IL-6 driven STAT3 signalling in circulating CD4+ lymphocytes is a marker for early anti-citrullinated peptide antibody-negative rheumatoid arthritis.

Anderson AE *et al*.

Online Supplementary Data

Prediction Rule.

Method for modifying Leiden prediction rule (as described in *Reference 17* of main document)**.**

Binary logistic regression was carried out amongst 32 UA patients in the described cohort whose outcome diagnoses were known, with diagnostic outcome (RA versus non-RA) as the dependent variable (see also main document text and *Supplementary Table S4* for demographic details of this cohort). For each individual in the cohort the Leiden prediction score was calculated according to available baseline clinical and serological parameters, and instructions outlined in *Reference 17*. In addition, the ratio of constitutive pSTAT3/pSTAT1 (MFI) in circulating CD4+ T-cells was calculated for each individual. These 2 variables were entered into the regression model as independent variables, and the results are depicted below.

	B	SE(B)	Wald	**p-value**	95% CI (B)
Leiden Score	**-0.60**	0.3	3.53	**0.067**	0.29-1.04
pSTAT3/pSTAT1	**-2.97**	1.4	4.25	**0.039**	0.003-0.87
Constant	7.38	2.7	7.41	**0.006**	-

B=regression coefficient; SE=standard error; CI= confidence interval.

Based on the above, and utilising the respective regression coefficients, we reasoned that for an individual UA patient the probability of RA development was related to the two covariates via the expression:

(-0.6[Leiden Score]) + (-2.97[CD4+ pSTAT3:pSTAT1).

In order to simplify the calculation for general use, we rounded regression coefficients to the nearest integer and removed negative charges. Since the revised metric was designed primarily for use amongst ACPA-negative UA patients (and all but one of the patients in our cohort were indeed ACPA negative), we then modified the Leiden prediction rule by stipulating that ACPA status was no longer considered, but that a value of 3 times the constitutive CD4+ T cell pSTAT3:pSTAT1 ratio was added to the accumulating score instead. Hence, for an individual, the modified metric is calculated as shown overleaf:

1. What is the age?	…Multiply by 0.2:		_____
2.What is the sex?	In case female	1 point	_____
3. What is the distribution of involved joints?			
	In case small joints hands and feet	0.5 point	_____
	In case symmetric	0.5 point	_____
	In case upper extremities	1 point	
Or:	In case upper and lower extremities	1.5 point	_____
4. What is the length of the morning stiffness (minutes)?			
	In case 30-59 min	0.5 point	_____
	In case ≥60 min	1 point	_____
5. What is the number of tender joints (out of 68 joints)?			
	In case 4-10	0.5 point	_____
	In case 11 or higher	1 point	_____
6. What is the number of swollen joints (out of 66 joints)?			
	In case 4-10	0.5 point	_____
	In case 11 or higher	1 point	_____
7. What is the C-reactive protein level (mg/L)?			
	In case 5-50	0.5 point	_____
	In case 51 or higher	1.5 point	_____
8. Is the rheumatoid factor positive?			
	If yes	1 point	_____
9. What is the CD4+ T cell pSTAT3:pSTAT1 ratio?			
	…Multiply by 3:		_____
		Total score:	_____

Table S1.

	ACPA-RA (n=18)	**ACPA+ RA** (n=24)	**Non-RA Inflam.** (n=44)	**OA / non-inflam.** (n=66)	**UA** (n=35)	p (Inflam. diagnoses[A])	p (All Diagnoses[B])
Age (years)	**68** (30-88)	**58** (27-81)	**55** (18-91)	**51** (27-86)	**52** (19-79)	0.016 ns	0.002 ns
% Female	**72**	**71**	**64**	**74**	**71**	ns	ns
Symptom duration. (weeks)	**12** (4 - >52)	**16** (4 - >52)	**9** (2 - >52)	**18** (3 - >52)	**12** (3- >52)	ns	ns
ESR (seconds)	**23** (1-71)	**22** (4-86)	**13** (1-113)	**8** (1-100)	**15** (1-78)	ns	0.001
CRP (g/l)	**10** (<5-91)	**10** (<5-56)	**8** (<5-189)	**<5** (<5-49)	**9** (<5-76)	ns	0.001
%ACPA+	**0**	**100**	**4**	**0**	**6**	<0.001	<0.001
%RF+	**33**	**75**	**9**	**9**	**17**	<0.001	<0.001
DAS28	5.15 (2.31-7.16)	5.00 (1.59-7.07)	n/a	n/a	n/a	ns	-

