TRANSLATIONAL SCIENCE

Multiomics analysis of rheumatoid arthritis yields sequence variants that have large effects on risk of the seropositive subset

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ABSTRACT

Objectives To find causal genes for rheumatoid arthritis (RA) and its seropositive (RF and/or ACPA positive) and seronegative subsets.

Methods We performed a genome-wide association study (GWAS) of 31 313 RA cases (68% seropositive) and ~1 million controls from Northwestern Europe. We searched for causal genes outside the HLA-locus through effect on coding, mRNA expression in several tissues and/or levels of plasma proteins (SomaScan) and did network analysis (Qiagen).

Results We found 25 sequence variants for RA overall, 33 for seropositive and 2 for seronegative RA, altogether 37 sequence variants at 34 non-HLA loci, of which 15 are novel. Genomic, transcriptomic and proteomic analysis of these yielded 25 causal genes in seropositive RA and additional two overall. Most encode proteins in the network of interferon-alpha/beta and IL-12/23 that signal through the JAK/STAT-pathway. Highlighting those with largest effect on seropositive RA, a rare missense variant in *STAT4* (rs140675301-A) that is independent of reported non-coding *STAT4*-variants, increases the risk of seropositive RA 2.27-fold (p=2.1×10^{-9}), more than the rs2476601-A missense variant in *PTPN22* (OR=1.59, p=1.3×10^{-160}). *STAT4* rs140675301-A replaces hydrophilic glutamic acid with hydrophobic valine (Glu128Val) in a conserved, surface-exposed loop. A stop-mutation (rs76428106-C) in *FLT3* increases seropositive RA risk (OR=1.35, p=6.6×10^{-11}). Independent missense variants in *TYK2* (rs34536443-C,

Key messages

What is already known about this subject?

- ► Although many genetic risk loci have been identified in rheumatoid arthritis (RA) overall, there are limited data available on the seropositive and seronegative subsets. Furthermore, most reported RA associations outside the HLA-locus are with common non-coding variants with low risk,which lack a compelling candidate gene mediating the effect on RA.

Key messages

What does this study add?

- In this largest genome-wide association study on RA to date, we studied both RA overall and the seropositive and seronegative RA subsets and found several unreported sequence variants with large effect on the risk of seropositive RA, while associations with seronegative RA were scarce. Through a genomic, transcriptomic and proteomic analysis, we identified candidate causal genes for most signals and show that the majority of those associated with seropositive RA are in the interferon alpha/beta and IL-12/23 signalling networks. Furthermore, most sequence variants that confer the largest risk of seropositive RA point to causal genes encoding proteins in the JAK/STAT-pathway and have not been reported in RA before. This includes a missense variant in the *STAT4* gene that confers 2.27-fold risk, larger than the lead signals at the well-known *HLA-DRB1* and *PTPN22* loci, and two unreported missense variants in the *TYK2* gene, affecting levels of the interferon-alpha/beta receptor 1 (IFNAR1).

How might this impact on clinical practice or future developments?

- These findings highlight how a multiomics approach can reveal causal genes. Our findings support treatment of seropositive RA with the already registered JAK and IL-6R inhibitors as well as CTLA4-Ig but also open for repurposing of other drugs that target proteins in the JAK/STAT-pathway, including inhibitors of FLT3, TYK2 and IFNAR1.

rs12720356-C, rs35018800-A, latter two novel) associate with decreased risk of seropositive RA (ORs=0.63–0.87, $p=10^{-9}$–10^{-27}) and decreased plasma levels of interferon-alpha/beta receptor 1 that signals through TYK2/JAK1/STAT4.

Conclusion Sequence variants pointing to causal genes in the JAK/STAT pathway have largest effect on seropositive RA, while associations with seronegative RA remain scarce.

INTRODUCTION

Rheumatoid arthritis (RA) is a heterogeneous clinical syndrome that affects around 0.5%–1% of the general population. It is characterised by inflammatory polyarthritis and progressive joint damage if insufficiently treated.[1] RA is divided into seropositive and seronegative RA, where around two-thirds of RA patients are in the seropositive subset, based on autoantibodies (rheumatoid factor (RF) and/or antibodies against citrullinated peptide antigens (ACPA)).[1,2] Although many risk loci have been identified in previous genome-wide association studies (GWAS), most reported RA associations are with common non-coding variants that confer low risk and lack a compelling candidate gene mediating the effect on RA.[1,3–6] The main exceptions are the shared epitope encoded by certain alleles of *HLA-DRB1* and two missense variants in the *PTPN22* (rs2476601-A) and *TYK2* (rs34536443-C) genes.[1,3]

Previous GWAS have focused on RA overall,[3–6] except for one study on ACPA-positive (n=1147) and ACPA-negative (n=774) RA that confirmed the strong association of HLA-DRB1 alleles with ACPA-positive RA but did not identify any genome-wide significant signals outside the HLA-locus[7] and another report on ACPA-negative RA only (n=1922) that identified two genome-wide significant signals.[8]

Here, we searched for sequence variants outside the HLA-locus affecting the risk of RA overall, the seropositive and/or seronegative subsets of RA, using the largest GWAS study population to date in RA (31 313 cases and ~1 million controls) from six countries in Northwestern Europe and searched for candidate causal genes through a genomic, transcriptomic and proteomic analysis.

METHODS

Study populations

Cases with RA were diagnosed by rheumatologists and/or captured through the nationwide Scandinavian rheumatology quality registries and/or the 10th revision of the International Statistical Classification of Diseases (ICD-10) code-based registration of all inpatient and outpatient healthcare visits (see four-digit based ICD-10 codes in table 1). If available, RF and anti-CCP measurement were used to define the seropositive/seronegative RA subsets, according to classification criteria.[2,9]

An overview of the study populations is provided in table 1. In the study populations from *Iceland* (3613 cases and 341 788 controls), *UK Biobank* (5798 cases and 402 767 controls of self-reported white British ancestry, confirmed by genetic analysis)[10] and *FinnGen* (https://www.finngen.fi/en/access_results version R4: 4701 cases and 125 923 controls), RA cases were compared with the remaining non-RA individuals, with the Icelandic study covering a large part of the Icelandic population and the latter two being nationwide genetic cohort studies. From *Sweden*, we included: (1) the population-based EIRA case–control study (www.eirasweden.se) with 3436 newly diagnosed cases and 3058 controls matched for age, sex and geographical area from mid and Southern parts of Sweden. In addition, we included 7488 controls from the parallel Swedish EIMS study (ki.se/imm/eims-epidemiologisk-undersokning-av-riskfaktorer-for-multipel-skleros); (2) the RA cohort from Umea (n=1935) and 1156 controls from Umea biobank, matched for age and sex (www.umu.se/en/biobank-research-unit); and (3) the Swedish Rheumatology Quality Register Biobank (n=3287, www.srq.nu).

