



Deposited via The University of Leeds.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/id/eprint/218251/>

Version: Accepted Version

Article:

Carter, L.M., Yusof, M.Y.M., Wigston, Z. et al. (2024) Blood RNA-sequencing across the continuum of ANA-positive autoimmunity reveals insights into initiating immunopathology. *Annals of the Rheumatic Diseases*, 83 (10). pp. 1322-1334. ISSN: 0003-4967

<https://doi.org/10.1136/ard-2023-225349>

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

Article Type

Original article

Title

Blood RNA-sequencing across the continuum of ANA-positive autoimmunity reveals insights into initiating immunopathology

Authors

Lucy Marie Carter^{1,2}, Md Yuzaiful Md Yusof^{1,2}, Zoe Wigston¹, Darren Plant³, Stephanie Wenlock⁴, Adewonuola Alase¹, Antonios Psarras^{1,5}, Edward M Vital^{1,2}

Affiliations

1. Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, Leeds, UK
2. NIHR Leeds Biomedical Research Centre, Leeds Teaching Hospitals NHS Trust, Leeds, UK
3. Division of Musculoskeletal and Dermatological Sciences, The University of Manchester, UK
4. Cambridge Genomic Sciences, University of Cambridge, UK.
5. Kennedy Institute of Rheumatology, NDORMS, University of Oxford, Oxford, UK

Correspondence

Dr Edward M Vital
Chapel Allerton Hospital
Leeds LS7 4SA
United Kingdom
Email: e.m.j.vital@leeds.ac.uk

Word Count: 4092

Figure Count: 6

Table Count: 1

Funding: This research was supported by Lupus UK and presents work undertaken within the NIHR Leeds Biomedical Research Centre. Dr.Md Yusof's work was supported by NIHR Doctoral Research Fellowship DRF-2014-07-155 and Wellcome Trust Institutional Strategic Fund Fellowship 204825/Z/16/Z. Dr. Vital's work was supported by NIHR Clinician Scientist award CS-2013-13-032. The views expressed are those of the author(s) and not necessarily those of the NIHR or the UK Department of Health and Social Care.

Financial Disclosures: LMC has received consultancy fees from UCB and Alumis. MYMY has received consultancy fees from Aurinia Pharmaceuticals and UCB and speaker fees from Alumis, Roche and Novartis. EMV has received consultancy fees from Roche, GSK, AstraZeneca, Aurinia Pharmaceuticals, Lilly and Novartis. He has also received research grants paid to his employer from Roche, AstraZeneca and Sandoz. All other authors have declared no competing interests.

Data Availability:

RNASeq FastQ data files will be made available at GEO from the point of publication. Further data underlying this article will be made available on reasonable request to the corresponding author.

Ethics statement:

Research ethics approval was obtained from The Leeds (West) Research Ethics Committee (REC: RR10/9608) and UK Health Research Authority (IRAS ref. 60762). Written informed consent was obtained from all patients.

Research in context

Evidence before study

- Only a small subset of ANA positive (ANA+) individuals progress to clinically manifest SLE, and type I interferon (IFN-I) pathway activation in blood and skin is associated with progression to classifiable SLE.
- ANA+ subjects, irrespective of later clinical trajectory display several significant immune abnormalities over healthy subjects, including dysfunction in plasmacytoid dendritic cells.

Added value of this study

- Preclinical ANA+ subjects display profound peripheral immune cell transcriptional dysregulation compared with healthy controls and are transcriptomically closer to patients with established SLE.
- Progression from ANA positivity to classifiable SLE was associated with the depth of downregulation in oxidative phosphorylation mitochondrial complex I, and specific upregulation in interferon pathway activation, with modular components which were responsive to both IFN-I and IFN-II.
- Non-progression associated with attenuated IFN-I activation but more diverse cytokine responses.

Implications of all the available evidence

- ANA positivity is a complex immune dysregulated state irrespective of subsequent clinical trajectory.
- Oxidative stress and metabolic dysfunction may occur early SLE pathogenesis in the ANA+ prodromal phase. An immune response skewed towards IFN-I activation promotes progression to manifest SLE while greater diversity in cytokine cascades appears protective.

Abstract

Objective:

Mechanisms underpinning clinical evolution to SLE from preceding ANA positivity are poorly understood. This study aimed to understand blood immune cell transcriptional signatures associated with subclinical ANA positivity, and progression or non-progression to SLE.

Methods:

Bulk RNA-sequencing of peripheral blood mononuclear cells isolated at baseline from 35 ANA positive (ANA+) subjects with non-diagnostic symptoms was analysed using differential gene expression, weighted gene co-expression network analysis, deconvolution of cell subsets, and functional enrichment analyses. ANA+ subjects including those progressing to classifiable SLE at 12 months (n=15) and those with stable subclinical ANA positivity (n=20), were compared with 15 healthy subjects and 18 patients with SLE.

Results:

ANA+ subjects demonstrated extensive transcriptomic dysregulation compared with healthy controls with reduced CD4+ naïve T-cells and resting NK cells, but higher activated dendritic cells. B-cell lymphopenia was evident in SLE but not ANA+ subjects. Two thirds of dysregulated genes were common to ANA+ progressors and non-progressors. ANA+ progressors showed elevated modular interferon signature in which constituent genes were inducible by both IFN-I and IFN-II in vitro. Baseline downregulation of mitochondrial oxidative phosphorylation Complex I components significantly associated with progression to SLE but did not directly correlate with IFN modular activity. Non-progressors demonstrated more diverse cytokine profiles.

Conclusions:

ANA positivity, irrespective of clinical trajectory, is profoundly dysregulated and transcriptomically closer to SLE than to healthy immune function. Metabolic derangements and IFN-I activation occur early in the ANA+ preclinical phase and associate with diverging transcriptomic profiles which distinguish subsequent clinical evolution.

Introduction

Systemic lupus erythematosus (SLE) is serologically characterised by autoreactivity to nuclear antigens including nucleic acids, histones and ribonucleoproteins, detectable by the presence of anti-nuclear antibodies (ANA) (1). Immunopathology is complex and may be modified by therapy. The earliest initiating events are still uncharacterised. ANA positivity may be established up to a decade before clinically manifest SLE (2) during which time prodromal symptoms and ANA subtypes binding extractable nuclear antigens progressively accumulate (3). Yet, ANA positivity is harboured by up to 25% of the wider population, and of these only a small fraction develops clinically apparent systemic autoimmune disease (4). Understanding the initiating pathology and immunological fingerprint of ANA positive (ANA+) individuals on a trajectory toward clinically manifest SLE could accelerate diagnosis and reveal therapeutic opportunities for intercepting disease which abort or ameliorate the early disease course, before establishment of chronic inflammation and immunological memory (5, 6).

