Exploring the Interferon Signature

Laboratory Elaboration and Clinical Perspectives

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Abstract

Type 1 interferonopathies encompass a group of autoinflammatory diseases in which overactivation of the type I interferon pathway is the keystone. In this article, we describe the elaboration and clinical aspects of the interferon signature (or score) at the level of these rare monogenic diseases, but also in more common rheumatologic conditions, discuss its potential implications for therapeutic options for patients, and open future perspectives of transcriptomic analysis in immunoinflammatory diseases.

Introduction

Type I interferon activity

Interferons (IFNs) are a group of cytokines involved in defence against viral infections of host cells by inhibiting viral replication, but also involved in protection against intracellular bacteria (e.g. mycobacteria for type II IFNs). IFNs are classified into three families: type I (IFNα/β/ε/τ/κ/ω/δ/ζ), type II (IFNγ) and type III (IFNλ) (1-3).

Type I IFNs, IFN-α and IFN-β, are the major cytokines of the host immune response. They are produced by most cell types. On one hand, they can be synthesised by stimulating pattern recognition receptors (PRRs) of the innate immune system, such as Toll-like receptors (TLRs), which recognise danger signals (Pathogen-Associated Molecular Patterns (PAMPs) constitutive of pathogens (e.g. nucleic acids or viral envelope glycoproteins) or Damage-Associated Molecular Patterns (DAMPs) released by damaged cells (e.g. stress signals such as cell death-related self-DNA or damaged proteins)). On the other hand, type I IFNs can be produced by other intracellular signalling pathways, such as the STING pathway or the helicase pathway, which are activated in infected cells (3-5). Once synthesised, these type I IFNs can act by binding specifically to their receptor: the Interferon-alpha/beta receptor (IFNAR1/IFNAR2). This binding activates the JAK/STAT (Janus kinases/signal transducers and activators of transcription) signalling pathway. This leads to the formation of a complex (ISGF3: Interferon-stimulated gene factor 3), which acts as a nuclear transcription factor that can induce the expression of ISGs (Interferons Stimulated Genes). These genes enable the production of antiviral proteins and pro-inflammatory cytokines (Figure 1) (4).

Molecular Mechanisms of Type 1 Interferonopathies

These IFNs therefore exert antiviral and antitumoral activity, via the JAK/STAT pathway, which is essential for the host, but overexpression of type I IFNs can be associated with autoinflammatory and possibly autoimmune processes giving rise to pathologies grouped under the term type 1 (6). Major groups encompass Aicardi-Goutières spectrum syndromes (AGS), familial chilblain lupus and different monogenic lupus, spondyolenchondrodysplasia, STING-associated vasculopathy with onset in infancy (SAVI) and COPA-associated inflammatory syndrome (COPA), proteasome-related autoinflammatory syndromes (PRAAS) (4, 5). These type 1 interferonopathies are rare monogenic genetic diseases characterised by autoinflammatory and autoimmune disorders due to the constitutive activation of the type 1 interferon antiviral axis signalling or a defect in its negative feedback control (5, 7). In the case of AGS and familial chilblain lupus, constitutive activation of signalling is caused by cytosolic accumulation of nucleic acids recognised as PAMPs or DAMPs. This cytosolic accumulation is due to the lack of enzymatic activity of the TREX1 and SAMHD1 proteins. In the case of PRAAS, it is the ubiquitinated proteins, accumulated in the cell due to defective immunoproteasome activity, which are recognised as DAMPs. In SAVI, chronic activation of the type 1 IFN response occurs via constitutive activation of the STING protein due to a gain-of-function mutation in *STING1*. Finally, Singleton-Merten syndrome, for example, is thought to result from constitutive activation of cytosolic MDA5 and RIG-I receptors due to of a mutation in the *IFIH1* and *DDX58* genes (Figure 1) (4). Other diseases with elevated interferon activity have been described, such as tricho-hepato-enteric syndrome, genomic instability syndromes (e.g.ataxia-telangiectasia, Bloom syndrome), X-linked reticulopigmentary disease, some mitochondriopathies (8). As in the case of polygenic “general” lupus, interferon production in these settings is possibly due to secondary lesional mechanisms with exposure of endogenous nucleic acids (excessive but adapted production of type I IFN) rather than to constitutive overexpression of the interferon pathway.

