Methods for type I interferon detection and their relevance for clinical utility and improved understanding of rheumatic diseases

L. Lamot[1,2], I. Niemietz[2,3], K.L Brown[1,2]

[1]Department of Paediatrics, Division of Rheumatology, The University of British Columbia, Vancouver;
[2]BC Children's Hospital Research Institute, Vancouver;
[3]Department of Microbiology and Immunology, The University of British Columbia, Vancouver, BC, Canada.

Lovro Lamot, MD, PhD
Iwona Niemietz, MPharm
Kelly L Brown, PhD

Please address correspondence to:
Dr Lovro Lamot,
Division of Paediatric Rheumatology,
Department of Paediatrics,
The University of British Columbia and
British Columbia Children's Hospital
Research Institute, Rm A4-122, Bay 14,
950 West 28th Ave.,
Vancouver (BC), V5Z 4H4 Canada.
E-mail: lovro.lamot@bcchr.ca

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ABSTRACT

Type I interferons (IFN) are a class of inducible and protective cytokines best known for immune defence against viruses and intracellular bacteria. Inappropriate stimulation or defective negative regulation of type I IFN expression however can lead to persistent type I IFN activity with detrimental effects. This is particularly relevant for a class of monogenic autoinflammatory diseases ("type I interferonopathies"), along with many other complex rheumatic diseases such as systemic lupus erythematosus (SLE), dermatomyositis (DM), systemic sclerosis (SSc), rheumatoid arthritis (RA) and Sjögren's syndrome (SS). Direct detection of type I interferon protein in biologic samples has proved challenging, thus indirect methods are often used to infer the presence of type I IFN via quantification of antiviral activity and/or induced expression of IFN-responsive genes. While some of these methods have been used to inform clinical care, none have proven feasible for everyday clinical practice. However, with new technologies emerging, this may soon change. This review provides a brief summary of the available methods to gauge the presence of type I IFN and their application for the improved understanding, diagnosis and monitoring of type I interferonopathies and other rheumatic diseases.

Introduction

Type I interferons are a family of structurally related cytokines that include interferon (IFN)-alpha (IFNα) and -beta (IFNβ). There are 13 distinct genes that encode IFNα and a single gene encoding IFNβ. Type I IFN were first recognised in 1957 as the soluble factor that protects cells from viral infections (1). Since, it has been established that type I IFN also regulate cell proliferation and differentiation, inhibit angiogenesis, promote apoptosis and have a breadth of immune-modulatory functions (2, 3). Having both anti-viral and immune-modulatory properties, recombinant type I IFN have been used for the treatment of Hepatitis B and Hepatitis C viral infections, multiple sclerosis and certain types of cancers; IFNα was the first cancer immunotherapy approved in the U.S. (4-8). Outside this context however, the persistent presence of type I IFN can have detrimental effects (9, 10).

Persistent type I IFN activity was first reported in Aicardi-Goutières syndrome (AGS), a genetic autoinflammatory disease that affects the central nervous system and resembles neurological sequalae of congenital viral infections. Recognition of elevated type I IFN in AGS founded a new group of monogenic autoinflammatory diseases called type I interferonopathies (11-15). Since, elevated type I IFN activity has been reported in other complex rheumatic disorders including, but not limited to, systemic lupus erythematosus (SLE), dermatomyositis (DM), systemic sclerosis (SSc), rheumatoid arthritis (RA) and Sjögren's syndrome (SS), as well as in patients with positive anti-nuclear antibodies (ANA) without defined disease (16, 17). Before the techniques to directly measure type I IFN in biologic samples were introduced, the association of elevated type I IFN activity with these various diseases was established with *in vitro* assays that measure the downstream effects of type I IFN (such as anti-viral and transcription stimulating activity). Continued use of such functional assays in parallel with emerging technologies to directly quan-

titate type I IFN in biologic samples will be instrumental to the improved understanding of type I IFN in health and disease and the management of type I IFN-mediated diseases (18, 19). In this review, we summarise past and current methodology for assessment of type I IFN with examples of potential clinical utility for rheumatic diseases.