We have established a prospective observational cohort of ANA+ individuals, referred to rheumatology by primary care physicians for non-diagnostic symptoms of concern. Up to 12% of these demonstrated accrual of clinical and immunological criteria sufficient to fulfil SLE classification at 1 year follow up (7), with diminishing rate of progression in subsequent years (8). We have demonstrated blood and skin type I interferon (IFN-I) signatures stratified the risk of clinical progression from baseline ANA positivity (7), and similar evidence for IFN-I inducible proteins has followed (9). We have also shown that ANA+ subjects display other immunological derangements including plasmacytoid dendritic cell (pDC) exhaustion and IFN-I production by non-haematopoietic sources (10). Thus, ANA positivity even in the absence of clinically overt autoimmune disease is a complex dysregulated state, but the wider factors modulating the risk of clinical progression are relatively unexplored.

The present work uses RNA-sequencing (RNA-seq) in our preclinical cohort to characterize peripheral blood immune cell transcriptional signatures of subclinical ANA positivity in contrast to healthy subjects and established SLE. We evaluate bulk differential gene expression and modular transcriptomic profiles associated with both progression and non-progression from ANA positivity to classifiable SLE to reveal new insights into early SLE and subclinical autoimmunity.

Methods

Subjects

Subjects were selected for RNA-seq analysis from our prospective cohort of ANA+ subjects recruited between November 2014 and May 2017 (7). Baseline eligibility included adults (≥ 18 years), ANA-positive (titre $\geq 1:80$ by indirect immunofluorescence), ≤ 1 clinical Systemic Lupus International Collaborating Clinics Classification Criteria (SLICC) 2012 criterion (11), symptom duration < 12 months, and naïve of immunomodulatory therapy (7). ANA+ progressors ($n=15$) were defined as subjects with accrual of criteria sufficient to fulfill 2012 SLICC criteria for SLE at 12 months follow up. Subjects lacking disease evolution to classifiable SLE at 12 months were defined as ANA+ non-progressors ($n=20$). Criteria non-progressors with clinically significant symptom burden, therapy requirement or features for undifferentiated or overlap autoimmune disease were excluded from selection. The nested cohort was selected for comparison of criteria progression vs clinically inert autoimmunity (ANA+ non-inflammatory symptomatology) on the basis of sample availability and adjudication of progression status end point by an independent clinician. Healthy control subjects ($n=15$) and patients with established SLE ($n=18$; Supplementary Table 1) comprising high and low disease activity patients, both on and off therapy were also analysed.

RNA-sequencing

Peripheral blood mononuclear cells (PBMCs) were isolated by a density gradient centrifugation using LeucoSep tubes (Greiner Bio-One). Total RNA was isolated using the Quick-RNA™ Miniprep Kit (Cambridge bioscience; Zymo research) with DNase treatment. Pooled cDNA libraries were sequenced using the HiSeq 4000 Illumina® platform (Illumina) at Cambridge Genomics Service core facility following ribosomal depletion (RiboZero Gold, Illumina).

Differential gene expression analysis

All analyses were performed in R v4.2.1 (Supplementary Methods 1). Differential gene expression analysis was performed in *edgeR* using generalized linear models (GLM) with age and sex covariables incorporated into design matrix and relevant pairwise contrasts made (12). Effective library sizes determined by trimmed mean of M-values (TMM) normalization. False discovery rate (FDR) correction was applied for multiple testing.

CIBERSORTx

Immune cell fractions were estimated by deconvolution of normalised logCPM (counts per million) expression data in CIBERSORTx (13) with the LM22 leukocyte gene signature matrix, in relative mode with quantile normalisation disabled and 100 permutations. Relative cell proportions were compared between groups by Kruskal-Wallis and Dunn's post-hoc test with Benjamini-Hochberg correction for multiple testing. Association between differentially expressed genes and cell proportions was assessed using regression analysis in Bioconductor package *limma* (14)

WGCNA

Weighted gene co-expression network analysis (WGCNA) was performed as previously described (15) in package *WGCNA* (v1.72-1) (16) using normalized logCPM expression levels of the input transcripts and parameters specified in Supplementary Methods 2 and Fig.S1. Module eigengenes identified using arbitrary

colour labels were compared between groups using one-way ANOVA and Tukey post-hoc tests.

Enrichment and protein:protein interaction analyses

Gene Ontology: Biological Process (GO:BP) and Molecular Function (GO:MF) enrichment analysis was performed in *ClusterProfiler* (v4.8.1) (17) with *org.Hs.eg.db* for genome wide background. Significant GO term enrichment was defined as $FDR \leq 0.05$. Protein:protein interactions were evaluated using the STRING database (18) and visualized in Cytoscape (v3.7.2) (19). Differential gene expression was overlaid on selected KEGG pathways using *pathview* (20). Transcription factor enrichment analysis was performed in ChEA3 (Chip-X Enrichment Analysis version 3) web tool using (21) the top integrated rank across available libraries.

Investigation of hub gene induction

PBMCs isolated from additional healthy volunteers (n=4) were cultured for 6 hours at 37°C with the addition of (a) media only, (b) IFN α 100ng/ul, (c) IFN β 100ng/ul, (d) IFN γ (100ng/ul), (e) IFN λ (100ng/ul), TNF (20ng/ul), IFN α (100ng/ul) plus TNF (20ng/ul), IL-1 (10ng/ul) or IFN α (100ng/ul) plus IL-1 (10ng/ul; all PeproTech, UK). After 6h incubation, total RNA was isolated and expression of selected Midnight Blue module genes was quantified using a customised 96.96 TaqMan® array as previously described (22). Ct values were normalised to reference gene peptidylprolyl isomerase A (PPIA). $\Delta\Delta Ct$ and fold change were derived for all experimental conditions versus unstimulated controls. See Supplementary Methods 3 for details.

Patient and public involvement (PPI)

The NIHR Leeds Biomedical Research Centre PPI group have regular insight and input into planning and conduct of pre-clinical and clinical ANA-RMD research in Leeds since 2015.