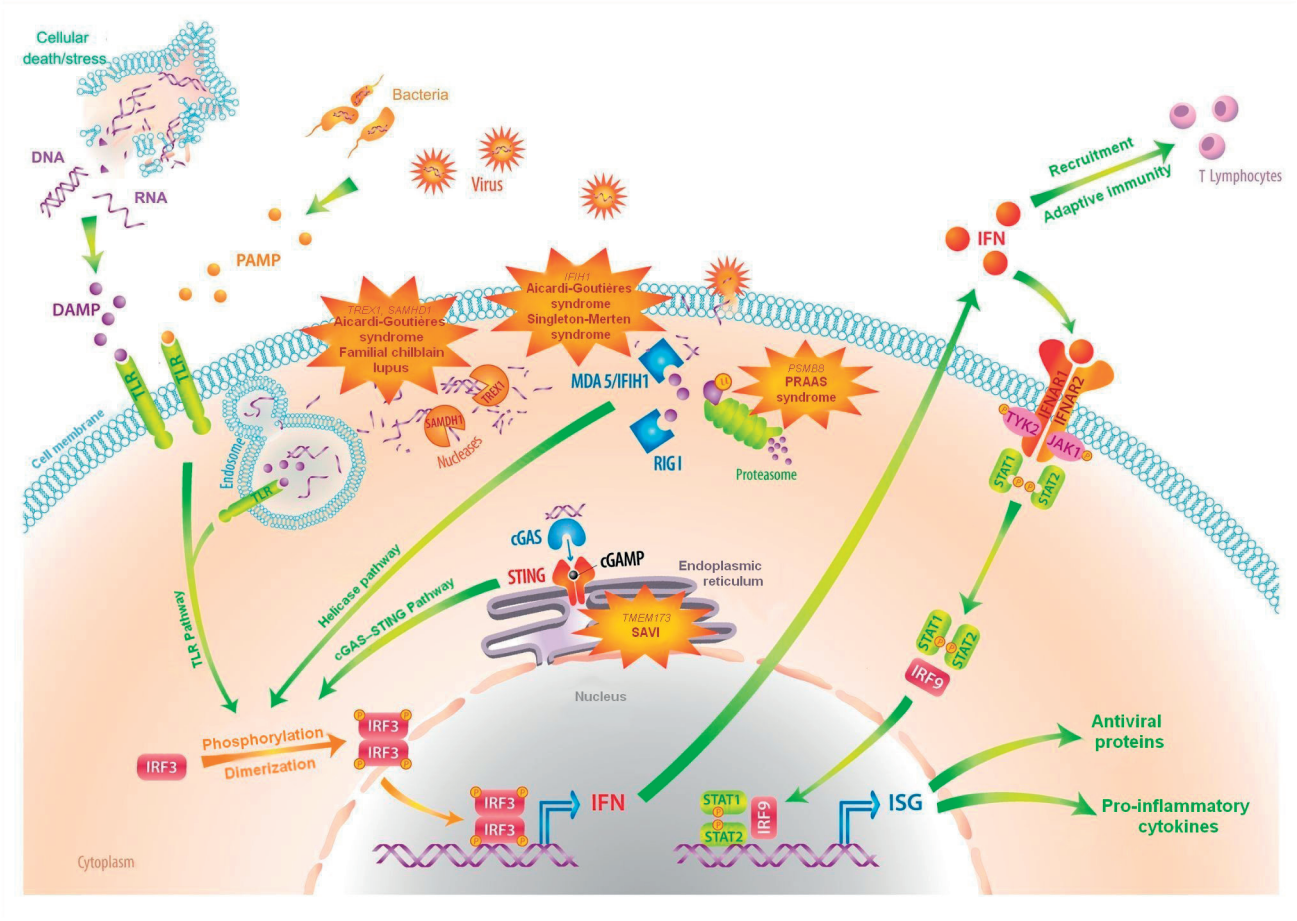
2. Diagnosis of Type 1 interferonopathies

Diagnosis of interferonopathies by conventional enzyme-linked immunosorbent assay (ELISA) is complicated. Indeed, interferons circulate in the blood at extremely low concentrations even following infection or interferonopathy (6, 9). Therefore, it is of interest to develop a routine diagnostic clinical test based on the interferon signature to indirectly assess type I IFN activity by measuring IFN-induced ISG gene expression. Techniques for routine measurement of IFN exposure to determine an IFN signature have recently been developed for the diagnosis and monitoring of type 1 interferonopathies (6).

Based on Pescarmona et al.'s 2019 landmark article, a series of quantitative PCR assays have been developed to assess the expression of a series of ISG genes for the diagnosis of interferonopathies. For this purpose, the RNA expression of 5 ISGs (*IFI27, IFI44L, IFIT1, ISG15, RSAD2*), previously described as the ISGs most overexpressed

Figure 1: Type 1 interferon signalling pathways and their mechanisms of overexpression in interferonopathies.