Induction of type I interferons and interferon-stimulated genes (ISGs)

Type I IFN is induced in a cell- and stimuli-specific manner (20). While IFNβ is secreted by many types of cells (*e.g.* fibroblasts, epithelial cells, dendritic cells, phagocytes and synoviocytes), the major sources of IFNα are plasmacytoid dendritic cells (pDC) and, to a lesser extent, mononuclear phagocytes (21). Type I IFN is readily produced following recognition of damage- or pathogen-associated molecular patterns (DAMPs and PAMPs; *e.g.* nucleic acids) by cell surface and intracellular pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). Receptor engagement initiates intracellular signalling that culminates in the activation of IFN regulatory factors (IRFs) and nuclear factor-κB (NF-κB) leading to the transcription of classic proinflammatory cytokine genes, IFN genes and a subset of IFN-stimulated genes (ISGs). These ISGs encode for proteins with a role in enhanced nucleic acid detection and/or amplification of IFN signalling (22).

De novo synthesised and secreted type I IFN acts in an autocrine and paracrine manner by binding to the IFNα/β receptor (IFNAR). This is a heterodimeric cell surface receptor present on all nucleated cells and comprised of subunits IFNAR1 and IFNAR2 (23). Canonical type I IFN signalling activates the Janus kinase (JAK) – STAT (signal transducer and activator of transcription) pathway. The result is sustained transcription of type I IFN genes and induced transcription of a new subset of several hundred ISGs (Fig. 1) with a wide range of activities including defense against viruses, bacteria and parasites, cell-to-cell communication and regulation of cellular homeostasis and processes leading to cell death. These activities are mediated through direct and indirect (*e.g.* via cytokines) pleiotropic effects of type I IFN signalling and are tightly controlled by positive and negative regulation (23-26). It is predicted that persistent type I IFN and continued perturbation of some of its immune modulating activities drive immune-mediated diseases, although the exact mechanisms, which may be numerous and disease-specific, have not been fully elucidated (23, 27).

To date, mutations in approximately 20 genes have been implicated in the dysregulation of type I IFN production and IFN-induced processes and pathways in the absence of infection (28). Causative variant-carrying genes are broadly divided into five categories according to the resultant cellular/molecular dysfunction: (i) abnormal accumulation of nucleic acids (*TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, POLA1*), (ii) abnormal chemical modification of nucleic acids (*ADAR1*), (iii) enhanced sensitivity or ligand-independent activation of nucleic acid sensing pathways (*TMEME173, IFIH1, DDX58*), (iv) impaired negative regulation of nucleic acid-induced type I IFN signalling (*ISG15, USP18, ACP5*) and (v) modulation of type I IFN responses independent of nucleic acid sensing (*PSMB8, PSMB4, PSMA3, PSMB9, POMP*) (29).

Detection of type I interferon

Enzyme-Linked Immunosorbent Assays (ELISA) are the most widely used method for detection of soluble proteins such as cytokines. An ELISA is a rapid and accurate immunoassay however sensitivity to detect type I IFN is limited to picomolar concentration (greater than physiologic concentrations of type I IFN) and a separate assay is required for individual type I IFNs (30-33). The recent introduction of Single Molecule Array (Simoa) digital ELISA technology has finally enabled detection of biologic concentrations of IFNα at the attomolar level, as well as quantification of all 13 IFNα species in one assay (33). In this method, single molecules of IFNα are captured with enzyme-linked patient-derived autoantibodies (tested for cross-reactivity with other IFN subtypes) coupled to paramagnetic beads that are detected as in an ELISA with an enzyme-generated fluorescent product (34, 35). Simoa has been used successfully to quantitate IFNα protein in otherwise healthy individuals in the absence and presence of viral infection as well as in individuals with monogenic interferonopathies (33). IFNα protein concentration in these samples correlated well with functional type I IFN activity assessed by a cytopathic protection assay and qPCR detection of six ISGs. Additionally, Simoa established that high circulating concentrations of IFNα were associated with increased clinical severity in SLE patients (33). Although promising, the application of Simoa technology for diagnosis and monitoring of rheumatic and other diseases needs further clinical validation in additional patient cohorts. Moreover, there are costs and risks associated with the implementation of this newly developed and single source technology which might delay its extensive use (36). These impediments might endorse clinical application of indirect methods for type IFN detection.

