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TRANSLATIONAL SCIENCE

Interferon-α-mediated therapeutic resistance in early rheumatoid arthritis implicates epigenetic reprogramming

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ABSTRACT

Objectives An interferon (IFN) gene signature (IGS) is present in approximately 50% of early, treatment naive rheumatoid arthritis (eRA) patients where it has been shown to negatively impact initial response to treatment. We wished to validate this effect and explore potential mechanisms of action.

Methods In a multicentre inception cohort of eRA patients (n=191), we examined the whole blood IGS (*MxA, IFI44L, OAS1, IFI6, ISG15*) with reference to circulating IFN proteins, clinical outcomes and epigenetic influences on circulating CD19+ B and CD4+ T lymphocytes.

Results We reproduced our previous findings demonstrating a raised baseline IGS. We additionally showed, for the first time, that the IGS in eRA reflects circulating IFN-α protein. Paired longitudinal analysis demonstrated a significant reduction between baseline and 6-month IGS and IFN-α levels (p<0.0001 for both). Despite this fall, a raised baseline IGS predicted worse 6-month clinical outcomes such as increased disease activity score (DAS-28, p=0.025) and lower likelihood of a good EULAR clinical response (p=0.034), which was independent of other conventional predictors of disease activity and clinical response. Molecular analysis of CD4+ T cells and CD19+ B cells demonstrated differentially methylated CPG sites and dysregulated expression of disease relevant genes, including *PARP9, STAT1,* and *EPSTI1*, associated with baseline IGS/IFNα levels. Differentially methylated CPG sites implicated altered transcription factor binding in B cells (GATA3, ETSI, NFATC2, EZH2) and T cells (p300, HIF1α).

Conclusions Our data suggest that, in eRA, IFN-α can cause a sustained, epigenetically mediated, pathogenic increase in lymphocyte activation and proliferation, and that the IGS is, therefore, a robust prognostic biomarker. Its persistent harmful effects provide a rationale for the initial therapeutic targeting of IFN-α in selected patients with eRA.

WHAT IS ALREADY KNOWN ABOUT THIS TOPIC

⇒ Type I interferons (IFNs) and the IFN gene signature (IGS) have received less attention in rheumatoid arthritis (RA) than in other rheumatic diseases such as systemic lupus erythematous.
⇒ Nonetheless, emerging evidence hints at a potentially important role for the IGS in early disease although, until now, it was unknown which IFN class was responsible.

WHAT DOES THIS STUDY ADD?

⇒ We demonstrate that IFN-alpha levels are transiently elevated in some early RA patients and are responsible for generating the IGS.
⇒ We validate the IGS as a robust prognostic biomarker associated with poor 6 month outcomes.
⇒ We also implicate IFN-α/IGS in epigenetic modification of circulating B and T lymphocytes, at genes associated with activation and proliferation, providing a potential mechanism for its persistent harmful effects.

HOW MIGHT THIS IMPACT ON CLINICAL PRACTICE

⇒ Our data provide a strong rationale for the use of therapies that target the IFN-α pathway and the IGS in selected early RA patients.
⇒ Our work also has implications for other conditions with high IFN-α levels, such as COVID-19, and the potential for persistent harmful sequelae.

INTRODUCTION

An interferon gene signatures (IGS) has been reported in multiple autoimmune conditions, including rheumatoid arthritis (RA).[1] It is a composite score of interferon response genes (IRGs) that are classically upregulated in response to type 1 interferons (IFN-I). IFN-I are released on detection of viral/bacterial genetic material by various nucleic acid receptors (NARs),[1 2] but the pathway by which IFN production is triggered in RA is unknown. Furthermore, there is an overlap between downstream signalling pathways for all interferon classes with upregulation of common

IRGs.[3] Historically, the direct measurement of IFN-I has been challenging,[3] creating uncertainty around which IFN class drives the IGS in RA thereby limiting understanding of its pathophysiological relevance.

To date, no association has been reported between the IGS and disease activity in established RA. However, longstanding patients with RA are frequently prescribed additional therapies, which modulate the IGS.[4 5] By contrast, we previously demonstrated in early RA (eRA) patients (naïve for disease-modifying antirheumatic drugs (DMARDs) and glucocorticoids), the IGS positively associates with baseline disease activity and, independent of conventional markers of disease activity, associates with worse clinical outcomes at 6 months.[6] The pathophysiological processes in eRA are distinct from those of established disease and the IGS is more prominent in eRA than in established RA.[6] The role of epigenetics in modifying phenotype is increasingly appreciated in autoimmunity[7] and RA has an early window of therapeutic opportunity. Thus, understanding and predicting heterogeneity to therapeutic response is important for early precision therapeutics.

In a large multicentre eRA cohort, we sought to: (1) confirm the IGS negatively impacts disease outcomes, (2) clarify which IFN classes are responsible for IGS generation, (3) seek evidence that IFN-α exposure contributes to a harmful epigenetic footprint at disease onset, potentially explaining its negative effect on longer-term outcomes.

METHODS

Patient cohorts

DMARD and glucocorticoid naive patients with eRA were recruited from the Newcastle Early Arthritis Clinic (NEAC) as described previously.[6 8] Data and samples relating to the 'Towards A CurE for RA' (TACERA) cohort,[9] an existing additional independent cohort of eRA was obtained from RA-MAP, a multicentre UK industry-academic partnership. All patients with eRA met the 1987[10] or 2010[11] RA classification criteria. TACERA patients were included according to the availability of clinical and transcriptome data (quality controlled) and biological samples. In some analyses, missing data sets, particularly longitudinal clinical data, reduced cohort size.

Clinical parameters including autoantibody titres (anticitrullinated protein/peptide antibody (ACPA) and rheumatoid factor (RF)), disease activity score (DAS-28) and its components were recorded. For TACERA at 6 months, DAS-28 was repeated, drug history recorded and additional biological samples were collected. All patients gave informed consent as described in Clark *et al* and RA-MAP Consortium.[8 9]

Patient and public involvement

This research was done without formal patient and public involvement.

Serum cytokines

Serum IFN-α was measured using the digital Simoa platform as described.[12] Monoclonal antibodies (specifically for all IFN-α subtypes) were isolated from autoimmune polyendocrinopathy candidiasis ecto-dermal dystrophy patients[13] and provided to D. Duffy by Immunoqure under a material transfer agreement. IFN-β, IFN-γ and IFN-λ1/IL29 (referred to hereafter as IFN-λ) were measured by MSD technology (Meso Scale Discovery, MD, USA) as per manufacturers' instructions.

Whole blood and cell-specific transcriptome/methylome

TACERA whole blood analyses used Tempus blood RNA tubes (Applied Biosystems). Peripheral blood mononuclear cells (PBMCs) were isolated using Leucosep separation tubes (Greiner) followed by MagMAX RNA isolation kits (Ambion). For subsequent microarray analysis, amplified RNA was hybridised to beadchips and scanned on an Illumina Beadstation 500 as described further in.[9] Full data are available via Gene Expression Omnibus database (GEO), http://www.ncbi.nlm.nih.gov/geo accession number GSE9747638. Existing paired microarray gene expression and DNA methylation data from CD4+ T cells and CD19+ B cells extracted from NEAC eRA patients was preprocessed as described in Clark *et al*,[8] GEO accession number GSE137634. In brief, this involved positive selection of CD4+ T cells/CD19+ B cells, RNeasy Mini kits or AllPrep DNA/RNA Mini kits (Qiagen) and Illumina Whole Genome 6 V.3/12HT BeadChip or a MethylationEPIC BeadChip for RNA and DNA, respectively. Additional method details included in online supplemental file 1.0.

Whole blood IGS

For both cohorts, the IGS was calculated as an average of whole blood or cell-specific expression of *MX1*, *IFI44L*, *OAS1*, *ISG15* and *IFI6*. IGS scores in the first or fourth quartiles were termed IGS high or low respectively.

Gene expression and DNA methylation analysis of eRA lymphocytes

Analyses included differential gene expression (DEGs) and differential methylation sites (DMSs) between IGS high and low eRA patients, effect of methylation on gene expression, pathway analysis of DEGs and enrichment analysis of DMSs within defined chromatin states. Full methods are provided in online supplemental file 1.0.

Modelling and statistical analysis

GraphPad Prism (V.5.0; GraphPad Software, La Jolla, Calif), JMP Statistical Visualisation (V.14; SAS Institute, Cary, North Carolina) and R Core Team (2020) software was used. Tests included Mann-Whitney U, Wilcoxon matched-pairs signed rank tests, simple linear regression, generalised linear models, multivariable and logistic regression. To adjust for potential confounding variables, tests of a significant association between IGS and 6 month outcomes were performed after adjustment for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), age, sex, baseline DAS28 and DMARDs, including glucocorticoids. R^2 values are reported as a measure of how well the regression model fits the observed data. Statistical significance when $p<0.05$.

