Cytokine 113 (2019) 446–452

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

# Comparison of RT-qPCR and Nanostring in the measurement of blood interferon response for the diagnosis of type I interferonopathies

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ARTICLE INFO

*Keywords:*
IFN score
IFN signature
Nanostring
ISG
Interferonopathy

ABSTRACT

Type I interferonopathies are characterized by an increase of circulating type I interferon (IFN) concentration. Type I interferonopathies refer to rare Mendelian genetic disorders such as Aicardi-Goutières Syndrome (AGS) as well as more frequent and polygenic auto-immune diseases like systemic lupus erythematosus (SLE). Yet, detection of type I IFN in these patients remains challenging as its amount is usually very low in patients' sera. Thus, the detection of interferon-stimulating genes has been proposed as an alternative for the detection of this cytokine but sensitivy, specificity and predictive values of the assay have not been reported so far. In this study, we propose two different methods based on Nanostring or RT-qPCR to measure in the clinical routine the IFN response, defined as a set of transcripts that are systemically induced by IFNs. The IFN signature is composed of 6 IFN stimulated genes (ISGs) and has a strong predictive value for the diagnosis of type I interferonopathies. The use of this simple test might represent a gold standard for the evaluation of various autoimmune diseases. Moreover, this test could also be used to monitor patients treated with drugs targeting type I IFN pathway. When comparing both methods - Nanostring and qPCR - in terms of analytical performance, they provided similar results but Nanostring was quicker, easier to multiplex, and almost fully-automated, which represent a more reliable assay for the daily clinical practice.

## 1. Introduction

Since their discovery in 1957 by Isaacs and Lindenmann [1], interferons (IFN) have been classified in 3 groups (type I, II and III) depending on their receptor binding preferences. Type I IFNs are the most represented family, including 13 IFNα and 1 IFNβ in humans [2]. These cytokines are mainly secreted by plasmacytoid dendritic cells [3] following the activation of intracellular molecular pathways downstream PRR (pattern recognition receptors) like TLR [4] (toll-like receptors), RLR [5] (RIG-I-like receptors), NLR (NOD-like receptors) or cGAS [6] (cyclic GMP-AMP synthase). Type I IFNs exert an antiviral and anti-tumor activity through JAK/STAT pathway activation downstream their heterodimeric IFNA Receptor [7] resulting in the transcription of hundreds of Interferon-Stimulated Genes (ISGs) [8]. The secretion of type I IFN is essential for the development of innate immunity and viral clearance but an excessive secretion could be detrimental and promote

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https://doi.org/10.1016/j.cyto.2018.10.023
Received 17 June 2018; Received in revised form 3 October 2018; Accepted 29 October 2018
Available online 07 November 2018

auto-immune process [9]. The key role of this pro-inflammatory cytokine in the pathophysiology of auto-immune diseases like systemic lupus erythematosus (SLE) [10], dermatomyositis (DM) [11,12], Gougerot-Sjögren syndrome [13] or scleroderma [14] is now well-established. More recently, a group of mendelian disorders due to mutations in genes involved in nucleic acid metabolism or PRR causing an excessive secretion of type I IFN has been classified as type I interferonopathies. Aicardi-Goutières syndrome (AGS) [15], STING-associated vasculopathy with onset in infancy (SAVI) [16], familial chilblain lupus [17], spondyloenchondrodysplasia (SPENCD) [18], proteasome-associated auto-inflammatory syndrome (PRAAS) [19] and Singleton-Merten syndrome (SMS) [20,21] are the main genetic type I interferonopathies described. Tetrasomy 9p is a very rare genetic disorder arising from a duplication of the short arm of the chromosome 9. As genes encoding for type I IFN are all located on this chromosome, tetrasomy 9p leads to an upregulation of type I IFN transcription in patients [22,23]. All these diseases present a range of non-specific symptoms including neurologic and cutaneous features like chilblain that can be inaugural or present within the first months of life. This set of diseases is characterized by an increase of circulating type I IFN. However, conventional ELISA techniques or inhibition of viral cytopathic effect fail to detect these cytokines (commercial ELISA kits can detect pg/mL of IFNα for the most sensitive) as they are expressed at very low serum concentration (fg/ml). An alternative solution is to evaluate exposition to IFN through the measurement of ISGs expression in circulating leukocytes. This assay has demonstrated a high sensitivity for type I interferonopathies diagnosis and other auto-immune diseases like SLE or DM [15].

The aims of this study were (1) to set up a clinical routine test to measure the interferon response and (2) to compare the results obtained with 2 techniques: RT-qPCR and Nanostring. For that, we quantified the expression of 6 ISGs previously found to reflect the overall exposure to IFNs in patients with Aicardi-Goutieres syndrome (AGS) [15], SLE and other interferonopathies [24]. Whereas RT-qPCR is a widely used technique, Nanostring is a novel digital barcode technology for direct multiplexed measurement of analytes which offer high levels of precision and sensitivity (< 1 copy per cell [25]). This technique is less time-consuming and more adapted to a routine use in a hospital laboratory.

