ manifestation, with the sole exception of CXCL16 with lung fibrosis. The results on CXCL16 are largely supported by the literature. CXCL16 is actively expressed in bronchial epithelial cells and lung fibroblasts [16], and increased concentrations can be found in bronchoalveolar lavage fluids from patients with interstitial lung disease [17].

One of the main challenges in the early diagnosis of chronic diseases is the possibility of an early intervention to attenuate disease progression. The non-prospective nature of our study does not allow establishing whether concentrations of vascular markers are predictive of the evolution of UCTD/SSc patients toward a definite or fibrotic disease and whether they might be used to evaluate the response to putative disease-modifying therapies in this subset of patients.

Due to the unique nature of the population we analysed and to the lack of data in the literature on UCTD/SSc patients, a direct comparison of our results with published research is almost impossible. Only one study determined CXCL8, sICAM-1 and sVCAM-1 in a smaller group of UCTD/SSc patients (n = 24) [7]; the results reported by these authors are largely comparable to ours as far as sICAM-1 and sVCAM-1 are concerned, while we could not replicate their CXCL8 findings. Most likely this difference lies in Luminex assay variance in relation to data storage and handling, as assayed CXCL8 concentrations rapidly decline after serum extraction [18]. In that study [7], samples were collected during a 12-year span while in our study the maximum interval was 3 years; this would easily explain the higher CXCL8 concentrations we observed and the variability of these findings. Overall, despite the advantages of using Luminex assay in small studies in a single laboratory (e.g. the small volume of serum needed, the efficiency in time and costs, the agreement with single ELISA measurements) the variance of results when comparing different populations or different analyte panels advise some caution in the interpretation of results [19].

Summarizing, our study confirms that vasculopathy is relevant to disease severity and stage in SSc. We showed, for the first time, that distinct SSc subsets have different degrees of vasculopathy and that markers of abnormal endothelial function are increased in the earliest stages of the disease where clinical and laboratory findings of advanced disease cannot yet be observed. These abnormalities progress with the appraisal of the first sclerodermatous manifestation in defSSc and further increase with the onset of fibrotic manifestations and evolution of the disease. Further longitudinal studies in UCTD/SSc patients are needed to evaluate the change of vascular markers in relation to the possible evolution of the disease and to capture the difference between those who are at risk for progression toward a defSSc and those who are not.

Funding: The present work was financed by a grant from Gruppo Italiano per la Lotta alla Sclerodermia, theme 'La diagnosi precoce della sclerodermia'.

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

References

1 Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med 2009;360:1989–2003.

2 Koenig M, Joyal F, Fritzler MJ *et al*. Autoantibodies and microvascular damage are independent predictive factors for the progression of Raynaud's phenomenon to systemic sclerosis: a twenty-year prospective study of 586 patients, with validation of proposed criteria for early systemic sclerosis. Arthritis Rheum 2008;58:3902–12.

3 Vigone B, Santaniello A, Marchini M *et al*. Role of class II human leucocyte antigens in the progression from early to definite systemic sclerosis. Rheumatology 2014;54:707–11.

4 Valentini G. Undifferentiated Connective Tissue Disease at risk for systemic sclerosis (SSc) (so far referred to as very early/early SSc or pre-SSc). Autoimmun Rev 2015;14:210–3.

5 LeRoy EC, Medsger TA Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol 2001;28:1573–6.

6 Camargo C, Sekiyama J, Arismendi M *et al*. Microvascular abnormalities in patients with early systemic sclerosis: less severe morphological changes than in patients with definite disease. Scand J Rheumatol 2015;44:48–55.

7 Vettori S, Cuomo G, Iudici M *et al*. Early systemic sclerosis: serum profiling of factors involved in endothelial, T-cell, and fibroblast interplay is marked by elevated interleukin-33 levels. J Clin Immunol 2014;34:663–8.

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

9 Liakouli V, Cipriani P, Marrelli A *et al*. Angiogenic cytokines and growth factors in systemic sclerosis. Autoimmun Rev 2011;10:590–4.

10 Cutolo M, Smith V. State of the art on nailfold capillaroscopy: a reliable diagnostic tool and putative biomarker in rheumatology? Rheumatology 2013;52:1933–40.

11 van den Hoogen F, Khanna D, Fransen J *et al*. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. Arthritis Rheum 2013;65:2737–47.

12 Clements PJ, Lachenbruch PA, Seibold JR *et al*. Skin thickness score in systemic sclerosis: an assessment of interobserver variability in 3 independent studies. J Rheumatol 1993;20:1892–6.

13 Goh NS, Desai SR, Veeraraghavan S *et al*. Interstitial lung disease in systemic sclerosis: a simple staging system. Am J Respir Crit Care Med 2008;177:1248–54.

14 Fransen J, Johnson SR, van den Hoogen F *et al*. Items for developing revised classification criteria in systemic sclerosis: Results of a consensus exercise. Arthritis Care Res 2012;64:351–7.

15 Miller MR, Hankinson J, Brusasco V *et al*. Standardisation of spirometry. Eur Respir J 2005;26:319–38.

16 Morgan AJ, Guillen C, Symon FA *et al*. Expression of CXCR6 and its ligand CXCL16 in the lung in health and disease. Clin Exp Allergy 2005;35:1572–80.

17 Day C, Patel R, Guillen C *et al*. The chemokine CXCL16 is highly and constitutively expressed by human bronchial epithelial cells. Exp Lung Res 2009; 35:272–83.

18 de Jager W, Bourcier K, Rijkers GT *et al*. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. BMC Immunol 2009;10:52.

19 Breen EC, Reynolds SM, Cox C *et al*. Multisite comparison of high-sensitivity multiplex cytokine assays. Clin Vaccine Immunol 2011;18:1229–42.