RESULTS

Patient cohorts

The TACERA cohort included 191 seropositive (ACPA and/or RF positive) patients with eRA. A separate NEAC cohort of mixed seropositive and seronegative (ACPA and RF negative) patients with eRA had paired transcriptome and methylome data from circulating B and/or T lymphocytes (n=41 and n=41, respectively, total cohort of n=54) with contemporaneous T and B cell data being available for 28. Patient demographics and clinical characteristics are shown in table 1. The demographic data and relevant methods of an additional smaller validation cohort of patients with NEAC (n=51) who had additional circulating inflammatory cytokines measured in addition to serum-IFN-α and the IGS are shown in online supplemental file 2.0.

Table 1 Early RA cohort demographics

			NEAC	
			NEAC lymphocyte methylome and transcriptome cohorts	
		RA-MAP TACERA cohort	CD4+ T cell	CD19+ B cell
Number (n)		191	41	41
Age, years		55 (20–84)	58 (27–74)	58 (27–74)
Female, n (%)		116 (61%)	26 (63%)	30 (73%)
RF positive, n (%)		155* (90%)	23 (56%)	26 (63%)
ACPA positive, n (%)		147* (85%)	17 (41%)	22 (54%)
DAS-28-CRP		5.27 (2.23–8.14)	4.61 (1.26–6.53)	4.36 (1.26–6.53)
CRP (mg/L)		8.65 (1-136)	9 (5–13)	9.5 (4–53)
Erythrocyte sedimentation rate (mm/h)		28 (2–113)	19 (7–32)	20.5 (2–86)
DMARDS initiated	Number with available data	175		
	MTX	144 (82%)		
	SSZ	10 (6%)		
	HCQ	91 (52%)		
	LFU	0		
	None	0		
	Glucocorticoid	124 (71%)		

*Missing data for 18 patients.
ACPA, anti-citrullinated protein/peptide antibody; CRP, C reactive protein; DAS-28, disease activity score; DMARDS, disease-modifying anti-rheumatic drugs; HCQ, hydroxychloroquine; LFU, leflunomide; MTX, methotrexate; NEAC, Newcastle Early Arthritis Clinic; RA, rheumatoid arthritis; RF, rheumatoid factor; SSZ, sulfasalazine; TACERA, Towards A CurE for RA.

Disease-modifying therapy (DMARD) and glucocorticoid naive patients with eRA were recruited at the time of diagnosis from two independent cohorts, RA-MAP TACERA and NEAC. The clinical characteristics and demographics are displayed. Median values with ranges are displayed for continuous variables. For the NEAC-matched methylation and transcription cohort, the total number of patients was 54 with contemporaneous T and B cell data being available for 28.

Baseline IGS but not baseline IFN-α is associated with 6-month clinical outcomes

We sought to examine the effect of the IGS and IFN-α on initial clinical outcomes. No significant association between baseline IGS and baseline disease activity (DAS-28, p=0.202) was observed among 171 individuals from the TACERA cohort for whom data were available. Nonetheless, in keeping with our previous findings,[6] DAS-28 at 6 months positively associated with the baseline whole blood IGS (n=165, linear regression, p=0.02, R^2=0.245), figure 1A. Furthermore, this effect was independent of sex, age and other known confounding variables, including baseline DAS-28, CRP, ESR, glucocorticoids and DMARDs initiated (n=165, multivariable regression analysis, p=0.017). Crucially the interaction term between baseline IGS and baseline DAS-28 is non-significant (p=0.368), indicating that this effect was independent of baseline DAS-28. This is demonstrated graphically by grouping patients according to baseline disease activity (low DAS-28 <3.1; moderate 3.2–5.1; high ≥5.1), where the relationship between baseline IGS and 6-month outcome is consistent across baseline disease activity groups, figure 1B. Smoking and ACPA status similarly demonstrated no significant impact on 6-month outcomes (p=0.399 and p=0.555, respectively) when included in the regression model. In summary, higher baseline IGS scores predicted smaller reductions in DAS-28 (and, therefore, reduced clinical improvements) at 6 months.

When classifying/scoring 6-month disease activity into EULAR response outcomes (good, moderate and none) patients with higher baseline IGS scores were less likely to achieve a good EULAR response at 6 months (p=0.034, logistic regression), figure 1C. This was again independent of the above variables.

Baseline IFN-α significantly positively associated with both baseline DAS28 (p=0.018) and ESR (p<0.0001), but not CRP (p=0.053) with similar but less marked associations seen at 6 months (DAS-28 p=0.048, ESR p=0.049, CRP p=0.146), demonstrating that IFN-α levels correlate with disease activity (online supplemental file 3). However, unlike the IGS, baseline IFN-α did not associate with 6-month DAS-28 (p=0.557, linear regression), figure 1D, nor when corrected for the above variables (p=0.57, multivariable regression analysis). IFN-β, -γ or -λ levels did not associate with disease activity at any time point or predict any clinical outcomes (p>0.2 for all, data not shown).

Circulating IFN-α drives the IGS in eRA

To elucidate which class of IFN is directly responsible for the IGS in eRA, circulating IFN-α, IFN-β, IFN-γ and IFN-λ were examined in relation to the IGS (TACERA cohort).

There was a strong positive association between the IGS and circulating IFN-α (n=164, R^2=0.417, p<0.0001, linear regression). Most IFN-β measurements were below the detection threshold but, where detectable, there was no significant association with the IGS (p=0.817, n=53). There was no association between the IGS and IFN-λ (p=0.345, n=117) nor with IFN-γ (p=0.065, n=158), figure 2A. An additional NEAC cohort (n=51) validated the significant association between IFN-α and the IGS (p=0.004), online supplemental file 2.0. In addition, there was no association between the IGS and either TNF-α, IFN-γ, IL6, IL-10, IL12-p70 and IL1β (p>0.1 for all) nor was there any association between IFN-α and any of the above cytokines (p>0.1 for all), online supplemental file 2.0.

As shown previously,[6] the IGS significantly fell between baseline and 6 months (n=165, p<0.0001, Wilcoxon signed rank test), figure 2B. Longitudinal serum IFN-α values mirrored the IGS, with a significant fall over 6 months (n=161, p<0.0001). IFN-β, IFN-γ or IFN-λ levels remained static over this period