For each technique an “IFN score” was calculated that consists in the median expression of the different ISGs and which can be used as a single diagnostic or prognostic value. The sensitivity and the negative predictive values of this IFN score in the diagnosis of type I interferonopathies were assessed in a cohort of pediatric patients hospitalized in rheumatology, neurology or nephrology department.

## 2. Material and methods

### 2.1. Participants

This study was carried out in pediatric patients hospitalized in rheumatology, neurology or nephrology department. Patient’s diseases were classified into two groups: interferonopathy or non-interferonopathy. JIA (juvenile idiopathic arthritis), vasculitis and undefined auto-inflammatory diseases were included in the non-interferonopathy group since a consistent upregulation of type I IFNs has not been reported in these diseases.

Control samples were collected from the Etablissement Français du Sang (Gerland, Lyon, France) from men or women of different ages without any medical condition or current treatment.

### 2.2. IFN score assessment

Total RNA extraction was performed on whole blood collected on EDTA using a Maxwell® 16 LEV SimplyRNA Blood Kit (Promega) and a magnetic particle processor (Maxwell 16; Promega) according to the manufacturer recommendations. The extraction kit included an individual DNAse treatment. Total RNA was diluted in 40 µL RNAse free water and concentration was quantified by spectrophotometry using a NanoVue (Biochrom). The first strand of cDNA was synthetized from 1 µg of total RNA using a SuperScript® VILO cDNA Synthesis Kit and Master Mix (Invitrogen).

**Table 1**
Primers used for ISGs and housekeeping genes quantification by RT-qPCR.

| Primer | Gene | Accession | Sequence |
|---|---|---|---|
| SIGLEC1 F | SIGLEC1 | NM_023068.3 | TGCTCACAGCATGGAAAAGT |
| SIGLEC1 R | | | TGTTGAGCAAGTTCTCCGTAGT |
| IFI27 F2 | IFI27 | NM_005532.3 | GTGGCCAAAGTGGTCAGG |
| IFI27 R2 | | | CCAATCACAACTGTAGCAATCC |
| IFI44L F2 | IFI44L | NM_006820.2 | TTGTGTGACACTATGGGGCTA |
| IFI44L R2 | | | GAATGCTCAGGTGTAATTGGTTT |
| IFIT1 F2 | IFIT1 | NM_001548.3 | GCCTAATTTACAGCAACCATGA |
| IFIT1 R2 | | | TCATCAATGGATAACTCCCATGT |
| ISG15 F2 | ISG15 | NM_005101.3 | GAGGCAGCGAACTCATCTTT |
| ISG15 R2 | | | AGCATCTTCACCGTCAGGTC |
| RSAD2 F | RSAD2 | NM_080657.4 | TGCTTTTGCTTAAGGAAGCTG |
| RSAD2 R | | | AGGTATTCTCCCCGGTCTTG |
| OAZ FqP | OAZ | NM_004152.2 | GGATAAACCCAGCGCCAC |
| OAZ RqP | | | TACAGCAGTGGAGGGAGACC |
| GAPDH F | GAPDH | NM_002046.6 | CACCCACTCCTCCACCTTTGAC |
| GAPDH R | | | GTCCACCACCCTGTTGCTGTAG |
| Actin F1 | ACTB | NM_001101.4 | CCAACCGCGAGAAGATGA |
| Actin R1 | | | CCAGAGGCGTACAGGGATAG |

qPCR assays were run in duplicate in 96-well plate using a FastStart Universal SYBR Green Master (Rox) kit (Roche). Standard curves were prepared for each gene generated by 10-fold serial dilutions in order to determine the efficiency (E) of each primer. We studied 6 ISGs that are among the highest ISGs expressed in AGS patients [15]: SIGLEC1 (sialic acid binding Ig like lectin 1), IFI27 (interferon alpha inducible protein 27), IFI44L (Interferon induced protein 44 like), IFIT1 (interferon induced protein with tetratricopeptide repeats 1), ISG15 (interferon-stimulated gene 15) and RSAD2 (radical S-adenosyl methionine domain containing 2). The expression of each target was first calculated using the formula $E^{-\Delta Ct}$ [26], and ISGs’ expression were normalized to the geometric mean of 3 housekeeping genes expression [27]: OAZ (ornithine decarboxylase antizyme), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-Actin (Table 1).

Concerning the Nanostring (NanoString Technologies) procedure, 200 ng of RNA were hybridized to the probes (a reporter probe and a capture probe) at 67 °C for 16–21 h using a thermocycler. Samples were then inserted into the nCounter Prep Station for the removal of excessive probes, purification and immobilization onto the internal surface of a sample cartridge for 2–3 h. Finally, the sample cartridge was transferred to the nCounter Digital Analyzer where color codes were counted and tabulated for each target molecule. Count number obtained for the 6 ISGs were normalized by the geometric mean of 3 housekeeping genes count number (β-Actin, HPRT1 (hypoxanthine phosphoribosyltransferase 1) and POLR2A (RNA polymerase II subunit A)) (Table 2) as well as the negative and positive controls values using nSolver software.