Table S1*. Clinical and serological characteristics of Newcastle early arthritis cohort stratified by baseline diagnosis. Except where indicated, median and range is given.* [A]*Kruskal Wallis ANOVA analysis confined to 3 groups with confirmed inflammatory diagnoses at inception: ACPA- RA, ACPA+ RA and non-RA IA.* [B]*Kruskal Wallis ANOVA, 5 groups, including OA / non-inflammatory arthralgia and undifferentiated arthritis (UA).*

Table S2

A	**Untreated RA** (n=22)	**Treated RA** (n=18)	**Healthy** (n=13)
Brisbane cohort, n (%)[A]	**4** (18)	**18** (100)	**13** (100)
Women, n (%)	**15** (68.2)	**10** (55.6)	**7** (54)
Age, years; mean (SD)	**57** (46-71)	**56** (41-71)	**31** (23-40)
Symptom duration, weeks	**16** (8-29)	**88** (48-192)	n/a
RF positive, n (%)	**14** (63.6)	**16** (88.8)	ND
CCP positive, n (%)	**14** (63.6)	**16** (88.8)	ND
Swollen joint count	**3** (0-7)	**0** (0-2)	n/a
Tender joint count	**7** (2-14)	**0** (0-5.5)	n/a
ESR, mm/h	**25** (11.8-55.5)	**16** (9.5-28.5)	ND
CRP, mg/l	**10** (5.8-22.5)	**3.5** (2-13)	ND
DAS28[B]	**5.35** (2.76-6.17)	**3.09** (2-4)	n/a
Treatment (% of cohort)			
Methotrexate		15 (83%)	
Hydroxychloroquine		14 (78%)	
Sulphasalazine		9 (50%)	
Azathiaprine		1 (5%)	
Low dose prednisolone		1 (5%)	

B	**Inception RA cohort**		
Baseline characteristic	Newcastle (n=7)	Brisbane (n=4)	**Total (n=11)**
Women, n (%)	5 (71)	2 (50)	**7** (64)
Age, years; mean (SD)	64 (53-75)	57 (46-68)	**61** (50-72)
Symptom duration, weeks	20 (8-52)	13 (12-42)	**18** (8-52)
DAS28	4.73 (2.01-7.15)	5.11 (2.11-6.7)	**4.73** (2.01-7.15)

***Table S2A**. Clinical characteristics of early / established RA patients and controls from Newcastle / Brisbane as per* Figure 1E. *[A]The majority (18/22; 82%) DMARD-naïve early arthritis patients were drawn from the Newcastle cohort, and all other patients from the Brisbane cohort. [B]There was a significant difference in DAS28 between untreated and treated RA patients ($p<0.05$, Mann-Whitney U test).*

***Table S2B**. Baseline clinical characteristics of inception RA cohort for which data depicted in* Figure 1F, *and showing respective contribution of patients drawn from Newcastle and Brisbane cohorts (the same 4 Brisbane patients contributed to the untreated RA group in Table 2A). Phosflow measurements were made in fresh blood in each case prior to, and 3 months following, initiation of DMARD therapy. DMARD therapy included methotrexate in all cases; all Brisbane patients received combination therapy with hydroxychloroquine and sulphasalazine (see reference 23, main document); Newcastle patients received a bolus of steroid (80mg im triamcinolone).*

Except where indicated, median values are presented (interquartile range). ND: not done; n/a: not applicable.

Table S3

		RA Patients			
		1	2	3	4
	Age (Yrs)	71	52	80	40
	Sex	F	M	F	F
	Disease Durⁿ. (Yrs)	17	13	14	5
DMARDs	Concurrent	none	MTX	none	MTX, HXQ
	Prior	RTX, MTX	RTX	Gold	RTX

Table S3. *Clinical characteristics of 4 patients commenced on tocilizumab therapy (see text). RTX: rituximab; MTX: methotrexate; HXQ: hydroxychloroquine.*