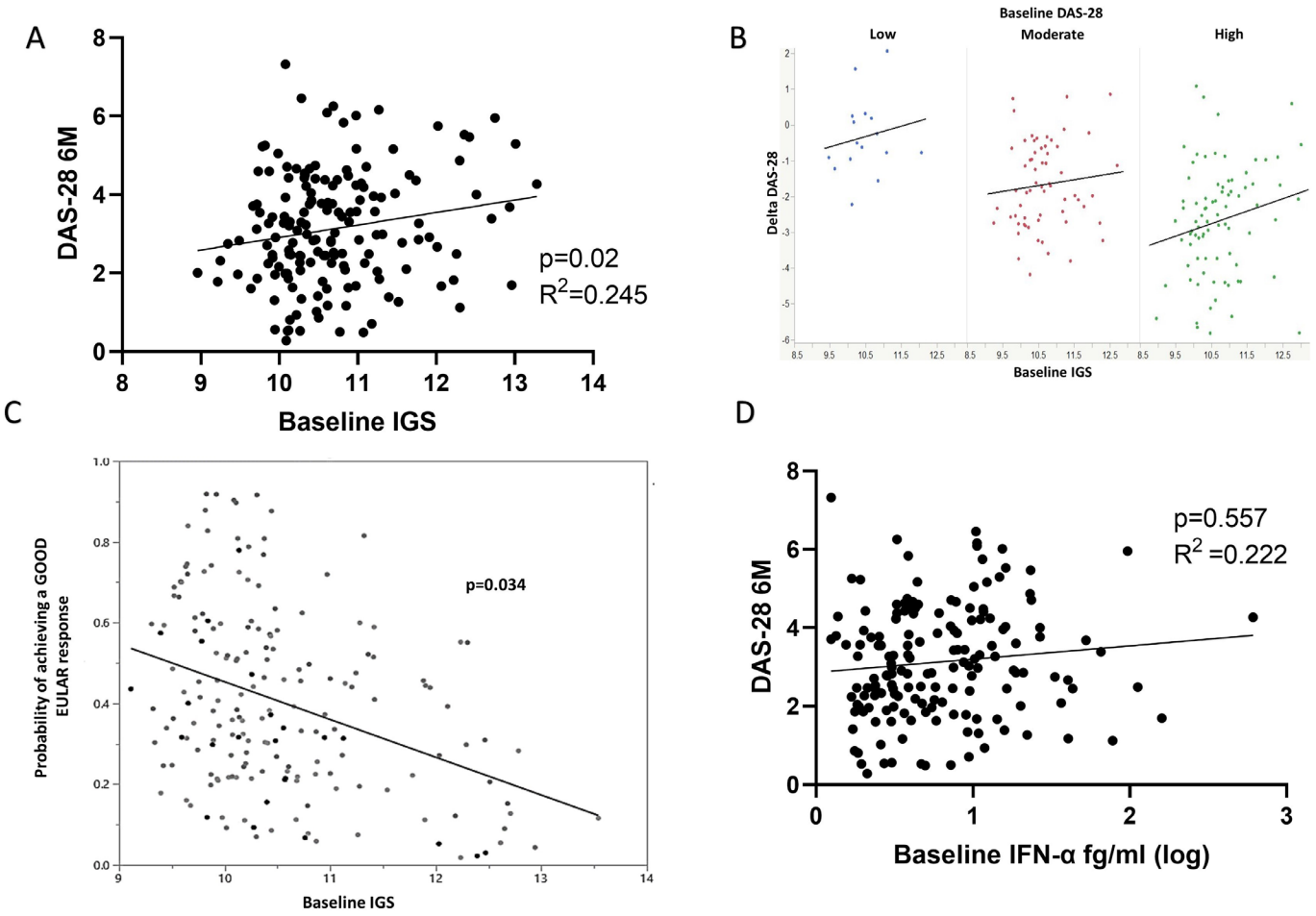

Figure 1 The IGS, circulating IFN-α and clinical outcomes. Early drug naïve RA patients (eRA, n=165) had their IGS calculated from whole blood microarray expression of *IFI6, OAS1, MxA, ISG15, IFI44L* and the impact of this baseline IGS on 6 month clinical outcomes sought. (A) Linear regression between baseline IGS and DAS-28 at 6 months (6M), p=0.02, R^2=0.245. (B) Graphical depiction of baseline IGS consistently impacting on change in DAS-28 at 6 months (Delta DAS-28) regardless of baseline DAS-28 (p=0.017, multivariable regression). Blue dots represent patients with baseline low DAS-28(<3.1), red moderate DAS-28 (3.2–5.1) and green high DAS-28 (>5.1). A negative Delta DAS-28 (Y axis) denotes a fall in DAS-28 and therefore response to therapy. (C) Relationship between the probability of achieving a good EULAR response at 6 months and baseline IGS. Nominal logistic regression, age, sex, DMARD, baseline DAS-28 and glucocorticoid administration corrected, p=0.034. (D) Linear regression between baseline IFN-α and DAS-28 at 6 months (6M), p=0.557, R^2=0.222. DAS-28, disease activity score; DMARDS, disease-modifying antirheumatic drugs; IFN, interferon; IGS, gene signature; RA, rheumatoid arthritis.

(p=0.275, p=0.819 and p=0.453, respectively), figure 2C. Furthermore, changes in circulating IFN-α correlated with the IGS (p<0.0001, multivariate analysis), but this was not seen for IFN-β, -λ or –γ (p>0.5 for all), online supplemental file 4. Finally, circulating IFN-α itself did not correlate with any other IFN (-β, -γ or –λ) measured at baseline (p>0.7 for all, data not shown). These data, in toto, suggest that the IGS is driven by circulating IFN-α in eRA.

To compare IFN-α levels in blood and target tissue, IFN-α was measured in matched synovial fluid and serum samples from five patients with RA. Full demographic and descriptive information (not represented in table 1) are shown in online supplemental file 5. There was no significant difference (p=0.8) between serum and synovial fluid IFN-α levels (median 3.93 fg/mL and 4.54 fg/mL, respectively), figure 2D.

IFN-α/IGS signalling pathways and effect on circulating haematological parameters

IFN-α production is triggered by NAR ligation. Whole blood mRNA expression of key NARs or their signalling proteins, TLR9, TLR7, TMEM173 (STING) and DDX58 (RIG-1) was, therefore, examined between IGS high and low eRA in the TACERA cohort. Expression of RNA sensing NARs RIG-1 and TLR7 was significantly increased in the IGS high patients (p<0.0001 for both). This was not seen for DNA sensing NARs nor their signalling components, TLR9 and TMEM173 (STING) (p=0.424 and p=0.609, respectively), figure 3A. Furthermore, circulating IFN-α and the IGS positively associated with whole blood expression of TLR7 (p=0.0002, R^2=0.191 and p<0.0001 R^2=0.216, respectively, linear regression) and RIG-1 (p<0.0001, R^2=0.216 and p<0.0001 R^2=0.458, respectively). Again this was unique to RNA sensing NARs with no significant association observed between either the IFN-α or the IGS and expression of TLR9 (p=0.926 and p=0.431) or TMEM173 (p=0.835 and p=0.738), figure 3B. TLR7 overexpression, particularly in relation to TLR9 expression, has been linked to autoimmunity and there was a significant positive association between ratio of whole blood TLR7:TLR9 and the IGS (p=0.0003, R^2=0.075) as well as with IFN-α (p=0.037, R^2=0.027) figure 3C. A similar pattern was observed in circulating PBMCs, online supplemental file 6.

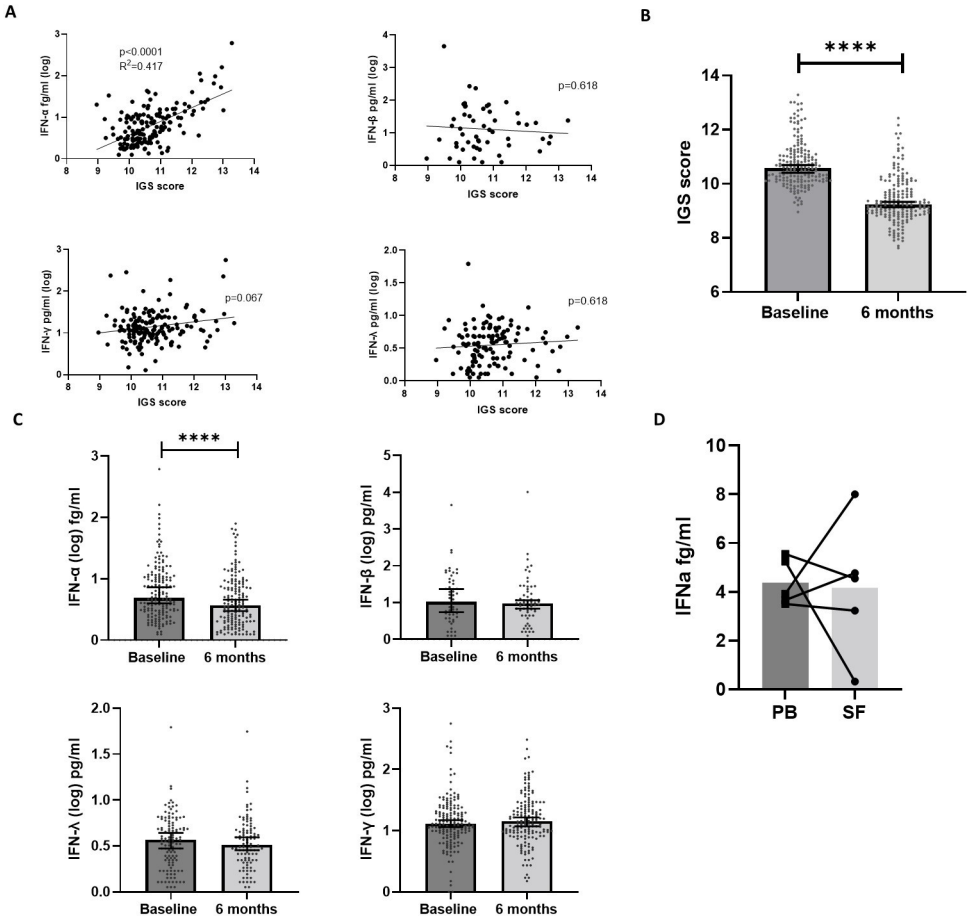

Figure 2 Circulating IFN-I, -II, –III, the IGS and longitudinal expression. (A) Linear regression was performed between the IGS and circulating IFN-α (n=163, p<0.0001, R^2=0.29), IFN-β (n=53, p=0.817, R^2=0.001), IFN-γ (n=164, p=0.067, R^2=0.034), IFN-λ (n=117, p=0.345, R^2=0.007). (B) Paired IGS scores between baseline and 6 months in eRA (n=165). Median values are depicted with 95% CIs and statistical analysis using Wilcoxon signed rank test performed on the differences between baseline and 6 months. (C) Comparisons between baseline and 6 month circulating levels of IFN-α, IFN-β, IFN-γ and IFN-λ. Median values are depicted with 95% CIs and statistical analysis using Wilcoxon signed rank test performed on the differences between baseline and 6 months. (D) Comparison of circulating peripheral blood (PB) IFN-α and synovial fluid (SF) IFN-α from five patients with RA, four of whom had established RA and one who had early RA, Wilcoxon signed rank test demonstrated no significant difference. Median values are depicted with paired samples demonstrated. ****p<0.0001. IFN, interferon; IGS, gene signature; RA, rheumatoid arthritis.

IFN-α is known to affect B cell function so associations with autoantibody titres were sought. RF titre strongly positively correlated with baseline IFN-α (p<0.0001, R^2=0.183) but not with baseline IGS (p=0.091). There was no association between RF titre and IFN-β (p=0.379) nor with IFNγ (p=0.230), but there was a weak positive association with IFN-λ (p=0.005, R^2=0.069), figure 3D and online supplemental file 7. ACPA titres did not correlate with either the IGS nor any interferon examined (p>0.1 for all), figure 3D and online supplemental file 5.