From *Denmark*, RA cases were identified in four study populations: (1) Danish Biomarker Protocol[11] (n=2544 with samples in the Danish Rheumatological Biobank and clinical data in the Danish Rheumatology Quality Register, DANBIO)[12] (2) the Copenhagen Hospital Biobank (n=3282), (3) the TARCID cohort (n=1826) and (4) the nationwide Danish Blood Donor Study (DBDS; 10 RA cases).[13] Controls for these 7662 cases were age-matched and sex-matched non-RA individuals from DBDS (n=86 964).

From *Norway*, 881 RA cases from the Oslo RA cohort and 28 517 population-based controls from the Norwegian Mother, Father and Child Cohort Study were included.[14,15]

Patients were involved in the design and conduct of several of the studies that are included in this report.

Genotyping and multiomics analyses

For a detailed methodological description, see online supplemental information 2. In short, genotyping of all cohorts except UK Biobank and FinnGen was performed at deCODE genetics using the Illumina technology, and the sequence variants for imputation were identified through whole-genome sequencing of 67 645 individuals.

We used logistic regression to test the association of ~64 million sequence variants with RA overall, the seropositive and

Table 1 RA study populations from six Northwestern European countries included in the present study*

	Total cases	Total controls	Sweden		Denmark		Iceland		Norway		UK biobank		FinnGen	
			Ca	Co	Ca	Co	Ca	Co	Ca	Co	Ca	Co	Ca	Co
RA overall	31 313	995 377	8658	9418	7662	86 964	3613	341 788	881	28 517	5798	402 767	4701	125 923
Seropositive RA	18 019	991 604	6455	9423	4850	86 964	1746	313 704	587	28 517	913	407 652	3468	145 344
Seronegative RA	8515	1 015 471	1852	9436	2652	86 966	1069	322 808	455	28 517	1051	407 514	1436	143 312
Serology lacking	4779	–	351	–	160	–	798	–	0	–	3834	–	0	–

*The following ICD-10 codes were used, in addition to clinical diagnoses validated by physicians, from case–control studies on RA or Scandinavian rheumatology quality and patient registers: RA overall (M05.8, M05.9, M06.0, M06.8, M06.9), seropositive RA (M05.8, M05.9 and/or positive rheumatoid factor (RF) and/or anti-CCP antibody measurement), seronegative RA (M06.0, M06.8 or M06.9 with negative RF measurement (and negative anti-CCP measurement if available). See Methods for further details.
Ca, number of cases; Co, number of controls; RA, rheumatoid arthritis.

the seronegative subset.[16] Sequence variants were split into five classes based on their genome annotation, and the significance threshold for each class was based on the number of variants in that class,[17] thereby adjusting for all ~64 million variants tested, maintaining an unadjusted significance threshold of 8×10^{-10}. The primary signal at each genomic locus has the lowest Bonferroni-adjusted p value. Conditional analysis was used to search for possible secondary signals (<500 kB from the primary signal, excluding HLA-locus). We tested whether primary and secondary signals were in strong linkage disequilibrium (R^2 >0.8) with top cis-eQTL variants for genes expressed in various tissues (online supplemental tables 5 and 6), and/or with levels of 4789 proteins in plasma (pQTL, SomaScan, Somalogic) in 35 559 Icelanders (online supplemental table 7).[18–21]

We used the Ingenuity Pathway Analysis software (QIAGEN Inc) to evaluate whether there is experimental evidence for direct or indirect interaction between the proteins coded by candidate causal genes, supporting biological connection.

RESULTS

Genome-wide association study

Of the 31 313 RA cases, 26 534 (84.7%) had information on serological status. Of these, 18 019 (67.9%) were seropositive and 8515 (32.1%) seronegative (table 1).

In separate meta-analyses of RA overall and the seropositive and seronegative RA subsets, we found in total 37 sequence variants at 34 non-HLA loci (online supplemental figure 1a–c), as summarised in table 2. Thus, we identified 25 lead signals for RA overall (online supplemental table 2), 33 for seropositive and 2 for seronegative RA (online supplemental table 3). When we searched for novel sequence variants, we adjusted for 82 independent sequence variants previously reported to associate with RA ($p<5\times10^{-8}$ in the largest meta-analysis to date),[4 6] and 15 of the 37 sequence variants are previously unreported. The 15 novel associations are at 12 loci and six of those loci are previously unreported. Little heterogeneity was observed between the study populations (see online supplemental tables 2 and 3 (P_{het}) and online supplemental figure 4 (average effect)).

Replication of previously reported signals

We replicated 53 of the 82 previously reported variants (online supplemental table 1, correcting for multiple testing, p value threshold=0.05/82 variants /3 phenotypes=2.03×10^{-4}). However, only 36 of the 82 variants were previously reported to be genome-wide significant in Europeans,[4 6] and we replicated 34 of these 36 variants (94%).

Comparison of RA subsets

The heritability estimates (total observed scale h2) were higher for seropositive RA (0.19 (0.022)) than for seronegative RA (0.099 (0.019)). For a substantial proportion of the RA-associated sequence variants, their effect was greater on seropositive RA than seronegative RA risk (table 2, figure 1). However, the genetic correlation between seropositive and seronegative RA was high (rg 0.87, SE 0.13, $p=4.5\times10^{-12}$ (online supplemental table 9).

Genomic, transcriptomic and proteomic analysis of lead signals

We searched for candidate causal genes with an omics approach (figure 2A) and evaluated the effect of lead signals (or correlated variants, R^2 >0.8) on amino acid sequence (online supplemental tables 2–4), mRNA expression (cis-eQTL (online supplemental tables 5 and 6) and/or plasma levels of proteins (pQTL (online supplemental table 7). This yielded a total of 27 candidate causal genes in RA overall and/or its subsets.