Detection of type I interferon-induced effects

Prior to the development of the Simoa technology, several bioassays were developed to indirectly quantitate type I IFN by measures of induced gene expression and anti-viral activity (Table I). The first indirect measure of type I IFN was a cytopathic protection assay in which type I IFN concentration was inferred by the concentration of patient CSF or serum that protected 50% of vesicular stomatis virus (VSV)-infected Madin-Darby bovine kidney cells (37, 38). This assay was used to characterise the first monogenic type I interferonopathy, AGS (39, 40). Subsequently, faster and more sensitive bioassays based on similar methodology were developed. For example, VSV recombinant replicons encoding the reporter proteins firefly luciferase or green fluorescent protein have been expressed in several mammalian and avian cell lines (41-43). Since the modified VSV genome lacks the envelope glycoprotein gene, the replicon particles cannot produce infec-

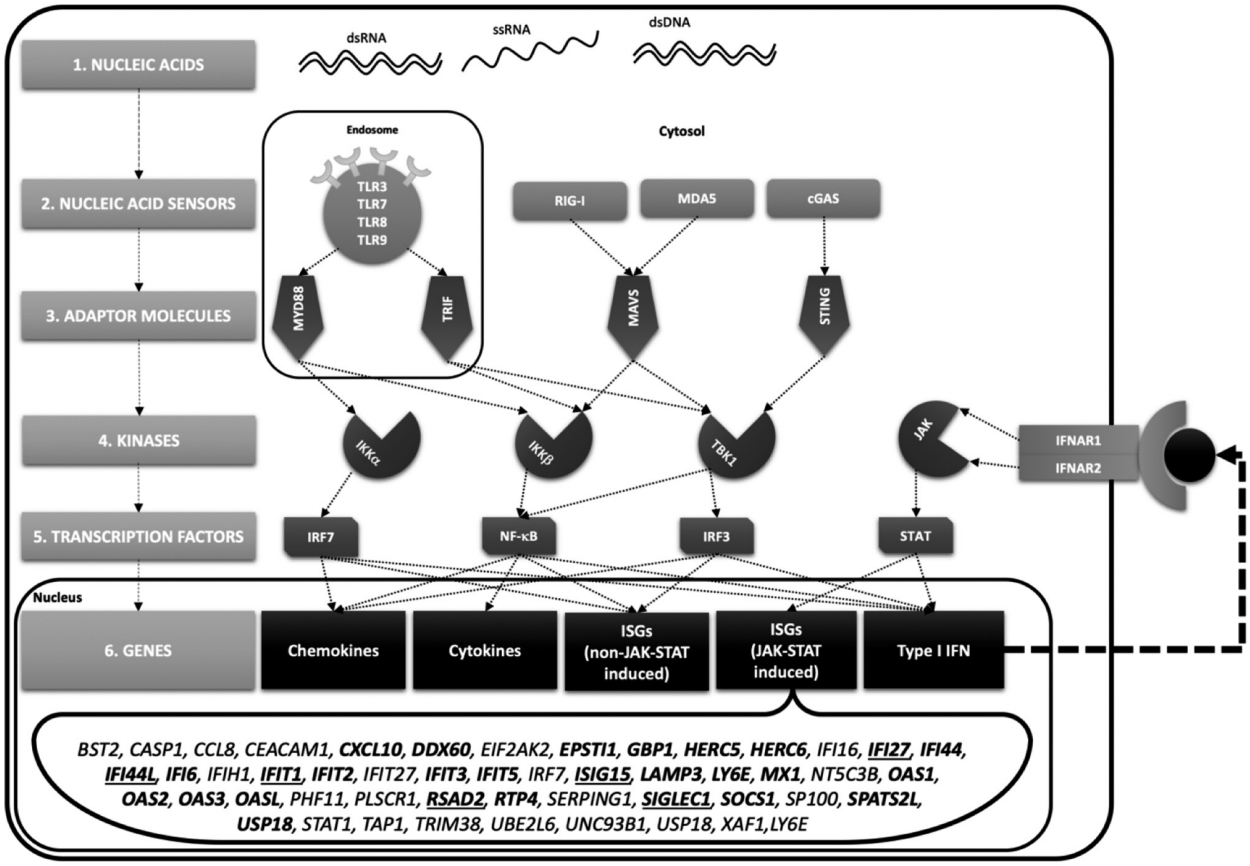

Fig. 1. Induction of type I interferons and interferon stimulated genes (ISGs) used for calculation of an interferon score (IS). Type I interferon (IFNα/β) is produced following the detection of intracellular nucleic acids by Toll-like receptors, RIG receptors and other nucleic acid sensors located in endosomes and the cytosol. These sensors initiate intracellular signalling via the recruitment of adaptor molecules (*e.g.* MYD88) and kinases (*e.g.* IKKα). Signalling results in the activation of specific transcription factors (namely IRFs and NFκB) and the expression of type I IFN as well as chemokines, cytokines and some interferon-stimulated genes (ISGs). Autocrine signalling ensues when secreted type I IFN is bound by cell surface IFNAR receptors and results in activation of JAK-STAT signalling and enhanced induction of more than 200 ISGs. The expression of ISGs utilised in the calculation of IFN scores is described in Table II; commonly it includes 6-genes (underlined) or 28-genes (bold).
dsRNA: double-strainded ribonucleic acid; ssRNA: single-strained ribonucleic acid; dsDNA: double-strained deoxyribonucleic acid; cGAS: cyclic guanosine monophosphate/adenosine monophosphate synthase; IFN: interferon; IFNAR: IFNα/β receptor; IKK: IκB kinase; IRF: interferon regulatory factor; ISGs: IFN stimulated genes; JAK: janus kinase; NF-κB: nuclear factor-κB; MAVS: mitochondrial antiviral-signalling protein; MDA5: melanoma differentiation-associated protein 5; MYD88: myeloid differentiation factor 88; RIG-I: retinoic acid-inducable gene I; STING: stimulator of IFN genes; TBK1: TANK-binding kinase 1; TLR: toll-like receptor; TRIF: TIR-domain-containing adapter-inducing IFNβ; STAT: signal transducer and activator of transcription.