IGS correlates with site-specific DNA methylation in B and T cells

Since both IFN-I levels and IGS fall at 6 months in the context of a continued apparent influence on disease activity, we hypothesised that IFN-I-mediated/associated epigenetic alterations may be a plausible mechanism, whereby gene expression programmes in lymphocytes become persistently dysregulated in eRA in response to IFN signalling. We, thus, examined genome-wide transcriptional and methylation data from CD4+ T and CD19+ B lymphocytes isolated from an independent cohort of NEAC patients with eRA (table 1).

Of 330 CpGs were differentially methylated between IGS high and low CD4+ T cells (57.2% hypomethylated in IGS high, online supplemental file 8) and 287 in CD19+ B cells (58.1% hypomethylated in IGS high, online supplemental file 8). Of the 287 DMSs in CD19+ B cells, 17 (5.9%) showed similar changes in CD4+ T cells, with 16 being hypomethylated in IGS high for both (figure 4A). In addition, 65 DEGs were identified between IGS high and IGS low in CD4+ T cells and 40 in CD19+ B cells. Twelve of these genes were increased for both T and B cells in IGS high patients (figure 4A), online supplemental file 9.

Pathway analysis of DEGs (online supplemental file 10) demonstrated increased expression of genes related to RIG-I and TLR signalling in CD19+ B and CD4+ T cells, respectively, with a significant increase in antiviral pathways and IFN-I signalling. In T cells, pathway analysis also demonstrated significantly increased gene expression linked to RA.

Analysis of genes whose expression correlated with DMSs at relevant loci demonstrated multiple IRGs, such as *IFI44L*, *RSAD2* and *Mx1*, online supplemental file 11. Of interest to RA pathophysiology, there was increased expression of *PARP9* and *EPSTI1* in B cells and *STAT1* in CD4+ T cells in IGS high patients, which negatively correlated with methylation DMSs

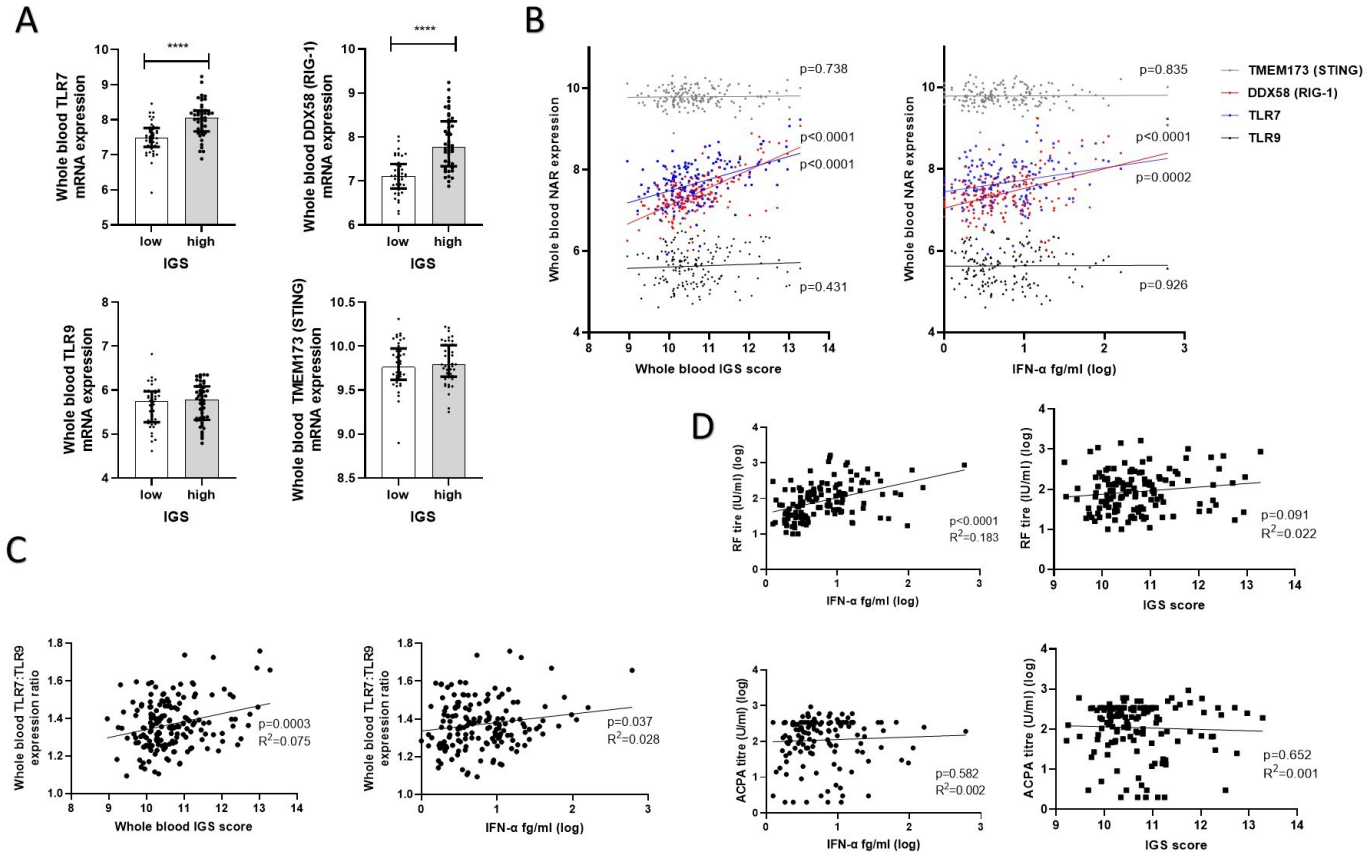

Figure 3 IFNs, signalling pathways and autoantibody titres. Whole blood expression of nucleic acid receptors (NARs) was examined in early RA TACERA cohort with respect to IFN-α/the IGS. (A) Expression of surface and cytosolic nucleic acid receptors, TLR7, TLR9 DDX58 (RIG-1) and TMEM173 (STING) were examined between IGS high and low patients, n=43 in each cohort. Median values with interquartile ranges are shown. Mann-Whitney U tests were performed. (B) Linear regression between the whole blood IGS or circulating IFN-α and whole blood mRNA expression of TLR7, TLR9 DDX58 (RIG-1) and TMEM173 (STING), n=164. P values are depicted in the figure. (C) Linear regression between whole blood ratio of TLR7: TLR9 mRNA expression and the whole blood IGS score or circulating IFN-α (fg/ml) in 164 eRA patients. (D) Linear regression comparing circulating IFN-α and RF and ACPA titres in seropositive eRA patients (n=132). ****p<0.0001. ACPA, anticitrullinated protein/peptide antibody; eRA, early rheumatoid arthritis; IFN, interferon; IGS, gene signature.

(figure 4B). We validated increased expression of CD4+ *STAT1* transcript in IGS high patients at baseline in the independent TACERA cohort (p=0.003, Mann-Whitney U test) and showed that this was maintained at 6 months (p=0.02), figure 4C. TACERA PBMC *PARP9* and *EPSTI1* was examined in lieu of a CD19+ B cell-specific transcriptome, which again confirmed significant gene upregulation in the IGS high cohort at baseline (p=0.0002 and p<0.0001, respectively, Mann-Whitney U tests), again sustained at 6 months (p<0.0001 for both), figure 4C.

To examine the potential effect of these methylation changes on gene regulation and expression, the CD4+ T and CD19+ B cell DMSs were overlapped with chromatin state information for E043 T cell line and E032 B cell line, respectively. DMSs, particularly hypomethylated DMSs, were enriched in putative enhancer regions and regions flanking transcription start sites for both cell types in IGS high patients (figure 4D). In IGS high CD19+ B cells, hypermethylated DMSs were enriched in the binding sites of several transcription factors, including GATA3, ETS1 and NFATC2, whereas hypomethylated DMSs were enriched in binding sites of polycomb protein EZH2. In IGS high CD4+ T cell hypermethylated DMSs were enriched, among others, for p300 TFBSs and hypomethylated DMSs for HIF1αHI (figure 4E). Full list of TFBS enrichment is found in online supplemental file 12.

DISCUSSION

In a large, multicentre cohort of DMARD and glucocorticoid nave patients with eRA, we identify, for the first time, IFN-α as primarily responsible for IGS generation. We additionally validate the IGS as a clinically relevant prognostic biomarker in RA for refractory disease.[6 14 15] This was independent of conventional markers of disease activity and suggests that IFN-related pathways drive disease persistence. We implicate lymphocyte epigenetic reprogramming as an underpinning mechanism.

Directly linking the IGS and IFN-α has been historically challenging due to difficulties directly measuring circulating IFN-α.[3 12] Therefore, our demonstration of the IGS in eRA positively associating with circulating IFN-α protein, and not other classes of IFN or other circulating cytokines, is an important step in dissecting the biological significance of the IGS and is in keeping with what has been reported in other rheumatic diseases.[16] Furthermore, both IGS and serum IFN-α levels fall in parallel with clinical response over 6 months, additionally supporting the role of IFN-α driving the IGS in eRA. Like others,[17] and in contrast to the IGS, we could not demonstrate an association of baseline IFN-α with longitudinal clinical outcomes. A potential explanation is that the IRG integrates activity over time of IFN-α, which itself has a short half-life.