Seropositive RA

Twenty-four of the 33 lead signals in seropositive RA pointed to 25 candidate causal genes, as shown in figure 2B ranked by effect. The one with the largest effect is a rare (MAF=0.14%) missense variant in the *STAT4* gene (rs140675301-A, Glu128Val) that associates with 2.27-fold increased risk ($p=2.1\times10^{-9}$, table 2 and figure 2B). Rs140675301-A is the first coding variant identified at the *STAT4* locus that associates with RA and has not been reported in any disease before. This signal is independent (online supplemental table 8) of the common lead *STAT4* intronic variant (rs4853458-A), which is strongly correlated (R^2=1) with other intronic variants in *STAT4*, previously reported to associate with RA[22 23] (figure 3A and online supplemental table 1). STAT4 contains six domains that have different functions, and the rare missense rs140675301-A variant leads to an amino acid change from negatively charged, hydrophilic, glutamic acid to non-polar hydrophobic valine at position 128 (Glu128Val) in a loop on the surface of the protein (figure 3B), between the N-terminal domain and the helical coiled coil domain. The coiled coil domain provides a carbonised hydrophilic surface that binds to regulatory factors.[24] The amino acid sequence and secondary structure of the loop is highly conserved between species (figure 3C) and within the family of STAT proteins,[24 25] indicating its importance for the function of STAT4. Tetramer formation of STAT at DNA binding sites is necessary for full transcriptional activation of many of its target genes,[26] and STAT without the N-terminal domain cannot form tetramers.[27]

The second largest effect on the risk of seropositive RA had the well-known missense variant rs2476601-A in the *PTPN22* gene, followed by a novel missense variant in the *TYK2* gene (rs35018800-A, Ala928Val), encoding tyrosine kinase 2, which is a member of the JAK/STAT-pathway like STAT4. This rare (MAF=0.60%) missense variant in TYK2 conferred reduced risk

Table 2 Sequence variants outside the HLA locus that associate with RA overall, seropositive (rheumatoid factor and/or anti-CCP antibody positive) and/or seronegative RA in GWAS meta-analysis within six Northwestern-European countries (table 1). Association results are shown for the lead signals for all three RA groups, and the heterogeneity between the seropositive and seronegative subsets.† Effect alleles with novel associations are marked with.*

					Seropositive RA		Seronegative RA		RA overall		
Chr	Position	Effect allele*	*Close gene*	Annotation	OR	*P value*	OR	*P value*	OR	*P value*	P_{het}
chr1	2 800 059	rs897628-T*	***TTC34***	Missense	0.90	*3.3E-16*	0.98	*0.18*	0.94	*1.9E-10*	*1.6E-05*
chr1	113 834 946	rs2476601-A	***PTPN22***	Missense	1.59	*1.3E-160*	1.29	*2.9E-27*	1.41	*3.9E-144*	*7E-13*
chr1	161 506 414	rs9427397-T*	***FCGR2A***	Missense	1.11	*2.2E-08*	1.02	*0.55*	1.07	*3.3E-06*	*0.026*
chr2	60 881 694	rs67574266-A	***REL,PUS10***	5-prime UTR	1.08	*6.2E-10*	1.01	*0.57*	1.05	*3.6E-07*	*2.0E-03*
chr2	111 119 036	rs72836346-C*	***BCL2L11***	Upstream gene	1.14	*2.5E-10*	1.01	*0.75*	1.10	*7.5E-09*	*1.4E-03*
chr2	191 073 180	rs140675301-A*	***STAT4***	Missense	2.27	*2.1E-09*	1.23	*3.4E-01*	1.63	*3.9E-06*	*0.017*
chr2	191 094 763	rs4853458-A	***STAT4,GLS***	Intron	1.11	*5.2E-14*	1.10	*1.1E-06*	1.10	*2.7E-19*	*0.71*
chr2	203 880 280	rs11571297-C	***CTLA4***	Regulatory	0.89	*2.9E-20*	0.95	*2.2E-03*	0.92	*4.4E-19*	*7.5E-04*
chr3	58 197 909	rs35677470-A	***DNASE1L3***	Missense	1.13	*2.0E-07*	1.16	*7.4E-07*	1.10	*1.8E-08*	*0.43*
chr4	26 083 889	rs10517086-A	*LINC02357*	Intergenic	1.11	*6.2E-16*	1.06	*1.8E-03*	1.09	*7.1E-18*	*0.025*
chr5	56 148 856	rs7731626-A	***ANKRD55***	Intron	0.87	*1.2E-26*	0.87	*8.4E-17*	0.88	*1.1E-39*	*0.83*
chr6	137 678 425	rs35926684-G	*TNFAIP3*	Regulatory	1.12	*4.3E-16*	1.02	*0.24*	1.09	*1.5E-14*	*1.3E-04*
chr6	159 085 568	rs2451258-C	.	Regulatory	0.91	*1.6E-12*	0.99	*0.75*	0.96	*1.2E-05*	*4.2E-05*
chr6	167 127 770	rs3093017-C	***CCR6***	Intron	1.11	*1.8E-18*	1.04	*0.03*	1.07	*7.0E-15*	*6.1E-04*
chr7	50 313 596	rs10261758-G*	*IKZF1*	Intron	1.07	*6.9E-07*	1.04	*0.04*	1.07	*3.6E-12*	*0.17*
chr7	128 938 247	rs2004640-G*	***IRF5***	Splice donor	0.92	*1.4E-11*	0.94	*1.9E-04*	0.94	*5.1E-13*	*0.25*
chr8	11 480 078	rs2409780-C	***BLK,FAM167A***	Regulatory	1.09	*1.1E-09*	1.05	*9.1E-03*	1.08	*1.3E-12*	*0.1*
chr8	100 105 506	rs1471293-A*	*RGS22*	5-prime UTR	1.08	*7.4E-10*	1.04	*3.4E-02*	1.05	*9.1E-08*	*0.039*
chr9	120 933 192	rs35942002-A	***TRAF1***	Upstream gene	1.09	*6.3E-13*	1.05	*9.1E-04*	1.06	*2.8E-09*	*0.1*
chr10	6 056 986	rs706778-T	***IL2RA***	Intron	1.09	*1.2E-11*	1.07	*3.7E-05*	1.07	*2.4E-12*	*0.36*
chr10	31 122 426	rs1538981-C	***ZEB1***	Regulatory	0.91	*8.1E-14*	0.99	*0.40*	0.94	*9.4E-12*	*9.4E-05*
chr11	64 340 005	rs479777-C*	*CCDC88B*	Upstream gene	0.93	*2.7E-09*	0.92	*7.4E-07*	0.94	*1.4E-10*	*0.68*
chr11	118 870 448	rs7117261-T	.	Regulatory	0.90	*2.0E-12*	0.94	*1.3E-03*	0.92	*7.6E-13*	*0.13*
chr11	128 627 057	rs73013527-C	*LOC105369568*	Intergenic	1.08	*2.7E-10*	1.04	*0.03*	1.06	*7.7E-10*	*0.045*
chr12	111 446 804	rs3184504-T	***SH2B3***	Missense	1.10	*7.6E-16*	1.08	*1.6E-06*	1.08	*1.1E-17*	*0.38*
chr13	28 029 870	rs76428106-C*	***FLT3***	Intron	1.35	*6.6E-11*	1.15	*0.03*	1.23	*1.7E-08*	*0.041*
chr13	39 788 092	rs8002731-C	***COG6***	Intron	0.92	*3.5E-10*	0.94	*2.1E-04*	0.93	*1.7E-14*	*0.35*
chr14	92 651 884	rs117068593-T*	***RIN3***	Missense	0.93	*3.2E-05*	0.94	*9.8E-03*	0.93	*1.9E-09*	*0.59*
chr15	69 751 888	rs11636401-G*	.	TF binding site	0.91	*2.0E-16*	0.95	*7.1E-04*	0.93	*4.3E-15*	*0.045*
chr16	85 982 485	rs9939427-A	***IRF8***	Intergenic	1.10	*5.2E-11*	1.06	*4.6E-03*	1.07	*1.7E-10*	*0.14*
chr16	88 981 246	rs62045818-C*	*CBFA2T3*	Upstream gene	0.93	*8.9E-10*	1.00	*9.3E-01*	0.96	*3.1E-05*	*5.7E-04*
chr17	39 908 216	rs11078928-C	***GSDMB***	Splice acceptor	1.07	*1.3E-07*	1.05	*1.3E-03*	1.04	*1.9E-05*	*0.34*
chr19	10 352 442	rs34536443-C	***TYK2***	Missense	0.69	*2.7E-27*	0.81	*1.6E-06*	0.75	*2.5E-29*	*4.0E-03*
chr19	10 359 299	rs12720356-C*	***TYK2***	Missense	0.87	*2.3E-09*	0.90	*7.5E-04*	0.90	*4.3E-10*	*0.38*
chr19	10 354 167	rs35018800-A*	***TYK2***	Missense	0.63	*1.4E-11*	0.86	*0.07*	0.77	*1.4E-07*	*3.7E-03*
chr21	35 340 290	rs8129030-T	.	Regulatory	0.92	*1.1E-11*	0.96	*0.01*	0.95	*2.3E-08*	*0.038*
chr21	44 236 891	rs11558819-T*	***ICOSLG***	Missense	0.91	*1.6E-09*	0.98	*0.26*	0.95	*1.2E-05*	*1.9E-03*