Table S4

Multiple Regression Analysis

A	Unstandardised coefficients:		**Standardised coefficients:**		
Variable	B	SE(B)	β	**p-value**	95% CI (B)
Log_{10}[**IL-6**]	101.7	27.1	**0.441**	**<0.001**	43.59-150.41
Log_{10}[**CRP**]	0.90	0.34	**0.182**	**0.01**	0.24-1.56
Log_{10}[**TNF**]	5.60	35.81	**0.011**	**0.88**	-65.06-76.25
Age	0.61	0.48	**0.083**	**0.21**	-0.337-1.562
Constant	97.00	27.07	**-**	**<0.001**	43.59-150.41

B	Unstandardised coefficients:		**Standardised coefficients:**		
Variable	B	SE(B)	β	**p-value**	95% CI (B)
Log_{10}[**IL-6**]	162.055	19.476	**0.558**	**<0.001**	123.63-200.49
Log_{10}[**CRP**]	1.004	0.380	**0.161**	**0.009**	0.25-1.76
Log_{10}[**TNF**]	21.118	40.491	**0.032**	**0.603**	-58.78-101.01
Age	0.522	0.548	**0.056**	**0.342**	-0.56-1.60
Constant	91.247	30.733	**-**	**0.003**	30.61-151.89

***Table S4 A** and **B**. Results of standard linear regression analysis to identify variables independently associated with CD4+ T cell pSTAT-3 amongst 187 EA clinic patients. The dependent variable was pSTAT3 (median fluorescence intensity) amongst total circulating CD4+ T cells (Table **S4 A**) or naïve (CD45RA+ CD62L+) CD4+ T cells (Table **S4 B**) . SE (B): standard error for B; CI: confidence interval. Where necessary variables were log10 transformed to satisfy normality conditions. See main article text.*

Table S5

	UA* – RA	**UA* – Non-RA**	**P value†**
	(n=12)	(n=20)	
Age	**55**	**48**	ns
(years)	(35-79)	(19-79)	
Female; n (%)	**8** (67)	**15** (75)	ns
Symptom duration.	**12**	**10**	ns
(weeks)	(3-52)	(3-52)	
Swollen joint count	**3**	**2.5**	ns
(n)	(0-18)	(0-15)	
ESR	**18**	**14**	ns
(seconds)	(4-65)	(1-78)	
CRP	**11**	**8**	ns
(g/l)	(5-53)	(<5-76)	
ACPA+ ; **n** (%)	**1** (10)	**0** (0)	ns
RF+ ; **n** (%)	**4** (33)	**1** (5)	ns
Diagnosis; n (%)			
RA	12	-	
PsA	-	6	
S-LIA	-	7	
ReA	-	3	
NIA	-	4	

Table S5*. Baseline characteristics and outcome diagnoses for 32/35 patients with undifferentiated arthritis (UA) patients in the cohort for whom all information is available; median follow-up since inception = 20 months (range 11-25); *All patients, classified here with reference to 2010 RA criteria* (Ref 15)*, were also determined UA with reference to pre-exiting 1987 criteria* (Ref 21)*. †Mann Whitney-U test. N.b. diagnoses for additional 3 patients in cohort remains UA; median follow-up for this group was shorter at 12 (9-21) months. RA: rheumatoid arthritis; PsA: psoriatic arthritis; S-LIA: self-limiting inflammatory arthritis; ReA: reactive arthritis; NIA: non-inflammatory arthralgia / OA.*

Table S6

A	Mean AUC	Median AUC	SD AUC
Leiden score	0.67	0.68	0.11
pSTAT3: pSTAT1	0.78	0.79	0.09
"Composite"	0.84	0.85	0.09

Table S6A. *Summaries of AUC across 1000 bootstrap samples for 3 parameters compared in* Figure 5D*, main document (see text).The mean values are identical to those derived in the primary analysis. "Composite" refers to composite risk metric derived from Leiden score modified to incorporate pSTAT3:pSTAT1 ratio (see text); AUC: area under curve; SD: standard deviation.*

Table S7

A.

		Actual outcome diagnosis		
		RA	Non-RA	Total
Predicted diagnosis	RA	9	2	11
	Non-RA	3	18	21
	Total	12	20	32

B.