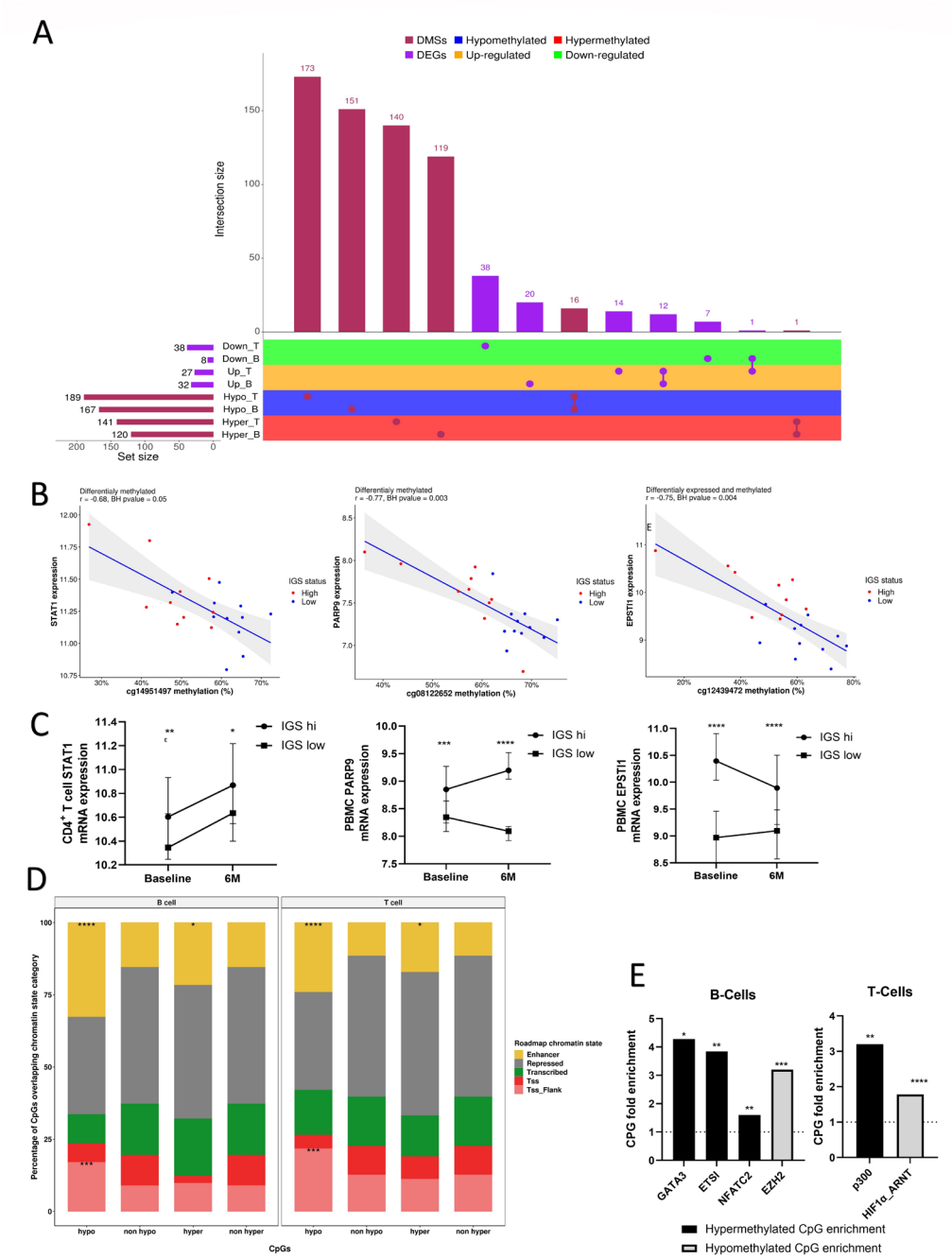

Figure 4 Differential methylation and expression of pathophysiologically relevant genes in eRA according to IGS. CD4+ T cells and CD19+ B cells were isolated from eRA patients (NEAC cohort) and their cell-specific transcriptome and methylome interrogated according to IGS status. (A) Upset plot[45] of differentially methylated sites (DMSs) and differentially expressed genes (DEGs) between IGS high and low early CD19+ B and CD4+ T cells and arranges the co-occurring variables into sets and with a bar chart of their frequency. The horizontal bar graph at the bottom left shows the total number of DEGs/DMSs that are altered in each cell subset between IGS high and IGS low cohorts. Joined red/purples circles to the right of these bar graphs indicate the same DEGs/DMSs were common to the IGS high/IGS low comparisons shown at the left. The vertical bar graph at the top quantitates the number of DEGs/DMSs with similar expression differences in the comparisons. 'Up' and 'Down' indicate increased expression or reduced expression in the IGS cohort respectively. (B) Scatterplots showing significant correlations (Benjamini-Hochberg (BH) adjusted p value (BHpval) <0.05) between gene expression and DNA methylation of exemplar genes in B and T cells of IGS high and low RA patients. R: Pearson correlation coefficient. (C) Baseline and 6 month (6M) expression of CD4+ T cell STAT1 and peripheral blood mononuclear cell (PBMC) PARP9 and EPSTI1 in IGS high and IGS low patients (n=41 for each) in a separate eRA cohort (RA-MAP TACERA). Median and error bars denoting 95% CI depicted. Mann-Whitney U tests performed between IGS high and IGS low cohorts at each time point. (D) Stacked bar plots indicating the relative distribution of the differentially methylated CPGs (DMS) between IGS high/low eRA patients as previously identified according to their chromatin state annotations. Chromatin states enrichments at DMSs that are hyper- or hypo-methylated in IGS high compared with IGS low RA patients are indicated for both cell types (Fisher's exact tests) along with standard expression for comparison. TSS: transcription start site; Tss_Flank: flanking a TSS. (E) Exemplar ENCODE and JASPAR transcription factor binding sites (TFBSs) that are significantly enriched (Fisher's exact test p<0.05) at CD4+ T and CD19+ B cell CPG sites detected as hyper-methylated or hypo-methylated in IGS high RA patients. CPG fold enrichment is displayed. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. eRA, early rheumatoid arthritis; IGS, gene signature; NEAC, Newcastle Early Arthritis Clinic.

Our finding of comparable IFN-α levels in serum and synovial fluids implicates a systemic source potentially influencing synovial pathophysiology. Larger studies are required to confirm this, and examination of other inflammatory arthritides will shed further light on the role of IFN-α in synovial pathology. RA is a heterogeneous disease and, despite the association of baseline IGS with clinical outcome reported here, not all patients with a low IGS at inception fared well. This could well reflect the dominance of alternative disease pathways in some patients but does not reduce the prognostic value of a high IGS.

Contrasting with other IFNs, increased baseline levels of IFN-α suggest a pathophysiological role in eRA and, potentially, in disease initiation. The permissive effects of IFN-α on lymphocyte activation and development of autoimmune characteristics are well documented[1] and murine transfer of IFN-α secreting dendritic cells propagated a persistent inflammatory arthritis.[18] IFN-α associated with raised ESR in our cohort and in other autoimmune conditions[12] but is also relevant prior to onset of inflammation as an IGS predicts progression to RA in 'at risk' cohorts.[19–21] Furthermore, autoantibodies predate clinical presentation, and we demonstrate a clear association between IFN-α and RF titres in eRA consistent with previous observations in autoimmunity.[12 17] In contrast, there was no association between IFN-α and ACPA. This dichotomy likely reflects differences in autoantibody sources and generation. RF-producing B cells demonstrate activation of IFN-I pathways, whereas ACPA-producing B cells do not.[22] These data, alongside observations in other diseases, highlighting the role of type 1 interferons at disease onset,[23] cumulatively suggest IFN-α may promote or accelerate breach of tolerance in susceptible individuals. Indeed, RA twin studies hypothesise that environmental and stochastic factors may be more important than genetic factors in determining development of disease relevant autoantibodies.[24]

We attempted to identify the receptor(s), and thus potential environmental triggers, responsible for IFN-α release. Although the expression of all examined NARs can be increased following IFN-I exposure,[25] only RNA sensing pathways increased in association with a raised IGS/IFN-α, reflecting previous observations in autoimmunity.[12 26] Furthermore, the IGS was associated with TLR7:TLR9 imbalance, itself associated with heightened autoimmunity risk and breach of tolerance.[2 27] We feel our data are more consistent with RNA sensing pathways triggering IFN-α production and release, but we accept these are associations, and understanding the primary trigger(s) of IFN-α release in eRA remains a pressing priority for future study.