*Sequence variants that remain significant after adjustment for previously reported sequence variants (online supplemental table 1). Bold indicates candidate causal genes (summarised in figure 2).

†We performed a meta-analysis using logistic regression analysis assuming a multiplicative model, reporting OR and two-sided p values adjusted for year of birth, sex and origin (Iceland) or the first 20 principal components (other countries). Variants were split into five classes based on their genome annotation and significance threshold based on the number of variants in each class. The adjusted significance thresholds are 1.3×10^{-7} for variants with high impact (splice donor, splice acceptor, stop gained, frameshift, stop lost, initiator codon), 2.6×10^{-8} for variants with moderate impact (missense, splice region, stop retained, inframe indels), 2.4×10^{-9} for low-impact variants (synonymous, 5′ UTR, 3′ UTR, upstream and downstream), 1.2×10^{-9} for other low-impact variants in DNase I hypersensitivity sites (intronic, intergenic, regulatory-region) and 5.92×10^{-10} for all other variants not in DNase I hypersensitivity sites. Primary signal at each locus (1 Mb) was selected based on conditional association analysis of all variants at each locus, using Bonferroni corrected p values (0.05×P/class-specific p value threshold). We report the coding signal when two markers are equivalent after conditional analysis. Secondary signals are sequence variants that remained GWAS significant after adjustment for the lead signal and other independent (secondary) signals at the locus. When different but correlated variants are lead in RA overall and seropositive RA, the seropositive RA signal is presented here. See further in online supplemental tables 2 and 3.
GWAS, genome-wide association study; Phet, a p value for test of heterogeneity between the effects in seropositive and seronegative RA subsets; RA, rheumatoid arthritis.

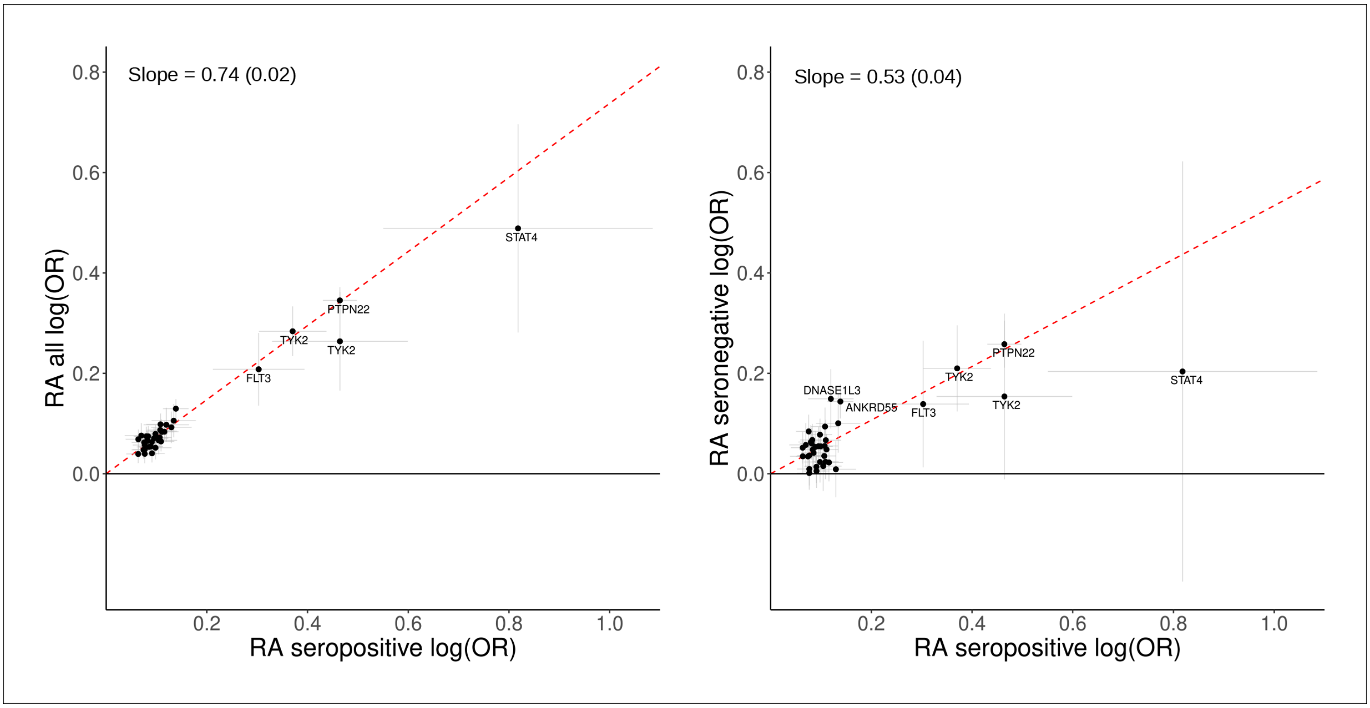


Figure 1 Effects of the lead sequence variants associated with seropositive RA (18 019 cases) compared with RA overall (31 313 cases, left graph) and seronegative RA (8515 cases, right graph). The x-axis and the y-axis show the logarithmic estimated ORs for the associations with the three phenotypes. All effects are shown for the RA risk increasing allele based on current meta-analysis of study population from six countries in Northwestern Europe (table 1). Error bars represent 95% CIs. The red line represents slope (SD) based on a simple linear regression through the origin using MAF (1-MAF) as weights. See further results in table 2 and online supplemental tables 2; 3.