For each technique, the relative expression was determined for each normalized ISG expression dividing by the median normalized expression of each ISG from a control group. Finally, the median of these 6 ISG relative expression was used to calculate the IFN score [15].

The threshold was first defined as the mean of control group IFN score + 2SD corresponding to a score of 2,3 for both techniques.

### 2.3. Cytokine stimulation

Whole blood was stimulated with 40,000 UI/mL of IFNα (Peprotech), IFNγ (Peprotech) or PHA (Cellestis) for 24 h at 37 °C + 5% $CO_2$.

PBMCs (peripheral blood mononuclear cells) were isolated from

**Table 2**
Probes used for ISGs and housekeeping genes quantification with Nanostring technology.

| Probe | Gene | Accession | Sequence |
|---|---|---|---|
| Probe A | SIGLEC1 | NM_023068.3 | CAACACTGCCTCATTCACATTCATAGGCTGGAGTCATCACAGATTCTGCGAACCTAACTCCTCGCTACATTCCTATTGTTTTC |
| Probe B | | | CGAAAGCCATGACCTCCGATCACTCCATAAAAAGTCAGATGTCACAGAGCTGTTTTCGTAGAGGCGGGCAGGACT |
| Probe A | IFI27 | NM_005532.3 | GAGCCCAGGATGAACTTGGTCAATCCGGAGAGTCCAGTTGCCTGTTGAGATTATTGAGCTTCATCATGACCAGAAG |
| Probe B | | | CGAAAGCCATGACCTCCGATCACTCGGAGCTAGTAGAACCTCGCAATGACAGCCGCAATGGCAGACCCAATG |
| Probe A | IFI44L | NM_006820.2 | CTTCTGCCCCATCTAGCCCCATAGTGTCACACAACATAAATGGCAGAGATCAAAGACGCCTATCTTCCAGTTTGATCGGGAAACT |
| Probe B | | | CGAAAGCCATGACCTCCGATCACTCCATACAACCTTTTAAGATGTGGGGAATGTCATCCATGCACAGTCCTGCTC |
| Probe A | IFIT1 | NM_001548.3 | TGTAGACGAACCCAAGGAGGCTCAAGCTTTCCAGATCTAATGCCTTTCTCCCAATTTGGTTTTACTCCCCTCGATTATGCGGAGT |
| Probe B | | | CGAAAGCCATGACCTCCGATCACTCCAGGGCCCGCTCATAGTACTCCAGGGCTTCATTCATATTTCCTTCCAATT |
| Probe A | ISG15 | NM_005101.3 | CTCAGAGGTTCGTCGCATTTGTCCACCACCAGCAGGACCTTTCGGGTTATATCTATCATTTACTTGACACCCT |
| Probe B | | | CGAAAGCCATGACCTCCGATCACTCTGCTGCTGCGGCCCTTGTTATTCCTCACCAGGATG |
| Probe A | RSAD2 | NM_080657.4 | CCGTCCCTTTCTACAGTTCAGAAAGCGCATATATTCATCCAGAATAAGGTCAACAGCCACTTTTTTTCCAAATTTTGCAAGAGCC |
| Probe B | | | CGAAAGCCATGACCTCCGATCACTCTTTATAGCTTCTTCTACACCAACATCCAGGATGGACTTGGAAGGGTCCTT |
| Probe A | HPRT1 | NM_000194.1 | TGAGCACACAGAGGGCTACAATGTGATGGCCTCCCATCTCCTTCATCACACACCGTGTGGACGGCAACTCAGAGATAACGCATAT |
| Probe B | | | CGAAAGCCATGACCTCCGATCACTCCAGTGCTTTGATGTAATCCAGCAGGTCAGCAAAGAATTTATAGCCCCCCT |
| Probe A | POLR2A | NM_000937.2 | ACTGGCCCAACAGGAAGACAGTAAGCGAAGGAGTCTTTGGCTTCTTGGAACCTGGAGTTTATGTATTGCCAACGAGTTTGTCTTT |
| Probe B | | | CGAAAGCCATGACCTCCGATCACTCCAGACGGCACAGAATATCCTTGGCTCTCTCAGCATCTCGAGCGG |
| Probe A | ACTB | NM_001101.2 | GATCTTGATCTTCATTGTGCTGGGTGCCAGGGCAGTGATCTCCTTCTGCACAGATAAGGTTGTTATTGTGGAGGATGTTACTACA |
| Probe B | | | CGAAAGCCATGACCTCCGATCACTCAGGATGGAGCCGCCGATCCACACGGAGTACTTGCGCTCAGGAGGAGCAAT |

whole blood by FICOLL® density gradient centrifugation (a lymphocytes separation medium (Eurobio)).