Results

ANA-positivity is a complex transcriptomically dysregulated state

Baseline characteristics of ANA+ subjects and accrued features of progression at 12 months are outlined in Table 1. After adjustment for age and sex, compared with healthy subjects, 37% of the transcriptome (3962/10618 genes) showed significant dysregulation in ANA+ subjects with $FDR \leq 0.01$ (Fig.1A). The degree of gene dysregulation compared with patients with established SLE was substantively lower (1.7%, 178/10618 genes $FDR \leq 0.01$, Supplementary Fig.S2). Relative immune cell frequencies estimated by RNA-seq deconvolution indicated ANA+ subjects collectively retained similar naïve and memory B cells fractions to healthy control subjects, and did not demonstrate the B cell lymphopenia evident in established SLE patients (Fig.1C;D naïve B cells $H=6.5$, ANA+ vs HC $p=0.98$, SLE vs HC $p=0.03$; memory B cells $H=5.9$, ANA+ vs HC $p=0.33$, SLE vs HC $p=0.05$). Like patients with SLE, baseline ANA+ subjects harboured significantly lower proportions of resting NK cells (Fig.1E; $H=22.6$, ANA+ vs HC $p < 0.001$, SLE vs HC $p < 0.001$), naïve CD4+ T cells (Fig.1F; $H=12.1$, ANA+ vs HC $p=0.006$, SLE vs HC $p=0.003$) and resting memory CD4+ T cells (Fig.1G; $H=6.18$, ANA+ vs HC $p=0.04$, SLE vs HC $p=0.04$). Monocytes, the largest cell fraction in this bulk analysis, showed no significant differences between groups ($H=5.6$, $p=0.132$) and there were no significant differences in any of these populations between baseline ANA+ subjects on a trajectory towards subsequent classifiable SLE (progressors), and those showing no clinical evolution over 12 months follow up (non-progressors). Estimated fraction of activated dendritic cells (Fig.1H), though small, was significantly higher than healthy control subjects ($H=13.3$) in both SLE patients ($p=0.002$) and baseline ANA+ subjects ($p=0.003$), with a substantial upward trend between ANA+ non-progressors and progressors ($p=0.067$).

Two thirds of dysregulated genes in ANA+ subjects are common to patients with imminent SLE and stable autoimmunity

To evaluate the divergence between stable ANA-positivity and impending SLE, the transcriptomic departure from healthy controls was compared for baseline ANA+ progressors and non-progressors. These shared similar demographic, symptom, clinical immunology and comorbidity profiles (Table 1), though with higher prevalence of arthritis among ANA+ progressors. The number of significantly dysregulated genes versus healthy subjects was numerically higher among non-progressors than among progressors (3309 vs 3133), but over two thirds of differentially regulated genes were common to both groups (Fig.1B). Differentially expressed genes (DEGs) showed the same direction of change in progressors and non-progressors (Fig.1B). Top 100 DEGs are tabulated in Table S2.

Gene downregulation in ANA+ subjects implicate metabolic derangement over ANA- healthy subjects

We next examined mutually dysregulated genes of greatest magnitude fold change common to both ANA+ progressors and non-progressors compared with healthy control subjects (Fig.1A, yellow). In keeping with the decline in deconvoluted T cell fractions among ANA+ subjects (Fig.1F,G), GO:BP enrichment for 94 genes with significant ($FDR \leq 0.01$) 2-fold mutual *down*regulation showed over-representation of terms associated with T cell fate (Fig.2A,B) and included transcription factor GATA3, regulators TCF7 and TOX as well as IL2 receptor beta subunit (Fig.2B, blue). A smaller network of mitochondrially encoded complex I subunits were also mutually downregulated (Fig. 2B, pink).

Baseline ANA+ subjects demonstrate an activated inflammatory response

259 genes demonstrated significant mutual 2-fold upregulation in ANA+ progressors and non-progressors compared with healthy subjects. GO:BP enrichment for these indicated activation of the inflammatory response, chemotaxis, and mitochondrial apoptotic processes (Fig.2D). Protein:protein interaction network centred upon TNF (Fig.2C, green). Other components included IL23A and FC gamma receptors 1A and 2A. This network was linked to a dense network of predominantly H2 histones via polyubiquitin and transcriptional modulators of immune activation including NF-kappa-B inhibitor alpha, CEBPB, and JUN.

Distinct cytokine profiles separate ANA+ progressors and non-progressors from healthy subjects

We next examined genes exhibiting discrete dysregulation unique to progressors or unique to non-progressors, to establish how these clinical trajectories separated transcriptomically at baseline. 147 genes demonstrated significant 2-fold upregulation uniquely among ANA+ progressors. These comprised a dense network of ISGs (Fig.3A,B) including MX1, OAS1, IRF7 (Fig.3B, blue). This network was linked to a second subnetwork centred upon potent neutrophil chemoattractant CXCL8 (Fig.3B, yellow), which also included key B-cell cytokine TNFSF13B (BAFF). A further group of histones was also identified (Fig.3B, red).

Two-fold downregulated genes unique to ANA+ progressors were enriched for chromatin remodeling, disassembly of the nucleosome and DNA breakage repair (Fig.3C, red, Fig.3D). As noted among downregulated genes common to ANA+ progressors and non-progressors, a network of progressor-specific downregulated genes comprised seven further mitochondrially encoded respiratory chain subunits, collectively enriched for oxidative phosphorylation, complex I assembly and response to oxidative stress (Fig.3C, purple, Fig.3E), suggesting greater perturbations to these processes among ANA+ progressors.

Interestingly, the 161 upregulated genes unique to non-progressors demonstrated a distinct cytokine profile to ANA+ progressors, suggesting divergent pattern of immune activation, with a network centred upon IL1B, which also included IL18 and chemokines CCL3 and CCL4 (Fig.3F,G). This network also included transcriptional regulators key to cellular stress response and DNA damage including ATF3 and IRF1 (Fig.3G). Eighty-three two-fold downregulated genes unique to ANA+ non-progressors (Supplementary Table S3) did not collectively show significant enrichment for any GO:BP or MF terms. The cellular associations of dysregulated genes differed between ANA+ progressors and non-progressors (Supplementary Table S4). A higher proportion of upregulated genes unique to ANA+ progressors showed significant association with naive CD4+, resting memory CD4+ and resting NK cells. A relatively higher proportion of ANA+ non-progressor unique upregulated genes associated with deconvoluted monocyte fraction. The majority of all DEGs showed associations with two or more deconvoluted cell populations (Fig.S3).

Modular transcriptional signatures stratify risk of progression from ANA-positivity to SLE

Since distinct profiles of DEGs separated ANA+ progressors and non-progressors from healthy subjects, we applied WGCNA to understand the context of these changes across wider networks. WGCNA employs pairwise correlations and hierarchical clustering to assemble highly interconnected genes into a modular network (Fig.S1).