of seropositive RA (OR=0.63, $p=1.4\times10^{-11}$), independently of a known missense variant in *TYK2* (rs34536443-C, Pro1104Ala, MAF 4.3%), which we also found to decrease the risk of RA overall (OR=0.75, $p=2.5\times10^{-29}$), and here, we extend this association to the seropositive RA subset (OR=0.69, $p=2.7\times10^{-27}$; table 2, online supplemental table 3 and online supplemental figure 2). In addition, we identified a common missense variant in *TYK2* that independently associated with reduced risk of seropositive RA (rs12720356-C, Ile684Ser, MAF=8.82%, OR=0.87, $p=2.3\times10^{-9}$). Analysis of the plasma proteome (online supplemental table 7) showed that the minor alleles of the variants encoding both Ile684Ser and Pro1104Ala in TYK2 are the only sequence variants that associate in trans with plasma levels of interferon alpha/beta receptor 1 (IFNAR1, Ile684Ser: effect=−0.19 SD, $p=7\times10^{-25}$; Pro1104Ala, effect=−0.13 SD, $p=6\times10^{-10}$). These variants did not associate with levels of any other plasma protein measured. Notably, both the missense variants in *TYK2* and *STAT4* are predicted to damage the function of the encoded protein (online supplemental table 4).

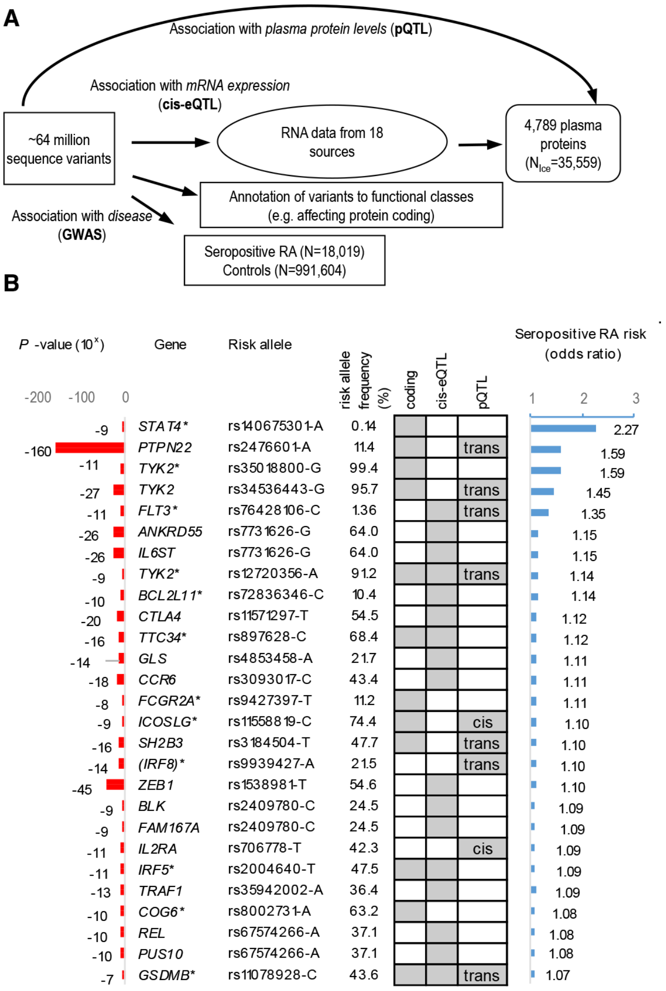
An intronic variant (rs76428106-C) in the *FLT3* gene, encoding another tyrosine kinase receptor that signals through the JAK/STAT-pathway, conferred 35% increase in risk of seropositive RA ($p=6.6\times10^{-11}$). This is in accordance with our previous report, where we discovered this variant in a GWAS on autoimmune thyroid disease and found that it also associated nominally with the risk of seropositive RA (OR=1.41, $p=4.3\times10^{-4}$) and with increased levels of 22 proteins in plasma (trans-pQTL), including the FLT3 ligand[18] (online supplemental table 7). rs76428106-C associated with increased mRNA expression of FLT3 in lung tissue (beta=0.82 SD, $p=1.3\times10^{-10}$, online supplemental table 6).

We performed a network analysis of the 25 seropositive RA candidate causal genes and found that 18 of them encode proteins that are linked in the same network (online supplemental figure 3), either through direct protein–protein interaction (eg, STAT4-TYK2, PTPN22-IRF5 and FLT3-SH2B3) or indirectly (eg, one affecting the level of another). Other molecules that are central in this network, and directly interact with proteins encoded by the candidate genes, are interferon alpha/beta and IL12/IL-23.

Among the other candidate causal genes, we also identified novel loss-of-function variants in genes encoding molecules in this network, although with more modest effect on seropositive RA risk (table 2 and figure 2B). This includes a splice-donor variant in the *IRF5* gene (rs2004640-G, OR=0.92, $p=1.44\times10^{-11}$) that encodes interferon regulatory factor 5. *IRF5* rs2004640-G association with decreased risk of seropositive RA was independent from previously reported non-coding variants at the *IRF5* locus (online supplemental table 1) and rs2004640-G is also associated with decreased mRNA expression of *IRF5* in several tissues (online supplemental table 6). Other novel coding variants pointing to putative causal genes were missense variants in *ICOSLG* (rs11558819-T, OR=0.91, $p=1.56\times10^{-9}$) encoding ICOS ligand and *TTC34* (rs897628-T, OR=0.90, $p=3.28\times10^{-16}$). *TTC34* encodes tetratricopeptide repeat protein 34 that has an unknown role in the pathogenesis of RA and belongs to another network that includes the remaining seven candidate causal genes for seropositive RA (online supplemental figure 3).