PBMCs were stimulated with IFNα (40000 UI/mL; Peprotech) and with ruxolitinib (pill diluted in DMSO (dimethylsulfoxyde); Novartis) in RPMI (Roswell Park Memorial Institute) complete media.

## 3. Results

### 3.1. ISG induction by type I vs type II IFN and effect of a JAK1/JAK2 inhibitor

In a first set of experiments, the IFN response was assessed by RT-qPCR. As ISGs are induced both by type I and type II IFNs, we wanted to compare the IFN score obtained after PBMC stimulation with both cytokines *in vitro*. Whole blood cells from healthy donors were stimulated with IFNα, IFNγ or PHA. PHA is a T cell mitogen that stimulates IFNγ production. ISGs were quantified using RT-qPCR and the IFN score was calculated for each condition. As seen in Fig. 1A, we observed that IFNα induced a strong response with an about 100-fold increase of the IFN score compared to the negative control. On the other hand, stimulation of whole blood with IFNγ, and at a lower extent with PHA, leads to a lower response with about a 10-fold increase of the IFN score.

As JAK inhibitors are increasingly used in the treatment of connective tissue disorders including interferonopathies [28], we next wanted to determine the effect of the addition of ruxolitinib, a JAK1/JAK2 inhibitor, on the induction of ISGs by IFNα. For that, whole blood was incubated overnight with IFNα and with different concentrations of ruxolitinib. We observed that the addition of 0,1 nmol/mL or 1 nmol/mL of ruxolitinib markedly decreased the IFN score in a dose-dependent manner (Fig. 1B). Furthermore, the use of a 10 nmol/mL concentration resulted in a complete normalization of the IFN signature comparable to the unstimulated condition.

These data show that ISGs are weakly induced by type II IFN and that this IFN score is more sensitive for type I IFN. Moreover, our data suggest that the IFN score can be used to monitor the efficacy of drugs like JAK inhibitors in treated patients.

### 3.2. The IFN score has an excellent negative predictive value (NPV) for the diagnosis of type I interferonopathy in a pediatric cohort

To test the performance of our test by RT-qPCR, we then measured the IFN score on 176 samples from 141 different pediatric patients and 32 healthy controls. Patients were classified into two groups (Table 3): the interferonopathy group included 47 samples from 31 different patients and the non-interferonopathy group included 129 samples from 110 different patients. When a patient was analyzed more than once, we considered all results independently. In the interferonopathy group, IFI27 and RSAD2 were the most highly induced ISGs (Fig. S1). In this group, patients treated for SLE presented the highest IFN scores (Fig. 2A).

To assess the clinical relevance of the IFN score, we next designed a ROC (receiver operating characteristic) curve based on IFN scores

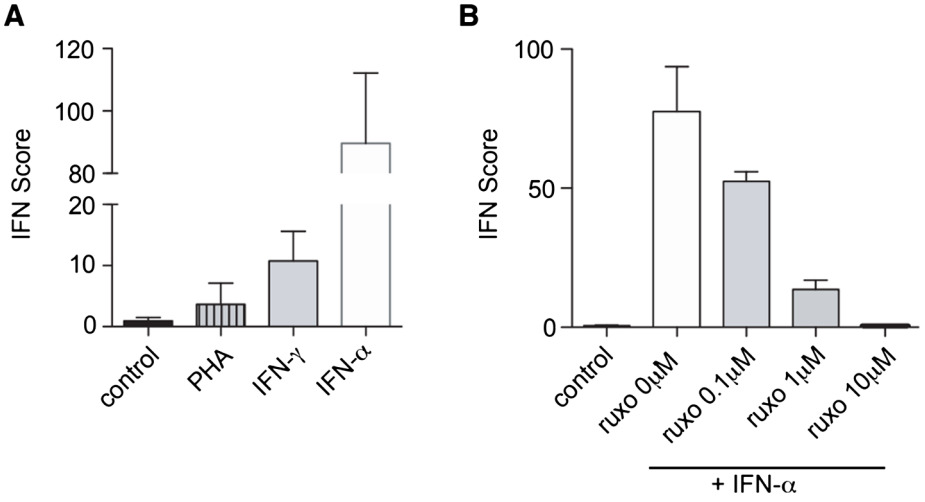


**Fig. 1.** IFN signature is more sensitive for type I IFN than type II IFN and is downregulated by pharmacological inhibition of JAK/STAT. (A) IFN score after stimulation with PHA, recombinant IFNα or IFNγ during24 hours. IFN score was calculated after RT-qPCR analysis as the median of six ISG relative expression compared to healthy controls. The data show the results of 3 different donors stimulated and analyzed independently. Bar graphs represent the means and error bars represent the standard deviations. (B) IFN score of healthy controls PBMCs stimulated with 40,000 UI/mL of IFNα +/− different concentrations of Ruxolitinib (0,1, 1 or 10 μM). IFN scores were calculated after RT-qPCR analysis as the median of six ISG relative expression compared to healthy controls. The data show the results of 3 different donors stimulated and analyzed independently. Bar graphs represent the means and error bars represent the standard deviations.