In ANA+ subjects, WGCNA identified 2 modules which significantly associated with progression to classifiable

SLE; Midnight Blue (positively correlated with progression $R=0.41$, $p=0.01$) and Light Yellow (negatively correlated with progression, $R= -0.43$, $p=0.01$). Neither module was significantly associated with other clinical variables including age, patient VAS or physician VAS (Fig.4A). To test whether WGCNA modules reflected specific cell population sizes, we examined correlation between module eigengenes and deconvoluted immune cell populations (Fig.4B). Midnight Blue module activity showed positive correlation with estimated activated dendritic cell numbers (Fig.4B; $R=0.39$, $p=0.02$) and negatively associated with the proportion of resting memory T-cells ($R= -0.47$, $p=0.004$). Light Yellow, which was negatively associated with clinical progression, was positively correlated with naïve B-cells (Fig.4B; $R=0.33$, $p=0.05$), while negatively associated with naïve CD4+ T-cells ($R=-0.37$, $p=0.03$) and activated dendritic cells ($R =-0.38$, $p =0.02$). An exploration of eigengene dissimilarity (Fig. S4), and metamodules within the ANA+ WGCNA network is presented in Fig.S5, Fig.S6 and Supplementary Table S5. Quantification of module eigengenes revealed a significant sequential decline in Light Yellow module activity from healthy control subjects across ANA+ non-progressors and ANA+ progressors (Fig.4C; $F=18.5$, both $p< 0.001$ vs HC), with a partial recovery in activity among patients with SLE ($p=0.002$ vs progressors, $p=0.002$ vs HC). Midnight blue module eigengene activity showed no significant difference between healthy control subjects and ANA+ non-progressors (Fig.4D; $F= 4.92$, $p=0.96$), but was significantly elevated over healthy controls in both ANA+ progressors ($p=0.050$), and showed a further increment in patients with SLE ($p=0.010$)

An IFN modular signature predicts progression from ANA positivity to SLE

We next examined functional enrichment for WGCNA modules associated with progression. Midnight Blue module, which demonstrated strongest positive association with progression, comprised a dense 140-gene network of ISGs (Fig.5A) with GO:MF enrichment for terms including helicase and nucleotidyltransferase activity (Fig.5B). Among these 140 genes, 20 were components of predefined blood IFN module M1.2 described by Chaussabel et al (23), thirty-one were components of IFN module M3.4 and two were constituents of IFN module M5.12. Seven located to modules annotated to inflammation, cell cycle and mitochondrial stress, while the remainder belonged to modules of undetermined function or lacked modular annotation in the Chaussabel et al network (23) (Supplementary table S6). Transcription factor enrichment among Midnight Blue constituents, was dominated by STAT1 and IRF9, which were themselves components of the module, with lesser enrichment BATF3 which is essential to dendritic cell development (Fig.5C).

Ten hub genes, best representing overall module expression profile were defined by ranked Module Membership (MM) and comprised PARP9, MX1, HERC5, RSAD2, TRIM22, ZBP1, IFIT1, SAMD9L, CMPK2, SAMD9 (Fig.5D), of which two (IFIT1 and TAP1) were components of our previously described IFN Score A (Supplementary Table S7). To further understand the regulation of these hub genes, we determined fold change in expression for four of these targets, following in vitro stimulation of PBMCs isolated from healthy volunteers. MX1 (Fig. 5E), HERC5 (Fig.5F), RSAD2 (Fig.5G) and IFIT1 (Fig.5H) all showed high level induction in response to type I IFNs, IFN α and IFN β , which was not replicated by type II IFN (IFN- γ) or type III IFN (IFN- λ). In light of the significant upregulation of TNF common to ANA+ subjects (Fig.3C), and IL-1 among ANA+ non-progressors, we investigated the ability of these cytokines to modify hub gene induction in response to IFN α . For selected Midnight Blue hub genes MX1 (Fig. 5E), HERC5 (Fig.5F), RSAD2 (Fig.5G) and IFIT1 (Fig.5H), potent induction in response to IFN α was neither substantively attenuated or amplified in vitro by co-stimulation with either TNF

or IL1.

Progression-predictive genes may be regulated by both IFN-I and IFN-II

Midnight blue constituent genes were next examined for their individual association with progression from ANA-positivity to SLE. Interestingly, genes demonstrating strongest association and significance for progression demonstrated relatively lower Module Membership, i.e. were not all within the hub of the module (Fig. 5D and Supplementary table S7). The ten genes with strongest association with progression comprised MTG2, GBP5, ZNF684, IFITM1, ZBP1, TAP1, ISG20, ERGIC2, GBP1, PARP9. Evaluation of expression for two of these targets following in vitro stimulation suggested a different interferon response profile. TAP1 (Fig.5I) and GBP1 (Fig.5J) showed more modest in vitro induction in response to type I IFNs, IFN α and IFN β , and unlike central hub genes, showed a similar degree of induction in response to IFN- γ . As with hub genes, MX1 (Fig.5E), HERC5 (Fig.5F), RSAD2 (Fig.5G) and IFIT1 (Fig.5H), TAP1 (Fig.5I) and GBP1 (Fig.5J) showed no substantive induction by type III IFN (IFN- λ) and neither IL1 nor TNF substantively modified in vitro fold change in response to IFN-I.

An oxidative phosphorylation modular signature is negatively associated with progression

The Light Yellow module, which inversely correlated with progression from ANA+ to SLE, demonstrated GO:BP functional enrichment for oxidative phosphorylation and mitochondrial ATP synthesis (Fig.6A). Similar to the earlier identified downregulated genes in ANA+ subjects, this 46-gene module included the same network of mitochondrially encoded respiratory Complex I components with NADH dehydrogenase activity (Fig.6B) as hub genes, ranked by module membership as follows MT-ATP6, MT-CO2, MT-ND4L, MT-ND4, MT-CYB, MT-ND, MT-CO1, MT-RNR1, MT-CO3, MT-ND3. Notable other members of this module were *STK17A*, a serine/threonine-protein kinase involved response to reactive oxygen species and regulation of cell death, and *ATRIP*, a 3' repair exonuclease with a key role in the DNA damage checkpoint and suppression of IFN activation through degradation of DNA fragments (Fig.6B). In view of downregulation in a subset of respiratory chain genes, we evaluated the location of differentially expressed genes (FDR \leq 0.01, logFC any magnitude) within the KEGG Oxidative Phosphorylation pathway. This suggested significant perturbations in various points of the pathway at transcriptomic level among ANA+ subjects (Supplementary Fig. S7).