Seronegative RA

Both signals in seronegative RA were also found in seropositive RA and pointed to causal genes: a missense variant rs2476601-A in *PTPN22* and intronic variant rs7731626-A in *ANKRD55* (table 2 and online supplemental tables 2; 3).



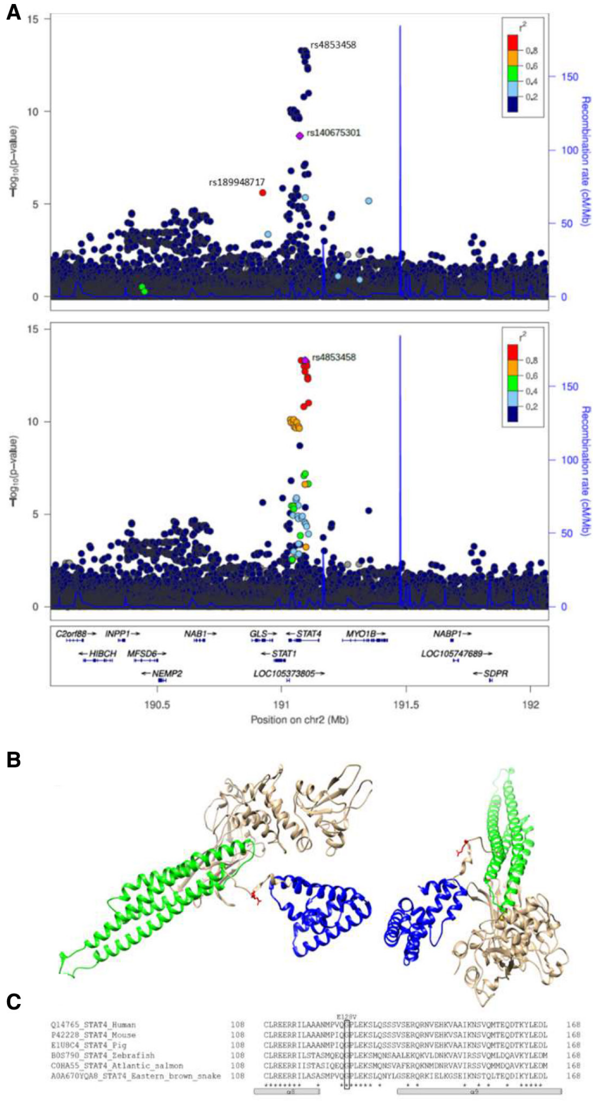
P-value (10^x)	Gene	Risk allele	risk allele frequency (%)	coding	cis-eQTL	pQTL	Seropositive RA risk (odds ratio)
-9	*STAT4**	rs140675301-A	0.14	▨			2.27
-160	*PTPN22*	rs2476601-A	11.4	▨		trans	1.59
-11	*TYK2**	rs35018800-G	99.4	▨			1.59
-27	*TYK2*	rs34536443-G	95.7	▨		trans	1.45
-11	*FLT3**	rs76428106-C	1.36		▨	trans	1.35
-26	*ANKRD55*	rs7731626-G	64.0		▨		1.15
-26	*IL6ST*	rs7731626-G	64.0		▨		1.15
-9	*TYK2**	rs12720356-A	91.2	▨	▨	trans	1.14
-10	*BCL2L11**	rs72836346-C	10.4		▨		1.14
-20	*CTLA4*	rs11571297-T	54.5		▨		1.12
-16	*TTC34**	rs897628-C	68.4	▨	▨		1.12
-14	*GLS*	rs4853458-A	21.7		▨		1.11
-18	*CCR6*	rs3093017-C	43.4		▨		1.11
-8	*FCGR2A**	rs9427397-T	11.2	▨			1.11
-9	*ICOSLG**	rs11558819-C	74.4	▨		cis	1.10
-16	*SH2B3*	rs3184504-T	47.7	▨		trans	1.10
-14	*(IRF8)**	rs9939427-A	21.5			trans	1.10
-45	*ZEB1*	rs1538981-T	54.6		▨		1.10
-9	*BLK*	rs2409780-C	24.5		▨		1.09
-9	*FAM167A*	rs2409780-C	24.5		▨		1.09
-11	*IL2RA*	rs706778-T	42.3			cis	1.09
-11	*IRF5**	rs2004640-T	47.5	▨	▨		1.09
-13	*TRAF1*	rs35942002-A	36.4		▨		1.09
-10	*COG6**	rs8002731-A	63.2	▨			1.08
-10	*REL*	rs67574266-A	37.1		▨		1.08
-10	*PUS10*	rs67574266-A	37.1		▨		1.08
-7	*GSDMB**	rs11078928-C	43.6	▨	▨	trans	1.07

Figure 2 Identification of sequence variants that associate with seropositive RA and the multiomics approaches used to recognise candidate causal genes. (A) schematic overview of the experimental approach used to identify sequence variants that associate with seropositive RA and their systematic annotation, applying multiomics approach to identify candidate causal genes, that is, based on whether lead variants or correlated variants (R^2 >0.8) affect protein *coding* (online supplemental tables 2–4), *mRNA expression* (cis-eQTL (online supplemental tables 5 and 6)) or *levels of proteins* in plasma (pQTL (online supplemental table 7)). (B) Out of 33 lead variant associations outside the HLA-locus (online supplemental table 3), 25 candidate causal genes were identified as listed, ranked by effect (OR). All effects are shown for the risk increasing allele based on GWAS in RA study populations from Northwestern Europe (table 1). Associations that are previously unreported in RA are marked with *. Grey boxes highlight where data point to a candidate causal gene. GWAS, genome-wide association study; RA, rheumatoid arthritis.

PTPN22 rs2476601-A associated with plasma levels of several proteins (trans-pQTL), and it was the only variant in the genome to affect the levels of these proteins (online supplemental table 7). *ANKRD55* rs7731626-A associated with a decreased risk of RA and its subsets and a decreased mRNA expression in whole blood of two neighbouring genes at the locus: *ANKRD55* and *IL6ST*.