tious progeny, which is an advantageous biosafety feature of these constructs (44, 45). While these bioassays infer biologic activity of type I IFN and enable investigations on type I IFN in the research laboratory, the methods are often not feasible in hospital laboratories due to associated cost and time (43, 46).

Alongside the development of antiviral activity assays, reporter gene assays (RGA) were established to quantitate type I IFN based on the ability of these cytokines to upregulate several hundred ISGs (47). Most common RGA constructs include HeLa, HeLa-derived cells (*e.g.* Wish) and Vero cells transfected with a plasmid carrying the luciferase gene under the control of a type I IFN inducible promoter (48, 49). Recently, cell lines like RAW-Blue ISG and B16-Blue IFNα/β have been engineered. In response to type I IFN, these cells produce a soluble gene product (*e.g.* secreted embryonic alkaline phosphatase) that can be quantitated using multi-well plate spectrophotometers or luminometers (50). Although this method provides a possible alternative for measuring type I IFN, it might still not be applicable to clinical settings due to lack of standardisation and complexity.

Interferon signature and score

Quantitative PCR (qPCR) and more recently NanoString technology have been used most extensively to evaluate differential expression of subsets of ISGs in whole blood and, to a lesser extent, in disease-related tissue (Table II) (51-53). A set of interferon-inducible genes, subsequently referred to as an 'IFN signature', was first defined in SLE patients using oligonucleotide arrays (54, 55). The signature was validated in other SLE cohorts and reported in various rheumatic diseases including but not limited to DM, SSc, RA and SS (56-65). As expected, an IFN signature was present in AGS patients (66). The six ISGs (*IFI27, IFI44L, IFIT1, ISIG15, RSAD2, SIGLEC1*) with the highest differential expression in AGS patients, when compared to healthy individuals, were used to calculate a type I 'IFN score (IS)' based on their relative magnitude of expression (52, 66). This six-gene assay was prospectively used

Table I. Methods available for indirect and direct detection of type I interferon (IFN) with attributes and selected studies relevant to uptake into clinical practice.