**Table 3**
Classification and IFN score of pediatric patients in interferonopathy and non-interferonopathy groups (SLE: systemic lupus erythematosus; DM: dermatomyositis; CTD: connective tissue disease; JIA: juvenile idiopathic arthritis). *Positive IFN score > 2,3.

| Diagnosis | | Number of samples/ patients | % Female (n) | Age (median, min–max) at time of sampling | Number (%) with positive IFN score* |
|---|---|---|---|---|---|
| Interferonopathy group | SLE | 26/18 | 83,3 (15) | 15,5 (6–53) | 24 (92,3) |
| | DM | 8/5 | 60,0 (3) | 9,5 (8–16) | 6 (75) |
| | Other CTD | 5/4 | 75,0 (3) | 11 (9–16) | 4 (80) |
| | Tetrasomy 9p | 2/1 | 100 (1) | 16 (16–16) | 2 (1 0 0) |
| | Monogenic interferonopathy | 6/3 | 66,7 (2) | 14 (1–14) | 6 (1 0 0) |
| Non-interferonopathy group | JIA patients | 19/15 | 60,0 (9) | 12 (7–19) | 9 (47,4) |
| | Other patients | 110/95 | 48,4 (46) | 11 (1–70) | 35 (31,8) |
| | Healthy controls | 32/32 | 34,4 (11) | 28 (23–61) | 1 (3,1) |

calculated with RT-qPCR data and patient classification (Table 3, Fig. 2B). The ROC curve had an AUC (area under the curve) of 0,856. In order to obtain a strong NPV, we established a threshold level at 2,3, which resulted in a sensitivity of 89,4%, a NPV of 95,9% and a specificity of 72,0%. This threshold was comparable to the calculated threshold based on the mean of the healthy control scores + 2SD (2,3). In the healthy controls group, only 1 sample showed a weakly positive IFN signature (3,2). In the interferonopathy group, out of 47 samples analyzed, 5 samples from 5 different patients were false negative (10,6%), but for one of these 5 patients a positive IFN score was measured from a second sample. In the non-interferonopathy group, 44 samples were falsely positive out of a total of 129 samples (34,1%).

### 3.3. IFN score measurement by Nanostring technology

The Nanostring is a new technology that could be more appropriate in the routine practice than RT-qPCR. In this context, we sought to compare the performance of these two techniques to measure the IFN score. The comparison between qPCR and Nanostring was made on 80 patient samples and 31 healthy controls. For Nanostring analysis, the interferonopathy group included 21 samples from 16 different patients and the non-interferonopathy group included 90 samples from 81 different patients. When a patient was analyzed more than once, all the results were analyzed. The correlation between Nanostring and RT-qPCR for the achievement of IFN signature was calculated on 111 samples (Fig. 3A and Fig. S2). The $R^2$ corresponding to the correlations of IFN score and relative expressions of each ISG were respectively 0,870 (IFN score), 0,885 (SIGLEC1), 0,818 (IFI27), 0,755 (IFI44L), 0,643 (IFIT1), 0,897 (ISG15) and 0,863 (RSAD2) (Table 4). Moreover, the reproducibility of this assay was demonstrated on 3 samples (1 negative, 1 medium and 1 high IFN score) tested on 3 different days (Fig. 3B). In the same way than RT-qPCR, the ROC curve obtained based on Nanostring data returned an AUC of 0,896 and we established a threshold level of 2,3 in order to keep a strong NPV. This threshold resulted in a sensitivity of 90,5%, a specificity of 63,3% and a NPV of 96,6% (Fig. 3C).

Two healthy controls scored weakly positive (2,6 and 2,8) and 8 patients had a negative IFN signature with RT-qPCR while it was weakly positive with Nanostring (2,4 to 3,5). Altogether, these results show that RT-qPCR and Nanostring-based techniques have similar performances (Table5).