Altered DNA methylation regulates the innate antiviral immune response and hypomethylation of IRGs, such as we report, has also been demonstrated in autoimmunity.[7] Pretreatment with IFN-α in vitro enhances subsequent B cell activation[28] and pretreatment of macrophages with IFN-α prevented the silencing of NF-κB via effects on chromatin, thereby abolishing TNF-induced tolerance to TLR ligation and potentiating the proinflammatory function of TNF-α.[29] Similar chromatin changes were identified in systemic lupus erythematous patients, a condition where IFN-α levels are increased, highlighting the in vivo relevance of IFN-α-related epigenetic modifications.[29] In addition, IFN-α treatment of salivary gland tissues reduced DNA methyltransferases, which catalyse DNA methylation, and upregulated TET3 which is involved in demethylation.[30] Stratifying eRA T and B cells by IGS, we identified multiple DMSs. These preferentially mapped to enhancers and flank regions, thereby supporting their biological relevance. They also reflected TFBS patterns that favoured proliferative responses. Namely, B cell CpG enrichment inferred increased binding of EZH2, which is increased in cell proliferation and lymphoma[31] and reduced binding of (1) GATA3, suggesting increased cell proliferation[32], (2) ETS1 which is required to prevent autoimmune responses[33] and (3) NFATC2, involved in anergy, which, when reduced, causes a hyperproliferative phenotype.[34] In T cells, there was inferred reduction in p300 binding, which would impair Foxp3$^+$ T-regulatory cell function[35]; and enrichment of HIF1-α, which promotes Th17 differentiation and reduced Foxp3$^+$ expression.[36] Additionally, CPG methylation changes in IGS high patients associated with increased gene expression of *PARP9*, *EPSTI1* (B cells) and STAT1 (T-cells) and remained significantly differentially expressed at 6 months between IGS high and low patients in a distinct cohort despite a sustained fall in IGS/circulating IFN-α. PARP9 (BAL-1) can modify B cell proliferation and altered *PARP9* methylation, and expression has been implicated in RA pathogenesis[37]; ETSI promotes pathological B cell activation in Primary Sjogren's Syndrome (PSS)[38]; and STAT1 in T cells is a key mediator of inflammatory cytokine signalling and important in focal RA inflammatory infiltrates.[39] Our interpretation of these data is that IFN-α-induced perturbation of DNA methylation influences the immune system early in the natural history of an identifiable subpopulation of patients with RA, leading to adverse outcomes.

Further work is needed to support this, namely (1) longitudinal measurements to determine whether the observed methylation changes (and gene expression of correlated transcripts) are sustained over time where the IGS/IFN-α levels are not and (2) ex vivo confirmation of the propensity for IFN-α to induce relevant epigenetic changes in relevant cell populations. Indeed, although we have focused on T and B lymphocytes in view of their known relevance to RA pathophysiology, these effects are likely to extend to other cell subsets.[40 41]

Cumulatively, these data incriminate IFN-α as a key cytokine underpinning prognosis in RA and support the hypothesis of an IFN-α-driven epigenetic programming at disease onset that perpetuates pathological signalling pathways and refractoriness to therapy. Such phenomena could underpin the well-recognised window of opportunity in eRA by persistent dysregulation of proinflammatory pathways after a period of unopposed activity. JAK inhibitors modify IFN signalling as well as IFN-induced epigenetic programming in PSS.[30] Targeted administration of these or similar therapies in the early stages of RA, or in 'pre-RA' at-risk groups, may provide a precision medicine approach ultimately reducing clinical progression and patient morbidity. However, before adoption of stratification by IGS, factors to address include IRG selection and interlaboratory standardisation, as recently reviewed in Cooles and Isaacs.[42]

Sustained immune dysregulation secondary to IFN-I may have additional implications. For example, IFN-I-induced epigenetic modifications[29] are also present in patients with COVID-19[43] and might have relevance to 'long-covid' syndromes as well as autoantibody development and, potentially, autoimmunity.[44]

In conclusion, we identify, for the first time, IFN-α as primarily responsible for the IGS in eRA and validate a high IGS as a clinically relevant prognostic biomarker in eRA, portending refractory disease and implying a therapeutic window of opportunity for drugs that target IFN-α signalling. We additionally implicate lymphocyte epigenetic reprogramming as an underpinning mechanism with relevance for other IFN-α enriched states.

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Contributors FAHC devised the experiments, developed the concepts and wrote the first manuscript draft and acts as guarantor. DWL and JT assisted with RA-MAP TACERA data analysis and modelling. NN, AGP and LNR assisted with epigenetic analyses and support. DD, NJM, BM, CMAL and VB assisted with analysis of serum cytokines. AGP, NT, AEA and JD assisted with T and B cell data collection. GRS, MRB, DW, SN, RH assisted with RA-MAP TACERA data processing, QC and curation. APC supervised RA-MAP TACERA data collection. JDI provided direction and oversight to the whole project. All authors approved the final manuscript.

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval Ethical approval was authorised by both the National Research Ethics Service London Central Committee (Reference number: 12/LO/0469) for RA-MAP study participants and North East—Newcastle & North Tyneside 2 Research Ethics Committee (REC reference: 12/NE/0251) for NEAC participants. All patients gave full informed written consent.

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Supplementary file 1.0: Additional methods

RNA extraction methods

As described in [1] RA-MAP blood was collected in Tempus Blood RNA Tubes (Applied Biosystems) and the RNA extracted using MagMAX RNA isolation kits (Ambion). This included removal of globin mRNA using GLOBINclear human 20 reaction kits (Ambion) according to the manufacturer's protocol. Additional blood for isolation of PBMCs was collected into EDTA Vacutainer collection tubes (BD) and separated using Leucosep separation tubes (Greiner). For subsequent microarray analysis, amplified RNA was hybridized to beadchips and scanned on an Illumina Beadstation 500. Illumina's GenomeStudio version 2011.1 with the Gene Expression Module v1.9.0 was used to generate signal intensity values. Non-normalised control and sample probe data were exported from GenomeStudio, background subtracted, and quantile normalised using Limma's neqc function [2]. The data were then filtered for probe signal intensity where probes were retained if they had a p value < 0.05 in at least 10% of samples. Probes were aggregated to genes using Limma's avereps function.

Cell specific RNA from the NEAC cohorts was extracted as described previously [3, 4]. In brief RNA was immediately extracted from total CD4+ T cells or B lymphocytes using an RNeasy Mini kit or AllPrep DNA/RNA Mini kit (both from Qiagen), and then subject to quality control using an Agilent 2100 Bioanalyzer (Agilent). The median RNA integrity number in the samples analyzed was 9.4. Complementary RNA generated from 250 ng total RNA (Illumina TotalPrep RNA Amplification kit) was hybridized to either an Illumina Whole Genome 6 version 3 (using CD4+ lymphocyte samples obtained prior to 2012) or a 12HT BeadChip (using CD4+ T cell samples obtained in or after 2012, and all B cell samples) (both from Illumina). For the subsequent methylation analyses four hundred nanogram of DNA was bisulphite-converted and DNAm quantified using the Infinium MethylationEPIC BeadChip (Illumina). After independent preprocessing and functional normalization of $CD4^+$ T- and B-cell data, probe filtering was performed and surrogate variable analysis used to estimate confounding variables (surrogate variable analysis package) as described in [4].

Expression and methylation analysis

Analysis of differential expression (DEG) and methylation (DMS) between IGS high and low eRA groups was performed using linear models using the limma R package. Differentially expressed genes (DEGs) were defined as genes with unadjusted p-value <0.05 and an absolute fold change >1.5. For methylation analysis, CpG sites exhibiting an absolute delta beta value of >0.1 and unadjusted p-value <0.05 were considered differentially methylated (DMSs). For both no multiple test correction was done for this as the basis for exploratory pathway analysis.

To assess the association between DNA methylation and gene expression, all CpGs remaining after pre-processing were first mapped to annotated genes based on the Illumina EPIC Human Methylation array annotation file, i.e. CpGs that were within a gene or its promoter region within 2kb. Only CpG-gene pairs containing a DMS or a DEG were analysed. Then, for each DEG or DMS, the paired gene expression and methylation profiles were tested for significant Pearson correlation coefficients (Benjamini-Hochberg, BH-adjusted p-value<0.05).

DMSs positions were overlapped with defined chromatin states corresponding to primary peripheral blood T helper (E043) and B cell (E032) epigenomes obtained from NIH Roadmap Epigenomics [5] using the LOLA R package [6]. The E043 and E032 15-state HMM models were downloaded from http://egg2.wustl.edu/roadmap and collapsed into five intuitive chromatin state annotations (Transcription start site, TSS flank, Enhancer, Transcribed and Repressed) as described in [4]. Transcription factor binding site (TFBS) enrichment analyses were performed using ENCODE phase 2 data generated using ChIPseq [7] and predicted TFBSs from the JASPAR database [8] (datasets downloaded from http://databio.org/regiondb). The 20bp region encompassing the DMSs were overlapped with TFBSs using the runLOLA function which applies Fisher's exact tests to calculate their significance (p-value < 0.05).