	Description	Pros (+)	Cons (-)	Studies
Immunoassays - direct				
ELISA	Standard sandwich ELISA with antibody capture	Ready-to-use kit; high throughput; rapid (~4h)	Not sensitive to biologic type I IFN concentrations; limited type I IFN subtype specificity	(30) (31) (32)
Single molecule array (Simoa) digital ELISA	ELISA based method with capture on magnetic beads by patient-derived antibodies	Sensitive to biologic concentration and specific for all type I IFN subtypes; high throughput	Requires special equipment; not well established	(33) (34) (35) (36)
Bioassays / Functional assays - indirect				
Antiviral activity				
Cytopathic protection assay	Cell-based assay with IFN suppression of the cytopathic effect of an infectious virus	Measures type I IFN bioactivity; robust	Time-consuming (>24h); high intra-assay variability; requires biosafety containment; complex	(37) (38) (41) (42)
Recombinant replicon assay	Cell-based assay with IFN inhibition of viral replication	Measures type I IFN bioactivity	Time-consuming (>24h); complex	(43) (46)
Transcription-stimulating activity				
Gene reporter assay	Cell-based assay with IFN-inducible genetic reporters of IFN activity	Simple to preform; high throughput; wide dynamic range	Lacks standardisation	(47) (48) (49) (50)
Interferon stimulated genes (ISGs) expression assay	qPCR or NanoString technology to quantitate ISGs expression	Reproducible; readily available (qPCR); high throughput; already used in many clinical studies	No consensus on ISGs subset for interferon score (IS) calculation	See Table II

to measure type I IFN activity in 82 patients with mutations in one of the genes known to be related to AGS (*TREX1*, *RNASEH2A*, *RNASEH2B*, *RNASEH2C*, *SAMHD1* and *ADAR*) (52). The IS was positive in 90% of the AGS patients and its positive or negative status correlated with patients' serum interferon activity measured by a viral cytopathic assay in 12 of 14 paired samples (52). Neutralisation assays with anti-IFNα and anti-IFNβ antibodies suggested that measurable anti-viral activity was primarily attributed to IFNα (52).

NanoString Technology, which uses molecular "barcodes" to detect and simultaneously count up to several hundred unique target gene sequences in a single hybridisation reaction, has enabled quantification of larger subsets of ISGs (typically 28 genes) rapidly and reproducibly (51, 67). The 28 target genes were selected from whole blood microarray expression profiles of two patients, one with CANDLE and one with chronic hepatitis C following IFNα therapy. The panel was subsequently validated in treatment-naive patients with genetically defined disease mediated by type I IFN (CANDLE, n=11; SAVI, n=7) or IL-1 (NOMID, n=16) and healthy individuals (n=26). An IS calculated from those 28 genes differed significantly between CANDLE and SAVI compared with NOMID and healthy individuals. There was a high correlation between repeated measurements and no variance in gender and age, or diurnal variations in a small number of tested samples. While it has yet to be determined whether there is a benefit of analysing a more extensive 28 gene panel compared to smaller subsets of 5-6 ISGs, it has recently been shown that both NanoString and qPCR measurement of 6 ISGs provides a similar result in terms of analytical performance (68).

In addition to the calculation of an interferon score from the expression of single sets of genes, a two-score system (IFN-score-A and -B) has been developed (69). In this system, the IFN-A and -B score include a separate set of genes (with some genes overlapping) selected from 31 ISGs associated with SLE. The scores were validated in a cohort of 279 patients with SLE, RA and undifferentiated connective tissue disease (UCTD), as well as in 49 healthy individuals. Values of both scores varied according to diagnosis and were associated with cutaneous and hematological activity in SLE patients. IFN-A score differentiated SLE patients from both RA patients and healthy individuals, while IFN-B score differentiated both SLE and RA from healthy individuals, indicating calculation of IS based on more than one set of genes could have a better disease specificity.