## 4. Discussion

Since the discovery of interferons and despite the discovery of interferonopathies in the past years, no reliable, sensitive and automated method to quantify type I IFN has been developed. Direct dosage of type I IFNs is complicated since this family includes 14 members (13 IFNα and one IFNβ) with low circulating levels. The SiMoA (single molecule array) is an ultra-sensitive technique based on a digital ELISA assay which allows the dosage of IFNα subtypes at the protein level [29]. However, this technique is expensive and difficult to implement in a routine hospital lab. The IFN signature, initially developed by Y. Crow [15] and based on the quantification of selected ISGs reflects the exposure of leukocytes to all members of the type I IFN family. The measurement of the "IFN score" represents a credible and cost-effective

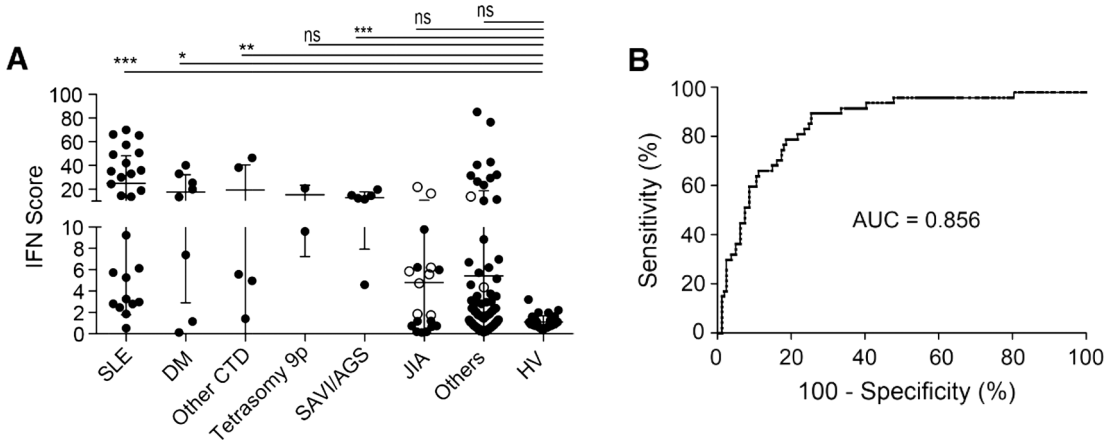


**Fig. 2.** SLE patients display the highest IFN score among the interferonopathy group (A) IFN score across interferonopathy and non-interferonopathy groups. White dots represent patients treated with anti-IL1 or anti-TNF therapy. IFN score was calculated after RT-qPCR analysis as the median of six ISG relative expression compared to healthy controls. Statistical analysis was performed using a Wilcoxon nonparametric test including a Bonferroni correction. (***p-value < 0,001; **p-value < 0,01; *p-value < 0,1; ns: non-significant). Bar graphs represent the means and the standard deviations. (B) ROC curve of the IFN signature assay based on RT-qPCR data and classification of patients (Table 3). This graph represent data of 47 interferonopathy samples and 161 non-interferonopathy samples. AUC = 0,856. With a threshold set at 2,3: Se = 89,4%, Sp = 72,0%, NPV = 95,9% and PPV = 48,3%.