	Value	(95% CI)
Prevalence[§]	0.38	(0.21 - 0.56)
Sensitivity	0.75	(0.50 - 0.88)
Specificity	0.90	(0.75 - 0.98)
+LR	7.5	(1.98 - 42.5)
-LR	0.28	(0.12 – 0.67)
PPV[§]	0.82	(0.54 – 0.96)
NPV[§]	0.86	(0.71 – 0.93)
Accuracy	0.84	(0.65 – 0.94)

Table S7. ***A***. *Contingency table cross-tabulating* predicted *diagnosis (based on calculated modified Leiden score, and employing an optimum score cut-off of 9.5, above which progression to RA is predicted) versus* actual *outcome diagnosis, for 32 UA patients in this study.* ***B***. *Diagnostic evaluation statistics based on contingency table presented in A. CI: confidence interval; +LR: positive likelihood ratio; -LR: negative likelihood ratio; PPV: positive predictive value; NPV: negative predictive value.* [§]*Prevalence value equates to proportion of UA patients in current sample (n=32) who actually progressed to RA, or* prior probability *of progression to RA. Calculations for PPV and NPV are valid where the value for prevalence is generalizable to the population (i.e. it is assumed that the rate of UA-RA progression of 0.38 is representative of the UA population in general).*

Figure S1

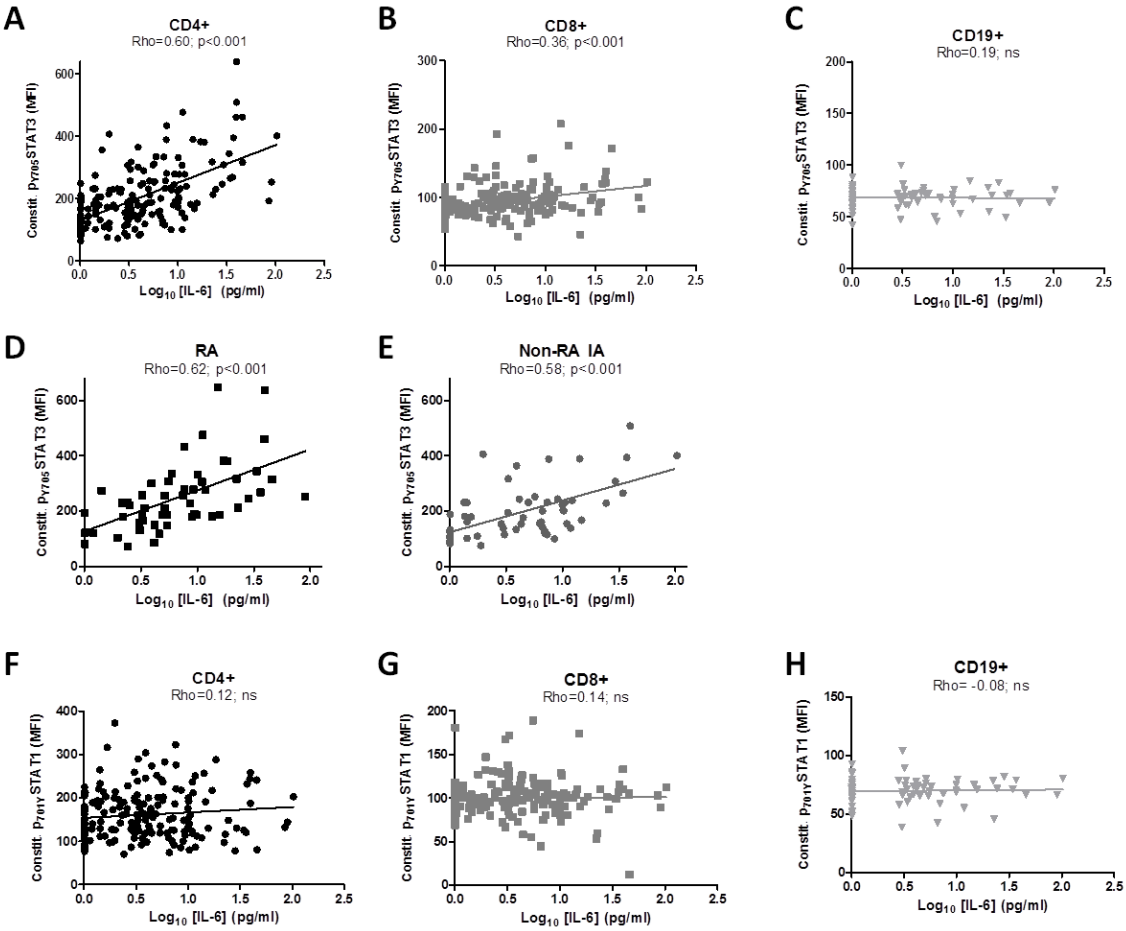

***Figure S1**. **A-C**. Individual plots depicted in Figure 2C of main article. **D-E**. Individual plots depicted in Figure 2D of main article. **F-H**. Individual plots depicted in Figure 2E of main article.*