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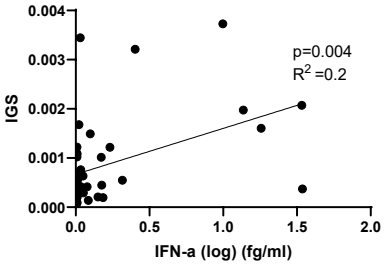
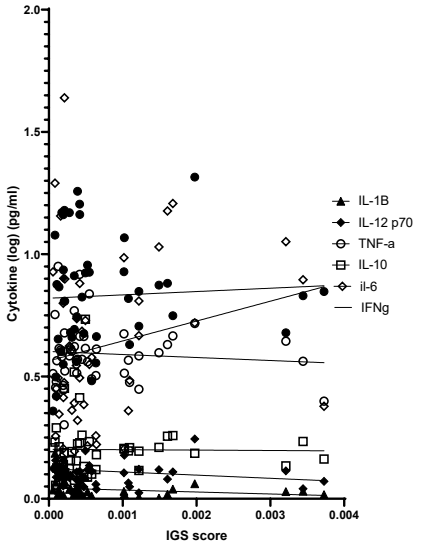
	NEAC Validation cohort
Number (n)	51
Age, years, median (range)	58 (30-87)
Female, n (%)	31 (61%)
RF positive, n (%)	32 (61%)
ACPA positive, n (%)	35 (67%)
DAS-28-CRP, median	4.39 (1.38-7.07)
C-reactive protein (mg/L), median (range)	8 (4-114)
Erythrocyte sedimentation rate (mm/h), median (range)	23 (2-68)

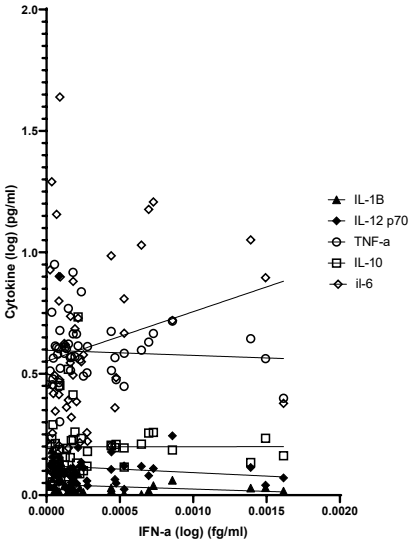
B



C



D

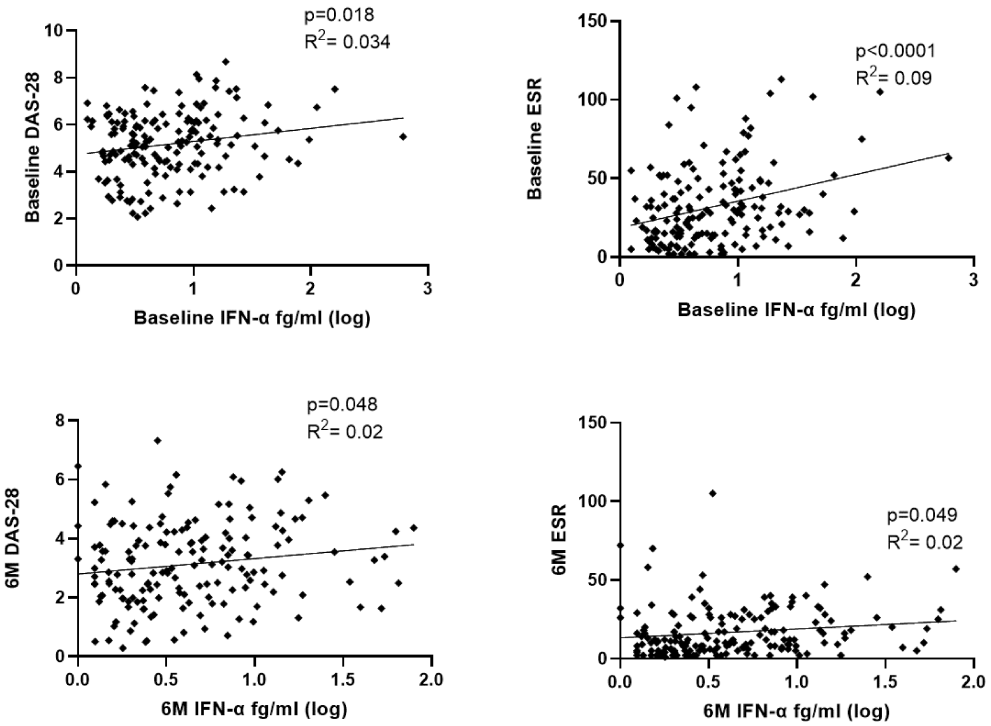


Supplementary file:

Cohort of early drug naïve RA patients recruited from NEAC with a new diagnosis of RA as per 1987 and 2010 classification criteria. **A** Demographic data for validation cohort shown. **B** Linear regression between circulating IFN-α and whole blood IGS in NEAC validation cohort (p=0.004, R^2 0.2, n=47). **C** Circulating levels of IFN-γ (IFNg), TNF-α (TNF-a), IL-6, IL-1β (IL1B), IL-12 p70 and IL-10 were measured

using MSD technology (Meso Scale Discovery) as per manufacturer's instructions. Linear regression between whole blood IGS score and circulating levels cytokines of IFN-γ (IFNg), TNF-α (TNF-a), IL-6, IL-1β (IL1B), IL-12 p70 and IL10 was performed. There was no significant association between any of the cytokines measured and the IGS (p=0.738, p=0.170, p=0.211, p=0.557, and p=0.939 respectively). **D** Circulating levels of IFN-α were measured using Simoa technology (see main methods for details) and linear regression performed between IFN-α and levels of other circulating cytokines, IFN-γ (IFNg), TNF-α (TNF-a), IL-6, IL-1β (IL1B), IL-12 p70 and IL10. Again there was no significant association, p=0.672, p=0.666, p=0.133, p=0.196, p=0.565, p=0.992 respectively.

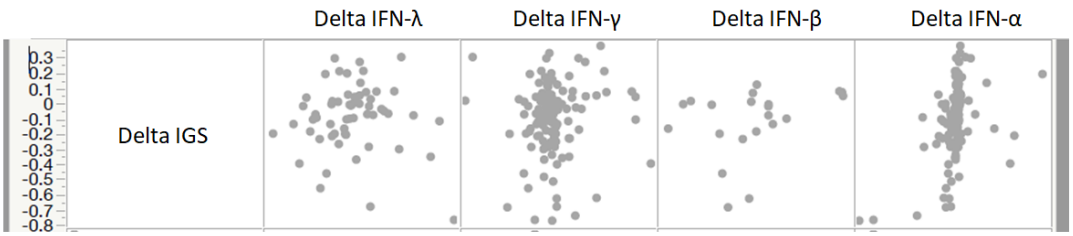
Supplementary file 2.0:



Circulating IFN-α and disease activity

Linear regression between baseline IFN-α and baseline DAS-28 and ESR and between 6 month (6M) IFN-α and 6 month DAS-28 and ESR (n=164).

Supplementary file 3.0



	p=0.551	p=0.483	p=0.566	p<0.0001
Spearman's rank co-efficient	0.077	0.138	0.401	0.463

Longitudinal changes in IFNs in reference to the IGS – significant association only seen with IFN-α

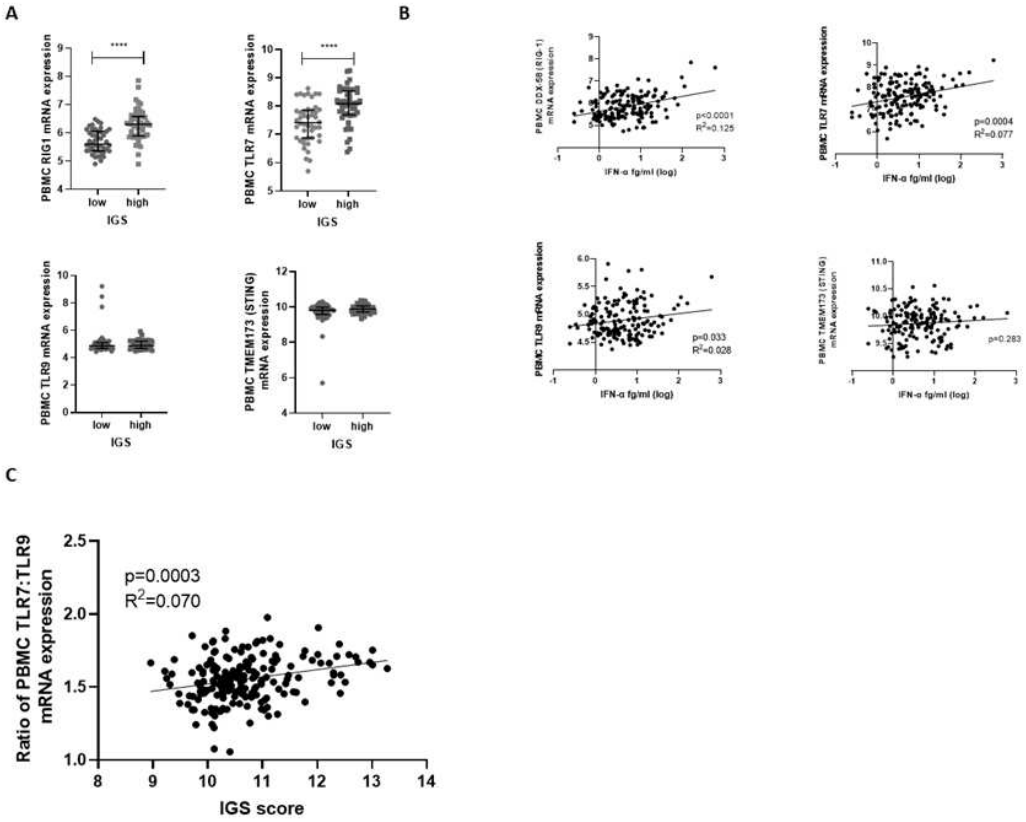
Multivariate analysis for the change (delta) in circulating IFN-α, IFN-β, IFN-γ and IFN-λ between baseline and 6 months in relation to changes in the IGS (delta) between baseline and 6 months. Significant correlations were seen only for changes in IFN-α and changes in the IGS ($p<0.0001$), spearman's rank correlation co-efficient 0.463.