RA overall

The lead signals pointing to causal genes in RA overall were also identified in the seropositive subset (table 2), with two



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Q14765_STAT4_Human                      108   CLREERRILAAANMPVQGPLEKSLQSSSVSERQRNVEHKVAAIKNSVQMTEQDTKYLEDL   168
P42228_STAT4_Mouse                      108   CLREERRILAAANMPIQGPLEKSLQSSSVSERQRNVEHKVSAIKNSVQMTEQDTKYLEDL   168
E1U8C4_STAT4_Pig                        108   CLREERRILAAANMPIQGPLEKSLQSSSVSERQRNVEHKVAAIKNSVQMTEQDTKYLEDL   168
B0S790_STAT4_Zebrafish                  108   CLREERRILSTASMQEQGPLEKSMQNSAALEKQKVLDNKVAVIRSSVQMLDQAVKYLEDM   168
C0HA55_STAT4_Atlantic_salmon            108   CLREERRIISTASIQEQGPLEKSMQNSVAFERQKNMDNRVAVIRSSVQMMDQAVKYLEDM   168
A0A670YQA8_STAT4_Eastern_brown_snake    108   CLREERRILASASMPVQGPLEKSLQNYLGSERQRKIELKGSEIKNSTQLTEQDIKYLEDL   168
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Figure 3 *STAT4* missense variant rs140675301 is associated with seropositive RA (18 019 cases), is not correlated with previously reported variants at the locus and leads to an amino acid change in a highly conserved area of the protein. (A) Locus plot for the association of variants at the *STAT4* locus with seropositive RA. The upper graph illustrates that the intronic variant rs4853458, that is the lead variant at the locus, is not correlated (r^2 <0.2) with the missense variant rs140675301, that is coloured in purple. The missense variant rs140675301 is only highly correlated (r^2 >0.8) with one variant, the intronic variant rs189948717 (coloured in red), that has less effect (seropositive RA: OR=1.81, p=3.69×10^{-6}). Neither of these variants have previously been reported in any disease. The lower graph highlights that the lead variant at the locus (rs4853458, coloured in purple) has many correlated variants, coloured by degree of correlation (r^2) with rs4853458. (B) Secondary structure of STAT4 (viewed from two angles) based on a structural model with STAT1 crystal structure (PDB code: 1yvl.1.A (Mao *et al*, *Molecular Cell* 2005;17:761–71) as template. Glu128Val (red) is located in a loop connecting the N-terminal domain (blue), important for tetramer formation of STATs and nuclear translocation, and the coiled coil domain (green), which provides a carbonised hydrophilic surface that binds to regulatory factors.[24] α-Helices are drawn as cylinders. Invariant residues are marked with asterix. (C) multiple sequence alignment of the conserved STAT4 loop between the N-terminal domain (α8) and the coiled coil (α9) domain, performed with Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). RA, rheumatoid arthritis.

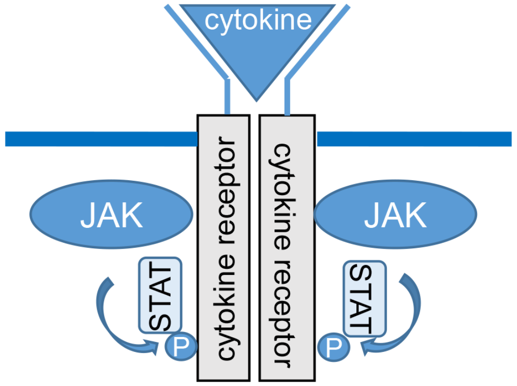
exceptions: missense variants in *DNASE1L3* (rs35677470-A) and *RIN3* (rs117068593-T) (online supplemental table 2). Both these missense variants are predicted to damage the function of the encoded protein (online supplemental table 4). *DNASE1L3* rs35677470-A is a known signal in RA, but the *RIN3* locus has to our knowledge not been reported to associate with any disease before. It encodes Ras and Rab interactor 3 that functions as a guanine nucleotide exchange factor of unknown relevance in RA.

DISCUSSION

In this largest GWAS study on RA to date, we studied both RA overall and the seropositive and seronegative RA subsets and found 37 sequence variants of which 15 were previously unreported. Several of these have large effect on seropositive RA risk, while only two signals were identified in the seronegative subset, both previously reported in RA overall. Through a multiomics approach, we identified candidate causal genes for most signals and show that the majority of those associated with seropositive RA are in the interferon alpha/beta and IL-12/23 signalling networks, with largest risk associated with sequence variants in genes encoding proteins in the JAK/STAT pathway.

Novel missense variant in the *STAT4* gene (rs140675301-A) confers 2.27-fold increased risk that is higher risk than any previously reported RA association, including the well-known *HLA-DRB1* shared epitope and the lead missense variant at the *PTPN22* locus. Although the STAT4 locus has been reported in genome-wide studies, this is the first *STAT4* coding variant found to associate with RA. This coding variant points directly to STAT4 as the causal gene at the locus. It has not been reported for any other disease before, and we found that it leads to an amino acid change in a surface loop of the protein that is highly conserved, thereby underscoring its importance for STAT4 function. *STAT4* encodes STAT4, a cytoplasmic transcription factor that regulates gene expression through the JAK/STAT-pathway.[28] It is phosphorylated in response to various cytokines and displacement of the N-terminal and coiled coil domains within the protein structure could interfere with DNA binding, transcriptional activation and/or target selectivity. As highlighted in the network analysis and illustrated in figure 4, both interferon alpha, IL-12 and IL-23, signal through STAT4 via TYK2/JAK1 and TYK2/JAK2.[29] Another RA-associated variant in STAT4 (rs7574865-T, R^2=0.99 to lead intron variant rs4853458-A)[23] increases IL-12-induced IFN-γ production in T cells.[30] STAT4 is expressed at inflammatory sites in activated peripheral blood monocytes, fibroblasts, dendritic cells and macrophages and also in synovial macrophages and dendritic cells from patients with seropositive RA.[28 31–34] Furthermore, reduced expression of STAT4 has been observed in RA patients that have responded well to disease-modifying treatment.[32] Thus, STAT4 may have a central role in the inflammatory cascade in joints of RA patients.

Tyrosine kinase 2, encoded by the *TYK2* gene, is another key molecule in the JAK/STAT pathway that regulates signal transduction pathways downstream of the receptors for several cytokines, including interferon alpha/beta and IL-23/IL12 as described previously. We found that three independent coding variants in *TYK2* associated with 25%–37% reduced risk of seropositive RA, and they associated with lower plasma levels of the IFNAR1 receptor for interferon-alpha/beta. Accordingly, one of the missense variants (Pro1104Ala) is located in the catalytic kinase domain of TYK2 and has previously been shown to reduce signalling through IFNAR1.[35]



cytokine	receptor	JAK	STAT
IFN-alpha	**IFNAR1****	**TYK2****/JAK1*	**STAT4**
IL-12	p35-p40	**TYK2****/JAK2*	**STAT4**
IL-23	p19-p40	**TYK2****/JAK1*	**STAT3/4**
FLT3-ligand	**FLT3****	JAK*	STAT5
IL-6	**IL-6R***	**TYK2**/JAK1/2*	STAT3

Figure 4 The JAK-STAT pathway. The figure and table shows which receptors, JAK and STAT subtypes certain cytokines bind to, highlighting proteins encoded by and/or affected by causal genes in seropositive RA, based on the multiomics analysis of sequence variants associated with risk of seropositive RA (shown in bold). Binding of a cytokine to its receptor activates the associated Janus kinases (JAK). The JAK in turn phosphorylates (P) the receptor, which provides a docking for signal transducers and activators of transcription (STATs) and other signalling molecules to bind to the receptor. STATs also become phosphorylated and translocate to the nucleus, where they regulate gene expression. *Protein targeted by drugs that are registered for RA. **Proteins targeted by drugs registered or in pipeline for other diseases. RA, rheumatoid arthritis.