Figure S2

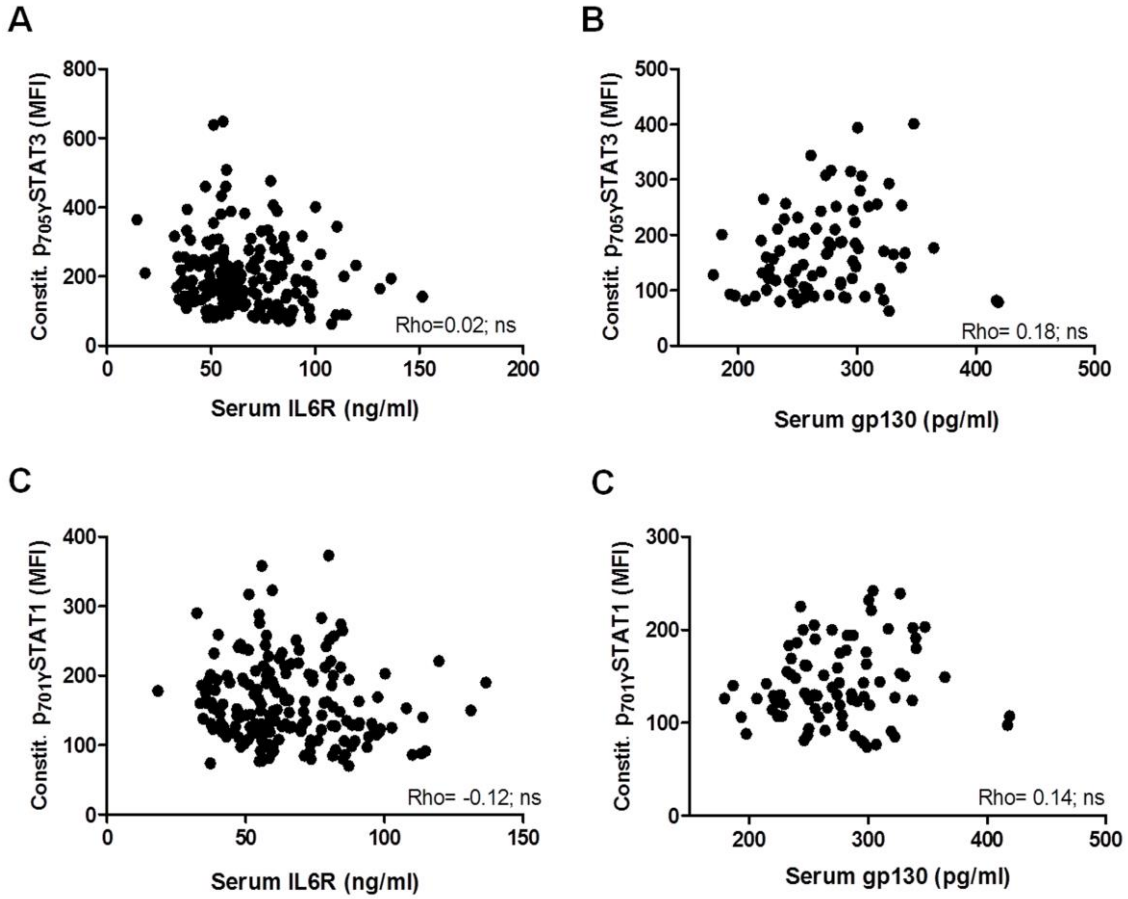

Figure S2. ***A-D***. *Lack of correlation between constitutive CD4+ T cell pSTAT3 or pSTAT1 with either sIL-6R or sgp130. Spearman's correlation coefficient (Rho) for each bivariate analysis; ns: not significant. Paired data available for 187 patients (sIL-6R) and 88 patients (sgp130).*