Even though both the interferon signature and score appear to be reliable readouts for type I IFN activity, similary to bioassays that utilise serum, they include some confounding factors such as differing abundance of circulating blood cell subpopulations (that naturally vary between individuals and can be altered substantially by certain medications). In addition, cell-specific

Table II. Selected major studies that apply various interferon score (IS) methodology in children and adults with rheumatic diseases.

Diseases	Technology	Genes	IS calculation	Studies
Adult patients				
SLE	qPCR	*LY6E, OAS1, OASL, MX1, ISG15*	Sum of standardised gene expression in patient relative to healthy individuals	(63)
SLE	Microarray qPCR	*EPSTI1, HERC5, IFI27, IFI44, IFI44L, IFI6, IFIT1, IFIT3, ISG15, LAMP3, LY6E, MX1, OAS1, OAS2, OAS3, PLSCR1, RSAD2, RTP4, SIGLEC1, SPATS2L USP18*	Median fold change of gene expression in patient relative to healthy individuals	(64)
SLE DM SSc RA	Microarray qPCR	*IFIT27, IFI44, IFI44L, IFI6, RSAD2*	Median fold change of gene expression in patient compared to healthy individuals	(65)
SLE RA	qPCR	*ISG15, IFI44, IFI27, CXCL10, RSAD2, IFIT1, IFI44L, CCL8, XAF1, IFI6, GBP1, IRF7, CEACAM1, HERC5, EIF2AK2, MX1, LAMP3, IFIH1, PHF11, SERPING1, IFI16, BST2, SP100, NT5C3B, SOCS1, TRIM38, UNC93B1, UBE2L6, STAT1, TAP1, CASP1*	2-score system (A and B) calculated from mean gene expression of two set of genes	(77)
Paediatric patients				
AGS IFNp [1] jSLE jDM sJIA COPA	qPCR NanoString	*IFI27, IFI44L, IFIT1, ISIG15, RSAD2, SIGLEC1*	Median fold change of gene expression in patient compared to healthy individuals	(52) (53) (74) (68)
FCL DADA2	qPCR	*IFI27, IFI44, IFI44L, IFIT1, ISIG15, RSAD2, SIGLEC1*	Fold change of gene expression relative to the to mean expression of healthy individuals	(75) (76)
CANDLE SAVI JIA jSLE DADA2	NanoString	*CXCL10, DDX60, EPSTI1, GBP1, HERC5, HERC6, IFI27, IFI44, IFI44L, IFI6, IFIT1, IFIT2, IFIT3, IFIT5, ISG15, LAMP3, LY6E, MX1, OAS1, OAS2, OAS3, OASL, RSAD2, RTP4, SIGLEC1, SOCS1, SPATS2L, USP18*	Sum of each gene count z-score relative to the mean and SD of healthy individuals and/or geomean of each gene count alone	(51)

[1] Interferonopathy patients with confirmed mutations in *TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR1, IFIH1, ACP5, TMEM173, C1Q, C2, ISG15, SKIV2L*.

SLE: systemic lupus erythematosus; jSLE: juvenile systemic lupus erythematosus; DM: dermatomyositis; jDM: juvenile dermatomyositis; SSc: systemic sclerosis; sJIA: systemic juvenile idiopathic arthritis; RA: rheumatoid arthritis; AGS: Aicardi-Goutières syndrome; IFNp: type I interferonopathies; FCL: familial chilblain lupus; DADA2: deficiency of adenosine deaminase 2; CANDLE: chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature syndrome; SAVI: STING-associated vasculopathy with onset in infancy.

ISGs expression and initiation of the JAK-STAT pathway by other stimuli (including other IFN and cytokines) cannot be excluded (69, 70). This might be particularly important for patients treated with biological agents, most notably IL1 inhibitors, since the clinical trial of anakinra in patients with systemic-onset juvenile idiopathic arthritis (sJIA) suggested de novo induction of ISGs and a positive IFN signature (71). Nevertheless, further studies are needed to examine this association.