To understand the relationship between the Midnight Blue and Light Yellow modules further, we explored correlation between leading hub genes in both modules (Fig.6C-D, Supplementary Fig.S8). In healthy subjects Midnight Blue hub gene PARP9 expression was low and was not significantly related to expression of the Light Yellow hub genes MT-CO2 (Fig.6C) or MT-ATP6 (Fig.6D). In ANA+ subjects the relationship between PARP9 and MT.CO2 adopted an L-shape configuration (Fig.6D) with a substantive subset of ANA+ subjects showing reduced MT,CO2 expression without elevation in PARP9 ($R = -0.134$, $p > 0.05$). ANA+ progressors in particular showed deeply reduced MT.CO2 expression across the range of expression levels of Midnight Blue Hub genes ($R = -0.415$, $p > 0.05$). Similar relationships were evident for comparisons involving other hub genes (Supplementary Fig.S8)

Discussion

Understanding the initiating immunopathology of autoimmune rheumatic disease conceptually offers the possibility of therapeutic interception in the pre-clinical phase of disease, to modify disease course and improve longer term outcomes (6). ANA positivity is an immunological prodromal phase to SLE established up to a decade before clinical diagnosis. We present the first transcriptome-wide exploration of preclinical ANA positivity and its diverging clinical trajectories towards stable subclinical autoimmunity and clinically manifest SLE, using differential gene expression and modular transcriptome approaches to reveal important novel insights into SLE pathogenesis.

Firstly, preclinical ANA positivity, irrespective of later clinical trajectory is a complex transcriptionally dysregulated state, with over one third of genes dysregulated compared with health control subjects, and is substantively closer in transcriptomic profile to established SLE than to the healthy immune system. Our previous observations demonstrate that, in common with established SLE, aberrant IFN production in the skin and pDC dysfunction also becomes evident in ANA+ individuals (10).

Secondly, ANA+ non-progressors demonstrate an equal degree of transcriptomic departure from healthy subjects, as those with impending SLE. Thus, subjects with stable subclinical ANA positivity do not simply lack activation of pathways propelling progression but appear to mount or retain counter regulatory responses.

Thirdly, a shared transcriptomic derangement in ANA+ progressors and non-progressors indicates a common immune pathology upon which clinical trajectory is established. Finally, we provide new insights into the chronology of events in clinical autoimmunity. Estimated cell proportions indicate the B-cell lymphopenia characteristic of SLE was not evident in ANA+ subjects at point of referral and appears to emerge relatively later in the clinical evolution, while activation of IFN-I and alterations in CD4+ T-cell and DC activation are evident earlier.

We and others have demonstrated that IFN-I pathway activation is a predictor of evolution from ANA positivity to SLE (7, 9) but factors implicated in non-progression have hitherto been unexplored. Mutually upregulated genes in progressors and non-progressors indicate they share a proinflammatory environment, but in those destined for classifiable SLE this is heavily skewed towards IFN-I responses while non-progressors demonstrate more diverse cytokine activity, with potential roles for IL-1 and TNF. Genetic factors may contribute to this since a number of influential SLE susceptibility loci relate to IFN activation (24). This observation might also suggest that non-progressors mount a more immunologically diverse homeostatic response when ANA reactivity is established. We previously identified an IFN gene expression score (7, 25), derived from the IFN-annotated blood gene expression modules described by Chaussabel et al (23, 26), which predicted progression to criteria SLE in ANA positive individuals. The current whole-transcriptome approach identifies a still wider network of ISGs which may underpin this effect. Indeed this network includes a subset of genes which did not carry IFN annotation in the whole blood transcriptome (23). Interestingly, Midnight Blue genes most predictive of progression, were located further from the eigengene, suggesting the possibility of subtle differences in their regulation compared with module hub. Genes induced by type I and II IFNs can overlap (27) due to harbouring both interferon-sensitive response element (ISRE) and gamma-activated site (GAS) elements, or through

induction of transcription factors with common downstream targets (28). We found progression predictive genes were among those sensitive to both IFN-I and IFN-II, and induction was not directly modified by IL-1 or TNF.

Current analyses indicated potential metabolic derangements in ANA+ individuals, with a gradient of perturbation between ANA+ non-progressors and ANA+ progressors. Most apparent was downregulation of mitochondrial Complex I / NADH dehydrogenase activity suggesting potential derangements in oxidative phosphorylation. Metabolic reprogramming has been extensively described across a range of cell types in SLE and other autoimmune disease (29). Evidence at transcriptomic level for altered cellular energy status and mitophagy in the subclinical phase is also emerging, with effects influenced by cell type and with disease transition (30). Mitochondrial stress is proinflammatory (31) but the relationship between mitochondrial dysfunction and IFN-I response appears complex and cell specific (29). Immune complexes involving ribonucleoprotein are potent inducers of mitochondrial reactive oxygen species (ROS) and superoxide (32), leading to loss of mitochondrial membrane potential and extrusion of oxidized mitochondrial DNA (mtDNA) (33) where it is a component of low-density granulocytes NETosis in SLE (32). mtDNA is a potent inducer of IFN-I via TLR9 (34) and cGAS-STING (35, 36). Chronic IFN exposure has been implicated driving downregulation of mitochondrial metabolism in SLE T-cells, through NAD⁺ depletion, leading to reduced viability and greater cell death following TCR stimulation (37). It has also been associated with defective mitophagy in monocytes and promoting differentiation to autoreactive DCs (35). SLE NK cells demonstrate enhanced oxidative phosphorylation but mitochondrial ultrastructural disruption and excess superoxide (38). Our work now suggests that immune cell mitochondrial dysfunction is an early event in SLE pathogenesis established in the ANA+ prodrome, and both the depth of this abnormality and the wider associated inflammatory environment appear to distinguish between clinical trajectories. At bulk transcriptomic level we did not identify a direct correlation between IFN and OxPhos modular signatures or hub genes, and the relationship between these two may vary with disease evolution. The precise alteration in cellular energy status cannot be accurately predicted from sequencing alone and may require interrogation of mitochondrial number, ultrastructure and redox function at single cell resolution to optimally delineate pathology.

This study has several limitations. This bulk transcriptomic approach cannot resolve cell-specific changes, and changes in smaller populations may be undetected. The physiological impact of gene dysregulation can only be inferred by these analyses and protein level functional studies are required to verify metabolic changes identified, particularly since metabolic reprogramming in SLE appears cell-type specific. We adopted 12 month follow-up as endpoint for progression status and, while total follow up now extends to 3 years (8), we cannot exclude the possibility of later progression in some individuals, or other confounding latent pathologies. While demographic profiles were similar between ANA+ groups, subjects were not ancestry matched and diverging immune pathology between ethnic groups is increasingly recognized (30, 39, 40). Our work nevertheless identified novel areas for focused research in SLE pathogenesis.

In conclusion, we demonstrate the complexity of immune dysregulation in ANA+ subjects is greater than previously known. Further study of the pathways we have identified could reveal the natural homeostasis which usually prevents SLE. Our data suggest interventions to prevent SLE targeted to high-risk individuals differ from those needed in treatment of established disease.