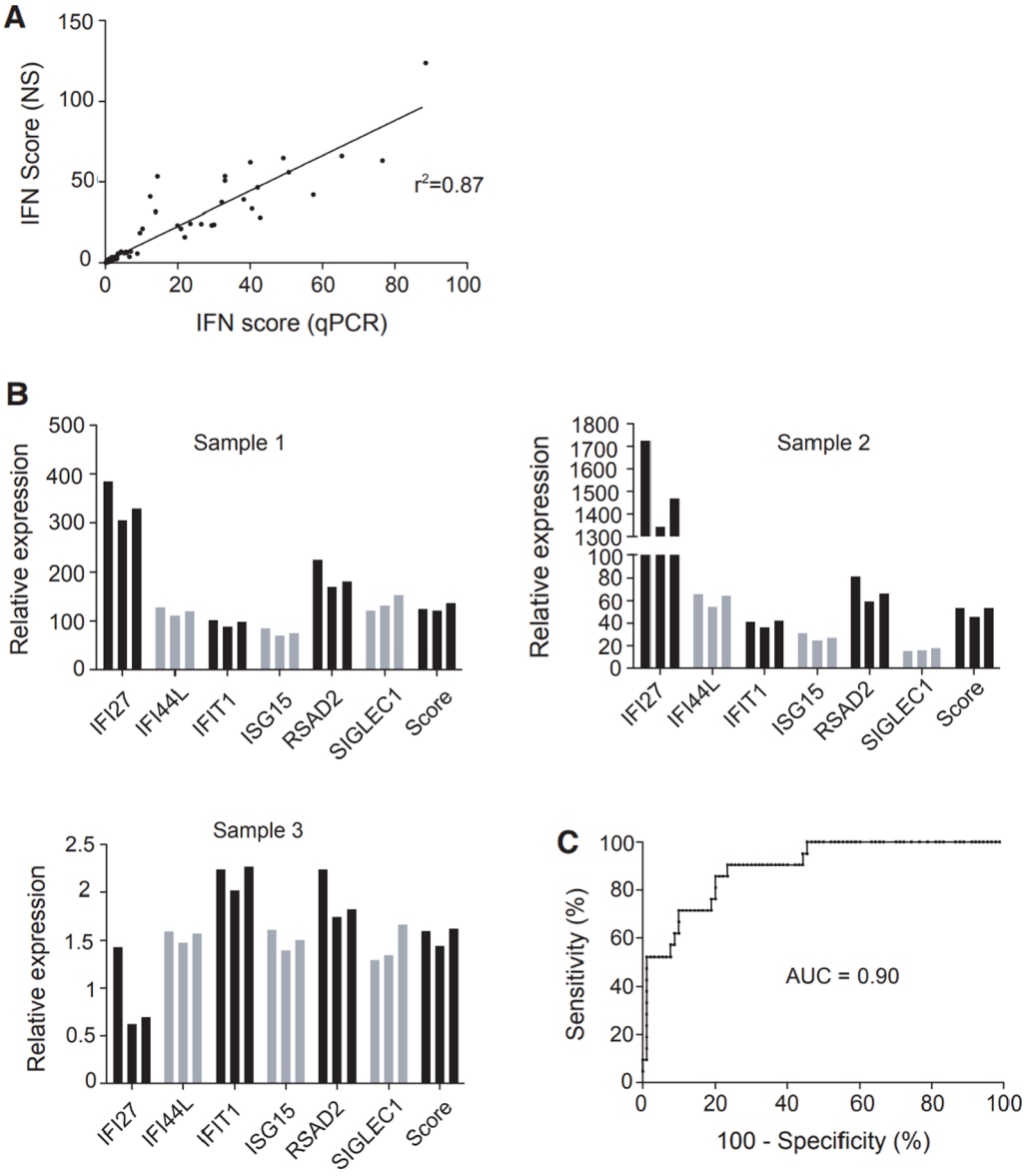


**Fig. 3.** Nanostring technology is a comparable method with RT-qPCR for IFN signature assay. (A) Correlation of IFN score calculated after RT-qPCR or Nanostring (NS) analysis on 111 samples. IFN score was calculated as the median of six ISG relative expression compared to healthy controls. $R^2$ = 0,8698. (B) Reproducibility of Nanostring analysis for ISG relative expression and IFN score on three different samples on three different days. C) ROC curve of the IFN signature assay based on Nanostring data and classification of patients (Table 3). This graph represents data of 21 interferonopathy samples and 90 non-interferonopathy samples. AUC = 0,896. With a threshold set at 2,3: Se = 90,5%, Sp = 63,3%, NPV = 96,6% and PPV = 36,5%.

**Table 4**
Correlation ($R^2$) between RT-qPCR and Nanostring technology for each ISG relative expression and IFN score (n = 111).

| ISG | $R^2$ |
|---|---|
| SIGLEC1 | 0,8848 |
| IFI27 | 0,8176 |
| IFI44L | 0,7554 |
| IFIT1 | 0,6426 |
| ISG15 | 0,8972 |
| RSAD2 | 0,8628 |
| IFN Score | 0,8698 |

**Table 5**
Comparison of RT-qPCR and Nanostring performances (AUC: Area Under the Curve; Se: Sensitivity; Sp: Specificity; NPV: Negative Predictive Value; PPV: Positive Predictive Value; SLE: Systemic Lupus Erythematosus; DM: Dermatomyositis; CTD: Connective Tissue Disease; JIA: Juvenile Idiopathic Arthritis).

| | | | RT-qPCR | Nanostring |
|---|---|---|---|---|
| AUC | | | 0,856 | 0,896 |
| Se (%) | | | 89,4 | 90,5 |
| Sp (%) | | | 72,0 | 63,3 |
| NPV (%) | | | 95,9 | 96,6 |
| PPV (%) | | | 48,3 | 36,5 |
| Positive IFN Score | Interferonopathy group | *SLE* | 24/26 | 11/12 |
| | | *DM* | 6/8 | 3/3 |
| | | *Others CTD* | 4/5 | 1/2 |
| | | *Tetrasomy 9p* | 2/2 | 2/2 |
| | | *Monogenic interferonopathy* | 6/6 | 2/2 |
| | Non-interferonopathy group | *JIA patients* | 9/19 | 2/7 |
| | | *Others* | 35/110 | 29/52 |
| | | *Healthy controls* | 1/32 | 2/31 |

alternative to the dosage of type I IFNs. Our results show that the selected ISGs are strongly induced by type I IFN and only weakly by type II IFN, suggesting that in most cases, the score reflects exposure to type I IFN and not type II IFN.

We found that the induction of ISGs in PBMC can be inhibited *in vitro* using JAK inhibitors like ruxolitinib. Ruxolitinib belongs to a new class of immunosuppressive drugs that target the type I IFN pathway and can decrease type I IFN concentration. This pharmacological class

comprises several drugs. For example, ruxolitinib, momelotinib or baricitinib are selective JAK1/JAK2 inhibitors while tofacitinib is a selective JAK1/JAK3 inhibitor. These drugs are differentially approved for the treatment of various diseases like myelofibrosis, rheumatoid arthritis or psoriasis and many others are currently tested in clinical trials. In this context, we propose that the IFN signature could be used as a biomarker to monitor these patients or to assess the efficacy of drugs during clinical trials [30].