Clinical application of IFN signatures and score in rheumatic diseases

Initial studies of type I IFN dysregulation were focused mainly on SLE patients (Table II). They showed that IFNα not IFNβ is the predominant inducer of ISGs expression, as well as correlation of high ISGs expression with increasing disease severity (72, 73). Five ISGs (*LY6E, OAS1*, *OASL, MX1* and *ISIG15*) were investigated by Feng *et al*. in 48 SLE patients, 22 patients with other rheumatic diseases (RA, n=14; Wegener Granulomatosis, n=8) and 48 healthy individuals (63). The magnitude of differential ISGs expression was highest in those SLE patients with active renal disease, as well as in patients with anti-dsDNA antibody positivity and hypocomplementemia. In another study, a type I IFN score was calculated from the expression of a different set of five ISGs (*IFIT27, IFI44, IFI44L, IFI6, RSAD2*) and was positive in 262 patients with SLE, as well as patients with RA (n=89), DM (n=44), polymyositis (PM) (n=33) and SSc (n=28) (65). The score correlated well between whole blood and disease-affected tissue (*i.e*. skin and muscle; for SLE, DM, PM and SSc patients), as well as with disease activity (SLE, PM and SSc patients) and ANA (DM patients) or ANA subtype measurements (SLE patients) (65). Subsequent studies have used a variety of ISGs to evaluate the association of IFN signatures and scores with a risk for disease development (for *e.g*. seropositive arthralgia patients before the development of arthritis, SSc patients before de-

velopment of skin fibrosis), presence of pathognomonic antibodies (*e.g.* ANA), disease activity and/or response to treatment in adults with rheumatic diseases (*e.g.* SLE, DM, SSc, RA and SS) (56-62). All these studies revealed a great potential of interferon signature and/or score for clinical application in various rheumatic diseases in adults.

In children, quantification of the expression of six ISGs (*IFI27, IFI44L, IFIT1, ISIG15, RSAD2, SIGLEC1*) was used to calculate and compare an IS in 992 samples from 489 patients with genetically confirmed or clinically well-defined inflammatory phenotype and 141 healthy adults and children (53). The expression of all six genes was consistently upregulated in patients with mutations in interferonopathy-associated genes (*TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR1, IFIG1, ACP5, TMEM173, C1Q, C2, ISIG15* and *SKIV2L*), patients with phenotypic features of an interferonopathy but negative for genetic mutations, and patients with mutations in genes currently not associated with interferonopathies (*DNASE1L3*, *PRKDC*, *CECR1*, *RNASET2* and *TRNT1*). The results were consistent in a subsequent study which showed that IS calculated from those six genes has a strong predictive value for diagnosis of type I Interferonopathies (68). A positive IS was also present in patients with juvenile SLE (jSLE), juvenile DM (jDM) and systemic juvenile idiopathic arthritis (sJIA) (53). Using the same or slightly modified sets of ISGs, the involvement of type I IFN has also been suggested in paediatric and adult patients with COPA syndrome, familial chilblain lupus (FCL), STING-associated vasculopathy with onset in infancy (SAVI) and deficiency of ADA2 (DADA2) (74-76).

Interestingly, the expression of five ISGs (*EPSTI1, IFI44L, LY6E, OAS3, RSAD2*) by NanoString technology was sufficient to demonstrate an increased interferon score in ANA positive individuals failing to fulfill diagnostic criteria for an associated rheumatic disease (SLE, SS, SSc, DM and mixed connective tissue disease). It was not however predictive of clinical progression to rheumatic disease during the one-year follow-up (17).

Conclusion

Various methods have been used over the years to assess activation of type I IFN in rheumatic diseases. Those methods can be categorised as direct measures of type I IFN concentration and indirect measures of type I IFN inferred from antiviral activity or induced gene expression. A number of research grade studies have shown that measures of type I IFN can aid diagnosis of patients with monogenic type I interferonopathies and correlate with disease activity in these and other, more complex, rheumatic disorders. The development of reliable clinical grade tests for type I IFN is critical for diagnosis and monitoring of disease severity, as well as treatment efficacy in these patient populations. Current methodologies, while promising, still require rigorous testing in a clinical setting.

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