TYK2 also mediates the signalling of IL-6, IL-10 and IL-4/IL-13.[36] IL-6 signals through the IL-6 receptor (IL-6R), thereby inducing IL6ST homodimerisation and activation of TYK2/JAK1/2 and STAT3 signalling pathway (figure 4), known to play a role in RA.[37] The intronic variant rs7731626-A in *ANKRD55* associated with a reduced risk of both seropositive and seronegative RA and also reduced expression of *ANKRD55* and *IL6ST*. The effect on *IL6ST* expression and its biological function points to *IL6ST* as a candidate causal gene at that locus. Accordingly, drugs inhibiting IL-6R are effective in RA.[38]

The FLT3 receptor is another activator of the JAK/STAT pathway that signals through STAT5[39] (figure 4), and an intronic variant in the *FLT3* gene (rs76428106-C) conferred 35% increase in risk of seropositive RA. This confirms a non-genome-wide significant signal in our previous report, in which we identified this variant as a strong risk factor for autoimmune thyroid disease and found that it generates a cryptic splice site, introducing a stop codon in 30% of transcripts that are predicted to encode a truncated protein, lacking its tyrosine kinase domains.[18] *FLT3* encodes fms-related tyrosine kinase 3 receptor, a key regulator in the development of monocytes and dendritic cells. The cell-surface receptor is expressed on common dendritic cells and lymphoid/myeloid progenitors that give rise to both classical and plasmacytoid dendritic cells, which produce large amount of interferons when activated.[40] As previously reported, *FLT3* rs76428106-C increases plasma levels of the FTL3 ligand,[18] and RA patients have increased levels of FLT3 ligand both in serum and synovial fluid of inflamed joints.[41 42] FLT3 ligand deficient mice are protected against collagen-induced arthritis,[42] and in a mouse model of collagen-induced arthritis, an oral inhibitor of FLT3/JAK2/c-Fms was found to block signalling through TYK2 and STAT4 and decrease both inflammation and bone resorption.[43]

Yet another variant affecting interferon signalling is a splice-donor variant in the *IRF5* (rs2004640-G) gene that encodes interferon regulatory factor 5 and reduced both RA risk and IRF5 expression. *IRF5*-rs2004640-G has not been reported in GWAS on RA before, although the locus is known, and a tentative association was reported in a meta-analysis of candidate gene studies (4818 cases, p=0.003).[44]

The size and homogeneous background of the study populations, with ~64 million sequence variants derived from over 67 thousand whole-genome sequenced individuals, increases the likelihood to detect rare and low-frequency sequence variants that associate with disease. Furthermore, we were able to test their functional relevance through analysis of RNA sequence and plasma proteome. However, it remains to be seen whether the sequence variants associate with RA in populations of another ancestries.

The SNP-based heritability estimate for seropositive RA was the same as in a previous study (0.19),[45] while lower for seronegative RA (0.099) where previous findings are scarce.[46]

In addition to the causal genes highlighted previously, the network analysis illustrated how majority of all candidate causal genes encode proteins in the interferon alpha/beta and IL-12/IL-23 signalling network. Furthermore, we observed a consistent direction of the effect on seropositive RA risk, gene expression and protein levels in plasma, indicating that increased signalling through the JAK/STAT-pathway is central in the inflammatory cascade in seropositive RA. Our findings are in line with the documented effectiveness of IL-6 receptor and JAK inhibitors (baricitinib, tofacitinib, filgotinib and upadacitinib) as well as CTLA4-Ig in RA.[1 36 38 47] Furthermore, there are inhibitors of other proteins in this pathway that are in development or already marketed for other diseases but have to our knowledge not been tested for treatment of RA, including FLT3 inhibitors used to treat acute myeloid leukaemia and other cancer forms,[48] TYK2 inhibitors that show promising results in clinical trials for psoriatic arthritis[49] and IFNAR1 inhibitors in systemic lupus erythematosus.[50]

In summary, through a large genome, transcriptome and proteome analysis of RA and its subsets, we identified new RA risk loci and highlight candidate causal genes at the majority of RA-associated loci. Most sequence variants have larger effect on the risk of seropositive than seronegative RA. Majority of those with largest effect on RA risk have not been reported before and point to candidate causal genes encoding proteins in the network of interferon alpha/beta and IL-12/IL-23 that signal through the JAK/STAT pathway. Together, these data thus shed light on the molecular mechanism affected by most non-HLA sequence variants that predispose to seropositive RA. In contrast, the genetic background of seronegative RA remains largely unexplained.

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Ethics approval This research has been conducted using the UK Biobank Resource (application licence number 24898, REC Reference Number: 06/MRE08/65), and the study was approved by the National Bioethics Committees in Iceland (approval no. VSN-15-045 and VSN-16-042), Sweden (approval no. 96-174, 2006/476-31/4, 2007/889-31/2, 2012/2070-31/2, 2015.1746-31.4 and 04-252/1-4), Denmark (Danish Data Protection Agency (general approval number 2012-58-0004 and local number: RH-2007-30-4129/ I-suite 00678) and the National Committee on Health Research Ethics (NVK-1700407, NVK-1803863 and H-2-2014-086)) and Norway (Regional Committees for Medical and Health Research Ethics, REC South-East C, 2019/ 28469, REK-13/05 and 2010/744). All data processing complies with the instructions of the Data Protection Authority in Iceland (PV_2017060950ÞS) and the Norwegian Data Inspectorate. Patients were involved in the design and conduct of several of the studies that are included in this report. Participants gave informed consent to participate in the study before taking part wherever applicable.

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Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as supplementary information. The GWAS summary statistics are available at https://www.decode.com/summarydata/. Sequence variants passing GATK filters will be deposited in the European Variation Archive (https://www.ebi.ac.uk/ena/data/view/). We used publicly available software (URLs listed further) in conjunction with the algorithms in the sequencing processing pipeline (whole-genome sequencing, association testing, RNA-sequence mapping and analysis, see methods description in Supplementary Information 2): BWA 0.7.10 mem (https://github.com/lh3/bwa);

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