Figure S3

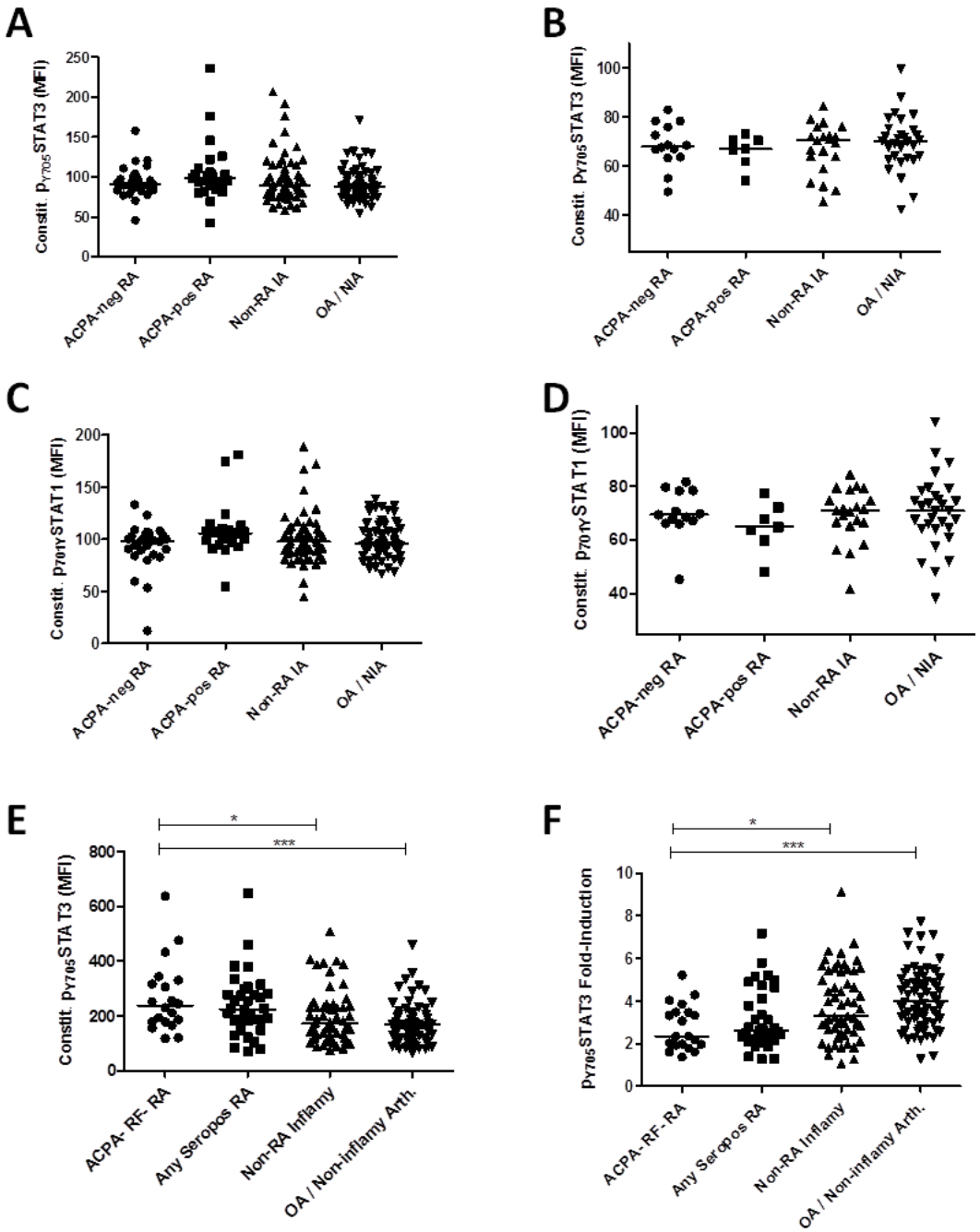

Figure S3 A-D*. No differences are seen between diagnostic groups with respect to constitutive pSTAT3 (**A**, **B**) or pSTAT1 (**C**, **D**) in CD8+ T-cells (left panels;184 patients) or CD19 B-cells (right panels; data available for 71 patients).* ***E-F****. Similar results to those presented in Figure 4 were obtained when RA patients were stratified according to whether they were RF* and *ACPA "double-seronegative"* versus *serpositive for* either *RF* or *ACPA; exemplar data shown with respect to constitutive pSTAT3 (**E**) and pSTAT3 fold-induction (**F**) in CD4+ T cells; compare with Figures 4B and C of main article. * and *** indicate p<0.05, and <0.001 (Dunn's post-hoc pairwise analysis following non-parametric ANOVA).*