Figure Legends

Figure 1. Differential gene expression and immune cell population sizes in ANA+ subjects, healthy controls and patients with SLE

(A), Volcano plot demonstrates extent and magnitude of gene dysregulation between baseline ANA+ subjects (n=35) versus healthy controls (n=15). Positive \log_2 fold change indicates upregulation in ANA+. Dashed vertical lines indicate 2-fold up / down regulation. Dashed horizontal line indicates significance at false discovery rate (FDR) adjusted p-value ≤ 0.01 . (B), Venn diagram shows numbers of significant (FDR ≤ 0.01) two-fold up- and downregulated genes in ANA+ progressors (P) and non-progressors (NP). Overlaps indicate gene dysregulation common to both ANA+ groups. (C-H), Violin plot series quantify immune cell fractions estimated by RNA-seq deconvolution in healthy controls (HC, blue, n=15), ANA+ non-progressors (NP, pink, n=20), ANA+ progressors (dark gold, n = 15) and patients with SLE (light tan, n = 18). (C), Naïve and (D), Memory B cells are significantly reduced in SLE but not in ANA+ subjects. (E), Resting NK cells, (F), Naïve CD4+ T cells, and (G), Resting memory CD4+ T cells are significantly decreased in ANA+ subjects and patients with SLE compared with healthy controls (HC). (H), Activated dendritic cells were significantly increased in both ANA+ subjects and patients with SLE. There were no significant differences between ANA+ progressors (P) and non-progressors (NP) * denotes multiple testing corrected p-value ≤ 0.05 , ** p ≤ 0.01 , *** p ≤ 0.001 compared with healthy controls (HC).

Figure 2. Characterisation of mutually two-fold dysregulated genes common to ANA+ progressors and non-progressors versus healthy subjects

(A), Dotplot shows GO:BP enrichment for 94 mutually two-fold downregulated genes common to ANA+ progressors (P, n=15) and non-progressors (NP, n=20). Point size indicates number of genes attached to GO:BP terms. Colour scale (p.adjust) shows significance of GO term enrichment after FDR multiple testing correction. (B), Network graphic shows major protein:protein interactions for mutually downregulated genes, which include targets associated with T-cell fate (blue) including *IL2RB* and *GATA3*, alongside a small subnetwork of mitochondrially encoded complex respiratory chain subunits (pink). (C), Protein:protein interaction analysis for 259 mutually two-fold upregulated genes common to ANA+ progressors and non-progressors versus healthy subjects, comprises a dense network of histones (peach), and a larger network centred upon TNF (light green). (D), GO:BP enrichment analysis for mutually two-fold upregulated genes indicates activation of inflammatory pathways.

Figure 3. Characterisation of two-fold dysregulated genes unique to ANA+ progressors and to ANA+ non progressors versus healthy subjects

(A), Dotplot shows GO:BP enrichment for GO:BP enrichment for 147 genes with two-fold upregulation unique to ANA+ progressors (n=15) versus healthy control subjects (n= 15). Point size indicates number of genes attached to GO:BP terms. Colour scale (p.adjust) shows significance of GO term enrichment after FDR multiple testing correction. (B), Network graphic shows major protein:protein interactions for these targets, which include a network of histones (red), a dense network of ISGs (blue) overlapped with a further network concentrated on CXCL8 (yellow). TNFSF13B (BAFF) and TNFSF10 (TRAIL) adjoined these networks. (C), Protein-protein

interactions for 68 targets uniquely 2-fold downregulated in ANA+ progressors comprised two discrete networks, one (light coral) with GO:BP enrichment for **(D)**, nucleic acid break repair, chromatin remodelling, and the second (pale lilac) comprising mitochondrially encoded respiratory chain subunits with **(E)**, GO:BP enrichment for oxidative phosphorylation. **(F)**, GO:BP enrichment dotplot and **(G)**, network diagram for 161 two-fold upregulated genes unique to ANA+ non-progressors (n=20) over healthy control subjects (n=15), indicate immune cell activation involving pro-inflammatory cytokines IL1B, IL18, CCL3 and CCL4 **(G)**, outlined black alongside transcription factors JUN, ATF3 and IRF1 **(G)**, outlined orange).

Figure 4. Modular analysis of the ANA+ transcriptome

(A), Heatmap shows clinical trait associations for gene expression modules derived from WGCNA. Rows denote module eigengenes (MEs) identified by colour, columns show clinical characteristics of interest. Cell values report correlation coefficient (Pearson's r) and p-value. Heatmap gradients indicate strength and directionality of correlations from positive (red) to negative (blue). Boxes indicate two modules, Midnight Blue and Light Yellow with significant positive and negative association respectively with progression from ANA+ to SLE. **(B)**, Heatmap shows module: cell type associations. Rows denote module eigengenes identified by colour as panel A, columns indicate cell fractions estimated by deconvolution. Cell values report correlation coefficient (r) and p-value. Boxes highlight Midnight Blue and Light Yellow modules. **C-D**, Boxplots with overlay jitter points show quantification of module eigengenes for **(C)** Light Yellow and **(D)**, Midnight Blue modules in healthy control subjects (HC, blue), ANA+ non-progressors (NP, pink), ANA+ progressors (P, dark gold) and patients with SLE (light tan). * denotes p -value ≤ 0.05 , ** $p \leq 0.01$, *** $p \leq 0.001$ compared with healthy subjects.

Figure 5. Characterisation of the progression-associated Midnight Blue module

(A), Network graphic shows major protein:protein interactions in the Midnight Blue module. Highlighted nodes indicate the five leading hub genes, by highest ranked Module Membership (pink) and leading enriched transcription factors (yellow). **(B)**, Dotplot shows GO:MF enrichment for the Midnight Blue module. Point size indicates number of genes attached to the GO:MF term. Colour scale (p .adjust) shows significance of GO term enrichment after FDR multiple testing correction. **(C)**, Horizontal bar chart shows transcription factor enrichment analysis using ChEA3, depicting five top scaled transcription factors with integrated rank across all libraries. Higher value 1-integrated Scaled Rank (x-axis) indicates higher degree of enrichment. **(D)**, Gene lists indicate Midnight Blue constituent genes ranked from by module membership and gene significance for progression from ANA+ to SLE. **(E-H)**, Boxplots show gene expression measured by Taqman® PCR as fold change (FC) from unstimulated healthy donor PBMCs for Midnight Blue hub genes **(E)**, MX1, **(F)** HERC5, **(G)**, RSAD2, and **(H)**, IFIT1 following 6hour in vitro incubation with IFN α (IFNA), IFN β (IFNB), IFN γ (IFNG), IFN λ (IFNL), IL-1, TNF or the combination of IFN α plus IL-1 or IFN α plus TNF, for 6 hours. **(I-J)**, Boxplots show in vitro stimulation responses under the same conditions for **(I)**, TAP1 and **(J)**, GBP1, non-hub genes with higher significance for progression from ANA+ to SLE.