A signalling cascade leads to IFN synthesis (via phosphorylation and dimerization of nuclear transcription factors IRF). The TLR pathway is driven by the recognition of molecular patterns by TLRs, cellular membrane or endosomal receptors. The cGAS-STING pathway begins with the detection of cytosolic DNA by the cGAS enzyme, leading to the synthesis of cGAMP, which binds to the endoplasmic reticulum transmembrane protein STING. The RIG-I and MDA5/IFIH1 helicase pathway is activated by recognition of intracellular DAMPs and PAMPs. The IFNs thus produced bind to their membrane receptors IFNAR1/IFNAR2 and generate an activation signal propagated throughout the cell via the JAK/STAT signalling pathway. JAK protein kinases lead to the formation of a nuclear transcription factor composed of STAT1, STAT2 and IRF9. It binds upstream of ISGs to induce their expression and thus the production of proteins responsible for the antiviral properties of type 1 IFNs. Chronic activation of the type 1 IFN response can be generated by various dysfunctions as in the case of AGS and familial chilblain lupus, SAVI (Munoz et al., 2015).



in interferonopathies, could be quantified (6, 10). This quantification analysis allows us to obtain an IFN score that can be used as a biomarker of theses specific diseases.

3. Development of diagnostic tests based on the interferon signature at the Gosselies Institute of Pathology and Genetics.

Methodology

Participants and Sample Collection

Control samples were collected from 23 anonymous, healthy individuals (adults of various ages and of both sexes, with no known medical conditions or infections). Positive samples were obtained from 13 patients with infections such as influenza or COVID-19. Additionally, two patients exhibited symptoms of type 1 interferonopathies, such as chronic vasculitis or calcification of the basal ganglia.

RNA Extraction from EDTA Blood

Total RNA was extracted from whole blood collected in EDTA tubes using the MAXWELL RSC48 robot and the 'Maxwell RSC miRNA Plasma and Serum kit' (Ref. AS1680, Promega) following the manufacturer's instructions. The extracted RNA was then quantified using a Nanodrop spectrophotometer (Thermo ScientificTM NanoDrop One).

Reverse transcription

For both RT-qPCR and ddPCR, reverse transcription was conducted in two steps. Total RNA (0.4 to 1.0 µg) was therefore first subjected to reverse transcription in 20 µl of 'SuperScript™ IV VILO™ Master Mix' (Ref. 11756050 Invitrogen) to synthesise first strand cDNA.

RT-qPCR Procedure

After reverse transcription, the cDNA was diluted to a concentration of 5 ng/µl RNA equivalent. RT-qPCR amplification was performed in duplicate for each sample using the FastStart Universal SYBR Green Master (Rox) kit (Ref. 4913850001, Roche). The qPCR amplification was conducted using the QuantStudio5 system with the following program: a 10-minute cycle at 95°C (activation of the FastStart Taq DNA polymerase) followed by 40 amplification cycles of 15 seconds at 95°C followed by 1 minute at 60°C.

Five interferon-stimulated genes (ISGs) were studied: *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). Three housekeeping genes (OAZ (ornithine decarboxylase antizyme), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-Actin) were used as internal controls. Primers were used at a final concentration of 0.3 µM in the PCR reactions.

ddPCR Procedure

For ddPCR, cDNA samples were diluted to a concentration of 0.2 ng/µl RNA equivalent. The ddPCR was performed in duplicate for each sample using the 'QX200 ddPCR EvaGreen Supermix' (Bio-Rad) for the same 5 ISGs. The final concentration of primers was 0.2 µM. The PCR mixes

(15 µl) were then added to 5µl of diluted cRNA in a 96-well plate. The plate was placed in the BioRad automated droplet generator to create the emulsion, followed by PCR amplification on the BioRad C1000 Thermocycler with the following program: starting with a 5-minute cycle at 95°C (enzyme activation) followed by 40 denaturation and annealing/extension cycles lasting 30 seconds at 95°C and 1 minute at 60°C respectively, followed by a signal stabilisation step lasting 5 minutes at 4°C and 5 minutes at 90°C with a ramp of 2°C/sec. The results were then read using the QX200 ddPCR reader.

Calculation the IFN score

Once the qPCR results were obtained, the IFN scores could be calculated. The relative abundance of each gene transcript is calculated using the formula: $E^{-Delta\ Ct}$ ($E = 10^{(-1/slope)}$ and represents the efficiency of the standard curve generated for each gene by 10-fold serial dilutions). ISG expression was normalised to the mean expression of 3 housekeeping genes: OAZ, GAPDH and β-Actin. The relative expression for each ISG is then calculated by dividing the normalised expression of patient ISGs by the median normalised expression of ISGs in the healthy controls. Finally, the IFN score is calculated using the median of the relative expression of the 5 ISGs.

An abnormal IFN score was defined as an IFN score more than 2 standard deviations above the mean IFN score of the control group.