Figure S4

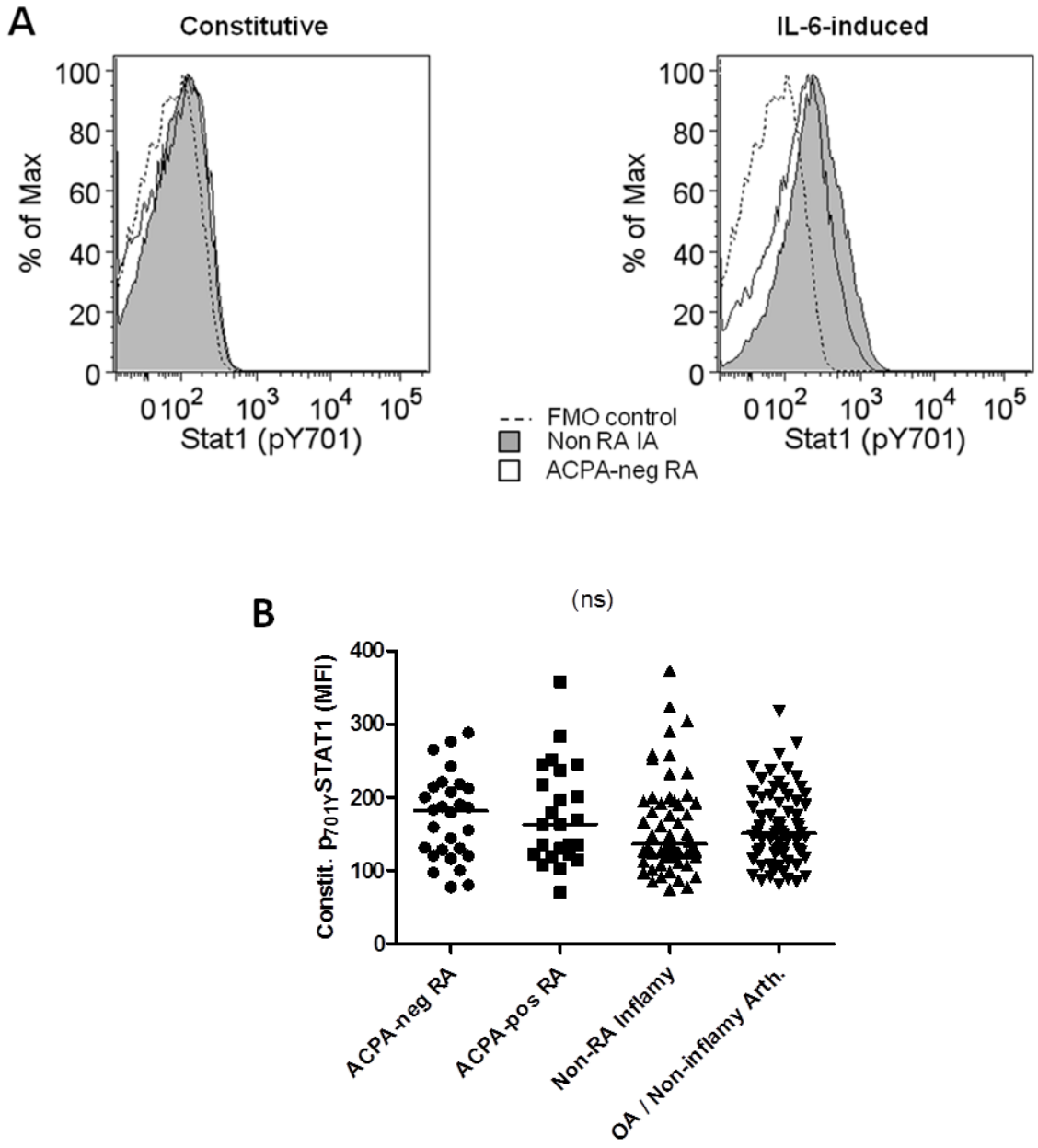

Figure S4. **A**: Representative flow cytometry histograms depicting constitutive and IL-6 induced pSTAT1 in the CD4+ T cell-gated population of whole blood from exemplar non-RA IA disease control (shaded plots) and ACPA-negative RA patient (non-shaded plots; dotted line denotes fluorescence-minus-one control). **B**: *ANOVA (Kruskall-Wallis)* reveals no relationship between constitutive pSTAT1 and diagnostic outcome in circulating CD4+ T cells of early arthritis patients. MFI: median fluorescence intensity.