In this study, we sought to compare RT-qPCR and Nanostring to perform this assay in routine. Compared to RT-qPCR, Nanostring technology does not require reverse transcription nor amplification of genetic material that are the two main causes of bias in the RT-qPCR. Moreover, the time required for the analysis is longer for the Nanostring than for RT-qPCR but involves much less technician time. Finally, as Nanostring is used for multiplex measurements of analytes, other interesting target genes like IL-1β, IL-6 or TNF-α can be easily added to the analysis.

The correlation between these two methods was strong for each of the ISGs and thus for the IFN score. Moreover, ROC curves established with each technique in our pediatric cohort demonstrated high sensitivity and negative predictive value for the diagnosis of interferonopathies. However, the specificity was somewhat weaker due to different causes of false positive. Type I IFN are pro-inflammatory, antiviral and antitumoral cytokines, thus acute (and some chronic) viral infections can induce positive signature. For example, in our cohort, a patient infected by influenza A virus presented the highest IFN score (85) among the non-interferonopathy group. Unfortunately, the IFN signature could not have been verified after virus eradication for this patient. Another child with chronic enterovirus infection demonstrated a positive IFN signature on 3 different samples. So, in case of a positive IFN signature measured during an acute viral infection, the analysis should be repeated after virus eradication. Similarly, in some types of cancer, malignant cells or intratumoral DCs can secrete type I IFN and induce an increase of type I IFN signature [31]. In systemic JIA patients (pediatric chronic inflammatory disease mediated by IL-1), signatures were generally negative, but a treatment with anti-IL1 molecules (anakinra) was sometimes associated with an increase of the IFN score. This observation was previously reported in other publications, suggesting an antagonism between IL and 1 and type I IFN [24,32]. This antagonism could occur through 2 different mechanisms: IFNα signaling, *via* STAT1 transcription factor, could suppress caspase-1-dependent IL-1β maturation, and IFN-α could induce the production of IL-10 in a STAT1-dependent manner in which IL-10 reduces the abundance of IL-1β [33]. In the same way, the onset of anti-nuclear antibodies and lupus symptoms have been frequently reported in patients treated with TNFα antagonists [34,35]. In our cohort, a patient treated with etanercept for a Behçet disease displayed a positive IFN signature (23,5) coupled with the onset of a Vespertilio erythema. The interruption of etanercept resulted in the negativity of the IFN signature 2 months later.

In the interferonopathy group, most patients were already treated with corticosteroids, hydroxychloroquine, methotrexate or other immunosuppressive drugs that could decrease IFN score [36,37] and lead to a false negative result. Among the 5 false negative results obtained in our cohort, one patient was treated with rituximab, an anti-CD20 monoclonal antibody, corticosteroids and hydroxychloroquine, a second with hydroxychloroquine and mycophenolate mofetil, another with mycophenolate and the last with corticosteroids and methotrexate. Two of these patients were followed for lupus nephritis and neurolupus in total remission at the time of sampling, suggesting an effect of the treatment on the IFN signature, while the 2 others were followed for juvenile dermatomyositis. Furthermore, the patient treated for lupus nephritis presented a positive IFN score on a second sample upon an acute phase of the disease. The last false negative patient was the only case of scleroderma. As far as possible, the IFN signature should be performed before the initiation of an immunosuppressive treatment and throughout an acute phase of the disease.

## 5. Conclusion

In conclusion, we describe in this study two comparable methods, RT-qPCR and Nanostring, to measure in routine exposure to IFNs. The panel of ISGs we used for the IFN signature includes less target genes than the one developed by Kim et al. [38] and confirm their results. Moreover, we compared the Nanostring to the reference method, RT-qPCR, for the realization of this analysis. We also assessed the sensitivity, specificity, NPV and PPV of this test in a pediatric cohort for the diagnosis of type I interferonopathies. The strong NPV of this test might avoid useless and expensive genetic explorations (whole exome sequencing) for patients with 2 successive negative signatures throughout an acute phase of the disease and without any immunosuppressive treatment. Furthermore, a positive IFN signature measured in the absence of viral infection could help in the early detection of type I interferonopathy since there is no other sensitive test to detect an increase of type I IFN concentration to date. Finally, the measurement of the IFN signature could be of interest to monitor patients treated by drugs targeting type I IFN pathway whose application should increase in the next years and for whom a good response to therapy is generally associated with a decrease of the IFN signature [39]. Further studies are needed to confirm this point.

## Acknowledgments

We thank the patients and healthy donors who participated to this study and the core facilities of the Lyon-Sud Hospital. The T.W. lab is supported by the FINOVI foundation, Agence Nationale de la Recherche, European Research council (ERC-Stg 281025), INSERM, CNRS, Université de Lyon, and ENS de Lyon.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.10.023.

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