Supplementary file 4.0

Synovial fluid/Serum IFN-alpha measurement

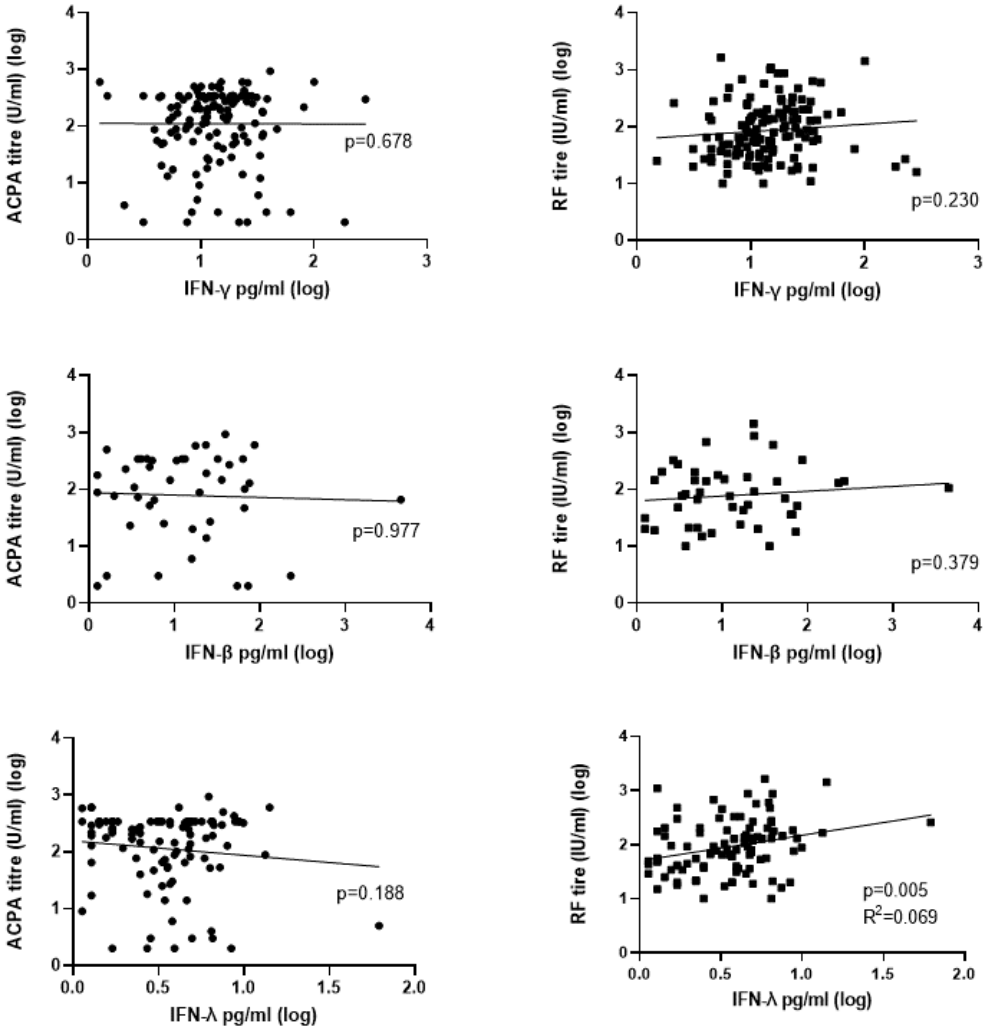
Patient demographics:

5 RA patients had matched synovial fluid and serum measurements of IFN-α performed. Median age 70, range 58-74 with a male:female ratio of 1:4. Three patients were seropostive for both ACPA and RF, one was seronegative and data was unavailable for one patient. Four patients had established RA (disease duration >12 months) and were receiving the following therapies, Adalimumab (n=1), Methotrexate and Sulphasalazine (n=1) and methotrexate and hydroxychloroquine (n=2). One was eRA and sampling taken at time of diagnosis pre initiation of DMARDs.



Supplementary file X.

Expression of cellular receptors and signalling proteins involved with IFN-α signalling were examined in circulating peripheral blood mononuclear cells (PBMCs) of early RA patients. (**A)** Expression of surface and cytosolic viral RNA receptors, TLR7 and RIG-1 (*DDX58)* between IGS high and low early RA patients (n=43 in each group). Median values with interquartile ranges are shown. Mann-Whitney U tests performed. **(B)** Linear regression between circulating IFN-α and TLR7, TLR9 DDX58 (RIG-1) and TMEM173 (STING) expression, p and R^2 values depicted in the figure, n=164 (**C**) Linear regression between PBMC ratio of TLR7: TLR9 expression and the whole blood IGS score at baseline in (n=164) eRA patients. p and R^2 values depicted in the figure **** p<0.0001.



Supplementary file 5.0

Linear regression comparing circulating IFN-β (n=53), IFN-γ (n=135) and IFN-λ (n=117) with and RF and ACPA titres in seropositive eRA patients.

TRIPOD Checklist: Prediction Model Validation

Section/Topic	Item	Checklist Item	Page
Title and abstract			
Title	1	Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted.	1
Abstract	2	Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions.	2
Introduction			
Background and objectives	3a	Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models.	4
	3b	Specify the objectives, including whether the study describes the development or validation of the model or both.	4
Methods			
Source of data	4a	Describe the study design or source of data (e.g., randomized trial, cohort, or registry data), separately for the development and validation data sets, if applicable.	5
	4b	Specify the key study dates, including start of accrual; end of accrual; and, if applicable, end of follow-up.	5
Participants	5a	Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centres.	5
	5b	Describe eligibility criteria for participants.	5
	5c	Give details of treatments received, if relevant.	n/a
Outcome	6a	Clearly define the outcome that is predicted by the prediction model, including how and when assessed.	6
	6b	Report any actions to blind assessment of the outcome to be predicted.	n/a
Predictors	7a	Clearly define all predictors used in developing or validating the multivariable prediction model, including how and when they were measured.	6
	7b	Report any actions to blind assessment of predictors for the outcome and other predictors.	n/a
Sample size	8	Explain how the study size was arrived at.	5
Missing data	9	Describe how missing data were handled (e.g., complete-case analysis, single imputation, multiple imputation) with details of any imputation method.	5
Statistical analysis methods	10c	For validation, describe how the predictions were calculated.	5
	10d	Specify all measures used to assess model performance and, if relevant, to compare multiple models.	6
	10e	Describe any model updating (e.g., recalibration) arising from the validation, if done.	n/a
Risk groups	11	Provide details on how risk groups were created, if done.	5
Development vs. validation	12	For validation, identify any differences from the development data in setting, eligibility criteria, outcome, and predictors.	5
Results			
Participants	13a	Describe the flow of participants through the study, including the number of participants with and without the outcome and, if applicable, a summary of the follow-up time. A diagram may be helpful.	n/a
	13b	Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome.	Table 1
	13c	For validation, show a comparison with the development data of the distribution of important variables (demographics, predictors and outcome).	n/a
Model performance	16	Report performance measures (with CIs) for the prediction model.	n/a
Model-updating	17	If done, report the results from any model updating (i.e., model specification, model performance).	n/a
Discussion			
Limitations	18	Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data).	10-12
Interpretation	19a	For validation, discuss the results with reference to performance in the development data, and any other validation data.	10-12
	19b	Give an overall interpretation of the results, considering objectives, limitations, results from similar studies, and other relevant evidence.	10-12
Implications	20	Discuss the potential clinical use of the model and implications for future research.	10-12
Other information			
Supplementary information	21	Provide information about the availability of supplementary resources, such as study protocol, Web calculator, and data sets.	5 & supp files
Funding	22	Give the source of funding and the role of the funders for the present study.	12

We recommend using the TRIPOD Checklist in conjunction with the TRIPOD Explanation and Elaboration document.