Figure 6. Characterisation of the Light Yellow module negatively-associated with progression

(A), Dotplot shows GO:MF enrichment for the Light Yellow module. Point size indicates number of genes attached to GO:MF term. Colour scale (p .adjust) shows significance of GO term enrichment after FDR multiple testing correction. **(B)**, STRING database graphic shows major protein:protein interaction within the module as

two discrete networks, the first a cluster of mitochondrially encoded complex I subunits (yellow) and the second (pale mint) including STING1 and MAVS, both implicated in induction of the IFN response. **(C-D)**, Scatterplots show the relationship between RNAseq gene expression as counts per million (CPM) for Midnight Blue hub gene PARP9 (x-axis) versus Light Yellow hub genes (C), MT-CO2 and (D), MT-ATP6 among health controls (HC, grey). ANA+ non-progressors (NP, blue), and ANA+ progressors (P, pink).

Table 1. Baseline characteristics of ANA+ progressors and non-progressors					
Characteristic	Non progressors		Progressors		P value
	n = 20		n = 15		
Demographics					
Age, mean (SD)	45.74 (17.14)		37.46 (12.53)		0.148
Female, n (%)	16 (84.2)		13 (100.0)		0.375
Number of SLICC 2012 criteria, mean (SD)	0.47 (0.61)		1.67 (0.98)		<0.001
Smoking history, n (%)	8 (40.0)		2 (15.4)		0.264
Family history autoimmune RMD, n (%)	5 (26.3)		7 (53.8)		0.227
Charleson comorbidity index, mean (SD)	1.1 (1.65)		0.4 (0.82)		0.112
Clinical assessment and symptomatology					
Patient global VAS (0-100), mean (SD)	43.10 (22.72)		55.00 (26.49)		0.170
Patient fatigue VAS (0-100), mean (SD)	62.25 (23.06)		71.29 (21.38)		0.373
Patient pain VAS (0-100), mean (SD)	38.05 (31.48)		33.00 (38.34)		0.747
HAQ score (0-3), mean (SD)	0.75 (0.41)		1.10 (0.65)		0.107
Physician global VAS (0-100), mean (SD)	12.30 (15.18)		34.33 (18.67)		0.001
Tender joint count, mean (SD)	2.16 (3.40)		4.31 (4.79)		0.147
SLICC Criteria	Baseline	Cumulative 12 months	Baseline	Cumulative 12 months	<i>Comparison of baselines</i>
Clinical					
ACLE, n (%)	1 (5.0)	1 (5.0)	3 (20)	11 (73.3)	0.399
Mucosal ulcers, n (%)	1 (5.0)	0	1 (6.7)	3 (20.0)	1.0
Alopecia, n (%)	0	0	0	4 (26.4)	-
Arthritis, n (%)	3 (15.0)	3 (15.0)	9 (60.0)	15 (100)	0.016
Serositis, n (%)	0	0	0	1 (6.7)	-
Pleural effusion, n (%)	0	0	0	1 (6.7)	-

Leucopaenia, or lymphopaenia n (%)	1 (5.0)	1 (5.0)	4 (26.7)	5 (33.3)	0.185
Immunological					
dsDNA antibody positive, n (%)	9 (45.0)	9 (45.0)	2 (13.3)	4 (26.7)	0.187
Sm positive, n (%)	1 (5.0)	1 (5.0)	3 (20.0)	4 (26.7)	0.276
Hypocomplementaemia, n (%)	1 (5.0)	1 (5.0)	2 (13.3)	2 (13.3)	0.673
Anti-phospholipid antibody positive, n (%)	1 (5.0)	1 (5.0)	1 (6.7)	1 (6.7)	0.64
Further baseline immunology					
Number of positive ENA, n (%)					0.074
0	2 (10.5)		0 (0.0)		
1	16 (84.2)		8 (66.7)		
2	0 (0.0)		2 (16.7)		
3	1 (5.3)		0 (0.0)		
4	0 (0.0)		2 (16.7)		
Sm/RNP positive, n (%)	1 (5.3)		4 (30.8)		0.145
RNP positive, n (%)	1 (5.3)		0 (0.0)		1.000
Chromatin positive, n (%)	1 (5.3)		3 (23.1)		0.341
Ro60 positive, n (%)	4 (21.1)		5 (38.5)		0.499
Ro52 positive, n (%)	6 (31.6)		2 (15.4)		0.533
Anti-dsDNA antibody titre, mean (SD)	22.32 (36.81)		10.83 (26.41)		0.357
Total IgG (g/L), mean	11.82 (3.06)		14.95 (7.20)		0.120

References

1. Crow MK. Pathogenesis of systemic lupus erythematosus: risks, mechanisms and therapeutic targets. *Ann Rheum Dis*. 2023;82(8):999-1014.
2. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, Harley JB. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med*. 2003;349(16):1526-33.
3. Heinlen LD, McClain MT, Merrill J, Akbarali YW, Edgerton CC, Harley JB, James JA. Clinical criteria for systemic lupus erythematosus precede diagnosis, and associated autoantibodies are present before clinical symptoms. *Arthritis Rheum*. 2007;56(7):2344-51.
4. Wandstrat AE, Carr-Johnson F, Branch V, Gray H, Fairhurst AM, Reimold A, et al. Autoantibody profiling to identify individuals at risk for systemic lupus erythematosus. *J Autoimmun*. 2006;27(3):153-60.
5. Choi MY, Costenbader KH. Understanding the Concept of Pre-Clinical Autoimmunity: Prediction and Prevention of Systemic Lupus Erythematosus: Identifying Risk Factors and Developing Strategies Against Disease Development. *Front Immunol*. 2022;13:890522.
6. Carter LM, McGonagle D, Vital EM, Wittmann M. Applying Early Intervention Strategies to Autoimmune Skin Diseases. Is the Window of Opportunity Preclinical? A Dermato-Rheumatology Perspective. *J Invest Dermatol*. 2022;142(3 Pt B):944-50.
7. Md Yusof MY, Psarras A, El-Sherbiny YM, Hensor EMA, Dutton K, UI-Hassan S, et al. Prediction of autoimmune connective tissue disease in an at-risk cohort: prognostic value of a novel two-score system for interferon status. *Ann Rheum Dis*. 2018;77(10):1432-9.
8. Yusof MYM, Hassan SU, Wigston Z, Psarras A, Arnold J, Carter LM, et al. POS1450 3-year analyses of At-Risk ANA-positive cohort: Prognostic value of clinical and interferon biomarkers towards autoimmunity. *Annals of the Rheumatic Diseases*. 2023;82(Suppl 1):1078-9.
9. Kim ST, Muñoz-Grajales C, Dunn SE, Schneider R, Johnson SR, Touma Z, et al. Interferon and interferon-induced cytokines as markers of impending clinical progression in ANA(+) individuals without a systemic autoimmune rheumatic disease diagnosis. *Arthritis Res Ther*. 2023;25(1):21.
10. Psarras A, Alase A, Antanaviciute A, Carr IM, Md Yusof MY, Wittmann M, et al. Functionally impaired plasmacytoid dendritic cells and non-haematopoietic sources of type I interferon characterize human autoimmunity. *Nat Commun*. 2020;11(1):6149.
11. Petri M, Orbai AM, Alarcón GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum*. 2012;64(8):2677-86.
12. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-40.
13. Newman AM, Steen CB, Liu CL, Gentles AJ, Chaudhuri AA, Scherer F, et al. Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nature Biotechnology*. 2019;37(7):773-82.
14. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47.
15. Zhang B, Horvath S. A general framework for weighted gene co-expression network analysis. *Stat Appl Genet Mol Biol*. 2005;4:Article17.
16. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*. 2008;9:559.
17. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, et al. clusterProfiler 4.0: A universal enrichment tool for