Figure S5

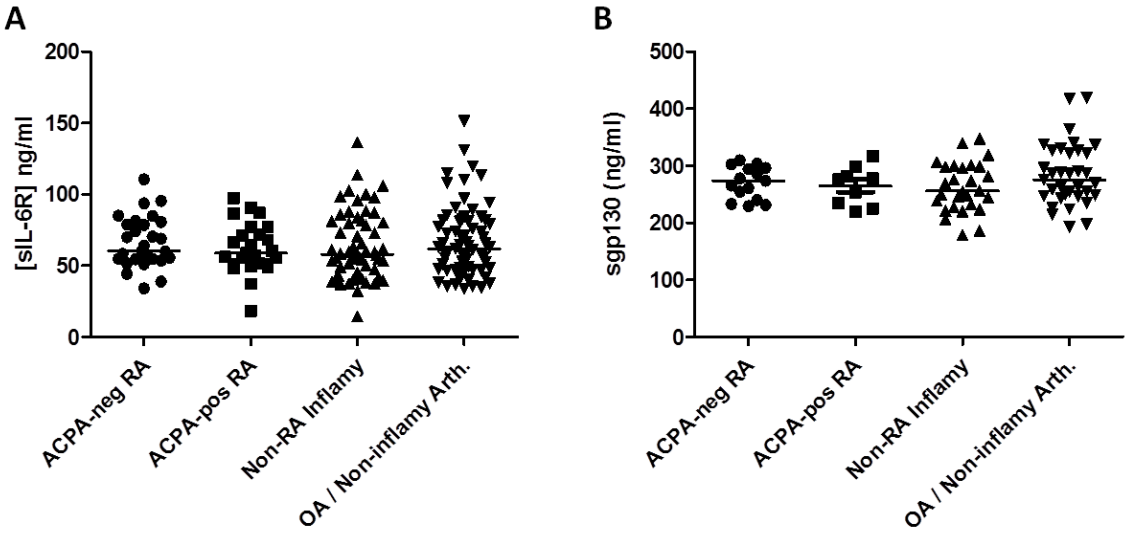

Figure S5. *No differences are seen between diagnostic groups with respect to serum concentrations of (**A**) soluble IL-6 receptor (184 individuals) or (**B**) soluble gp130 (data available for 88 individuals.*

Figure S6

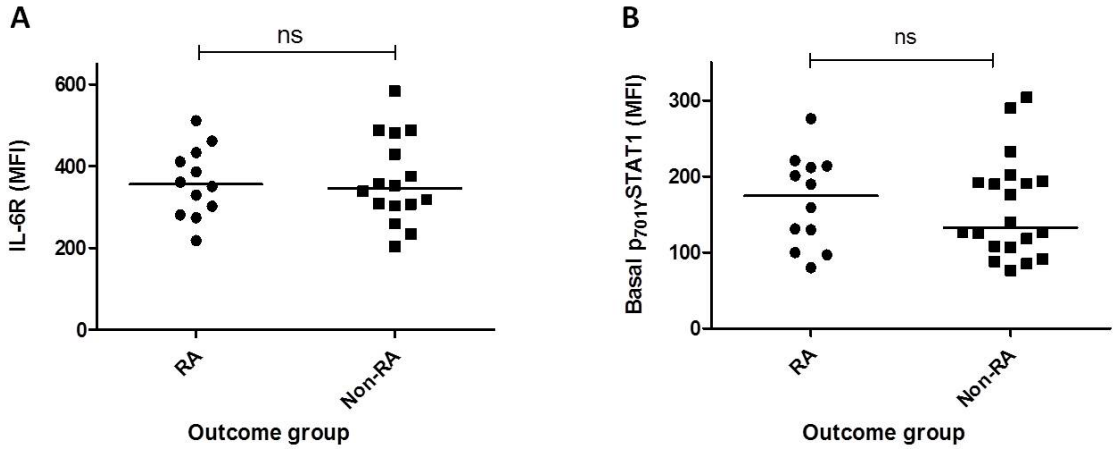

Figure S6. *Surface IL-6R (**A**) and intracellular pSTAT1 (**B**) in circulating CD4+ T cells of patients presenting with undifferentiated arthritis are each comparable between those who evolve into classifiable RA and those with alternative diagnoses at follow-up; see text. No significant differences are seen between comparator groups (Mann-Whitney U test).*