interpreting omics data. *The Innovation*. 2021;2(3).

18. Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F, Hachilif R, et al. The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res*. 2023;51(D1):D638-d46.
19. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13(11):2498-504.
20. Luo W, Brouwer C. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. *Bioinformatics*. 2013;29(14):1830-1.
21. Keenan AB, Torre D, Lachmann A, Leong AK, Wojciechowicz ML, Utti V, et al. ChEA3: transcription factor enrichment analysis by orthogonal omics integration. *Nucleic Acids Research*. 2019;47(W1):W212-W24.
22. Carter LM, Wigston Z, Laws P, Vital EM. Rapid efficacy of anifrolumab across multiple subtypes of recalcitrant cutaneous lupus erythematosus parallels changes in discrete subsets of blood transcriptomic and cellular biomarkers. *British Journal of Dermatology*. 2023;189(2):210-8.
23. Chaussabel D, Quinn C, Shen J, Patel P, Glaser C, Baldwin N, et al. A modular analysis framework for blood genomics studies: application to systemic lupus erythematosus. *Immunity*. 2008;29(1):150-64.
24. Goulielmos GN, Zervou MI, Vazgiourakis VM, Ghodke-Puranik Y, Garyfallos A, Niewold TB. The genetics and molecular pathogenesis of systemic lupus erythematosus (SLE) in populations of different ancestry. *Gene*. 2018;668:59-72.
25. El-Sherbiny YM, Psarras A, Md Yusof MY, Hensor EMA, Tooze R, Doody G, et al. A novel two-score system for interferon status segregates autoimmune diseases and correlates with clinical features. *Sci Rep*. 2018;8(1):5793-.
26. Chiche L, Jourde-Chiche N, Whalen E, Presnell S, Gersuk V, Dang K, et al. Modular transcriptional repertoire analyses of adults with systemic lupus erythematosus reveal distinct type I and type II interferon signatures. *Arthritis Rheumatol*. 2014;66(6):1583-95.
27. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci U S A*. 2003;100(5):2610-5.
28. Barrat FJ, Crow MK, Ivashkiv LB. Interferon target-gene expression and epigenomic signatures in health and disease. *Nature Immunology*. 2019;20(12):1574-83.
29. Psarras A, Clarke A. A cellular overview of immunometabolism in systemic lupus erythematosus. *Oxford Open Immunology*. 2023;4(1).
30. Bylinska A, Smith M, Thomas K, Guthridge C, Slight-Webb S, Macwana S, et al. OP0109 Single-cell RNA-Seq reveals differences in metabolic pathways of myeloid cells in the progression of SLE. *Annals of the Rheumatic Diseases*. 2023;82(Suppl 1):72-3.
31. Collins LV, Hajizadeh S, Holme E, Jonsson IM, Tarkowski A. Endogenously oxidized mitochondrial DNA induces in vivo and in vitro inflammatory responses. *J Leukoc Biol*. 2004;75(6):995-1000.
32. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat Med*. 2016;22(2):146-53.
33. Caielli S, Athale S, Domic B, Murat E, Chandra M, Banchereau R, et al. Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. *J Exp Med*. 2016;213(5):697-713.
34. Wilkinson MGL, Moulding D, McDonnell TCR, Orford M, Wincup C, Ting JYJ, et al. Role of CD14+ monocyte-derived oxidised mitochondrial DNA in the inflammatory interferon type 1 signature in juvenile

dermatomyositis. *Ann Rheum Dis.* 2023;82(5):658-69.

35. Gkirtzimanaki K, Kabrani E, Nikoleri D, Polyzos A, Blanas A, Sidiropoulos P, et al. IFN α Impairs Autophagic Degradation of mtDNA Promoting Autoreactivity of SLE Monocytes in a STING-Dependent Fashion. *Cell Rep.* 2018;25(4):921-33.e5.
36. West AP, Khoury-Hanold W, Staron M, Tal MC, Pineda CM, Lang SM, et al. Mitochondrial DNA stress primes the antiviral innate immune response. *Nature.* 2015;520(7548):553-7.
37. Buang N, Tapeng L, Gray V, Sardini A, Whilding C, Lightstone L, et al. Type I interferons affect the metabolic fitness of CD8+ T cells from patients with systemic lupus erythematosus. *Nature Communications.* 2021;12(1):1980.
38. Fluder N, Humbel M, Ribi C, Comte D. OP0020 The cellular metabolism of SLE NK cells in primarily altered at the level of mitochondrial homeostasis. *Annals of the Rheumatic Diseases.* 2023;82(Suppl 1):13-.
39. Carter LM, Alase A, Wigston Z, Psarras A, Burska A, Sutton E, et al. Gene expression and autoantibody analysis reveals distinct ancestry-specific profiles associated with response to rituximab in refractory systemic lupus erythematosus. *Arthritis Rheumatol.* 2022.
40. Catalina MD, Bachali P, Yeo AE, Geraci NS, Petri MA, Grammer AC, Lipsky PE. Patient ancestry significantly contributes to molecular heterogeneity of systemic lupus erythematosus. *JCI Insight.* 2020;5(15).