Technique validation steps

Primer Validation and RT-qPCR Efficiency

RT-qPCR was initially performed on a few control samples to validate the primers selected for studying ISG expression. Two negative controls were included: first, a matrix-free control (water sample) to check for contamination by foreign nucleic acids and primer dimer formation and secondly, a reverse transcriptase-free control and secondly, a reverse transcriptase-free control was prepared at the time of the reverse transcription step, without adding reverse transcriptase to the total RNA. This control was used to assess the amount of DNA contamination in the samples.

PCR efficiency was calculated for each gene using a standard curve generated by 10-fold serial dilutions. The PCR efficiency was then calculated using the formula: $E = 10^{(-1/\ slope)}$-1. qPCR reactions were considered good if the efficiency was between 90% and 110%. This efficiency of the standard curve will also allow, in a second step, to calculate the relative abundance for each gene transcript. Melting curves were also observed to confirm the specificity of our qPCR for the different genes in each sample.

Clinical relevance of the calculated IFN score

In order to assess the clinical relevance of the IFN score, calculated with the RT-qPCR data and their classification as positive or negative control, a ROC (Receiver Operating Characteristic) curve (Figure 2), a graphical representation of the performance of a binary classification model, was generated (11). The area under the curve (AUC) of the ROC curve is an important measure that is used to quantify the overall performance of the classification model. An AUC of 1 indicates that the classification model is perfect, while an AUC of 0.5 indicates that the classification model is random. Here, the area under the curve measured was 0.844, which is associated with reliable performance for this model.

Threshold confirmation and calculation of sensitivity and specificity

Subsequently, the ROC curve (Figure 2) was then used to confirm the threshold for defining whether the scores obtained are considered normal or abnormal. In this case, it is especially important that both the probability of the disease not being present when the test is negative, and the probability of the disease being present when the test is positive, are high. These probabilities are known respectively as negative predictive value (NPV) and positive predictive value (PPV). A threshold score of 3.2 was set, giving a sensitivity of 84.6%, a specificity of 91.3%, a NPV of 92%, and a PPV of 84.6%. This threshold was comparable to the threshold calculated from the mean scores of healthy controls + 2SD.

Test reproducibility

The reproducibility of the test was demonstrated on 3 samples (2 positive and one negative) and assessed on 3 different days, by 3 different laboratory technicians, on 3 different qPCR machines. The results obtained validated the intermediate accuracy of the test.

Test accuracy

Additional RNA samples, 5 negative controls and 6 positive controls, were obtained from the CHU de Lyon and were used as controls with

Figure 2: ROC curve for IFN signature assay based on RT-qPCR data and classification of positive controls and healthy controls.

The ROC curve represents the rate of true positives (Sensitivity) on the y-axis versus the rate of false positives (1 - Specificity) on the x-axis. Each point on the curve represents a different threshold value used to classify positive and negative instances. By varying the threshold, we can adjust the trade-off between the true positive rate and the false positive rate. The blue diagonal reference line represents a random classifier with no discriminatory power. The curve is based on the scores of 23 healthy controls and 13 RTqPCR positive controls. The AUC is 0.844. With a threshold set at 3.2: sensitivity (Se) = 84.6% and specificity (Sp) = 91.3%.

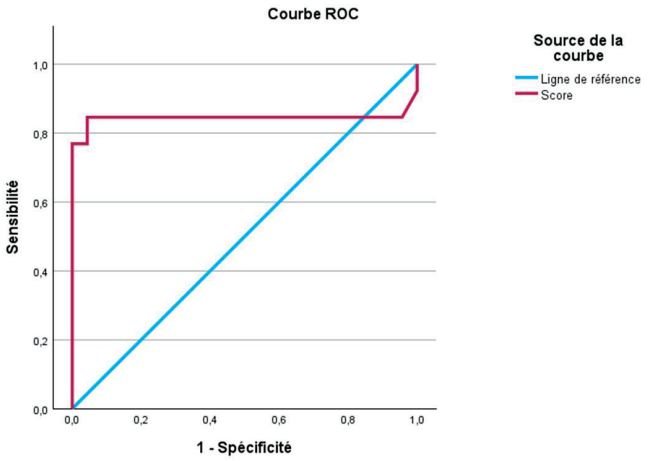


Figure 3: ROC curve for IFN signature assay based on ddPCR data and classification of positive control and healthy controls.

The ROC curve represents the rate of true positives (Sensitivity) on the y-axis versus the rate of false positives (1 - Specificity) on the x-axis. Each point on the curve represents a different threshold value used to classify positive and negative instances. The blue diagonal reference line represents a random classifier with no discriminatory power. The curve was constructed based on the scores of 9 healthy controls and 6 positive controls obtained by ddPCR. The AUC is 0.833. With a threshold set at 3.3: sensitivity (Se) = 83.3% and specificity (Sp) = 100%.

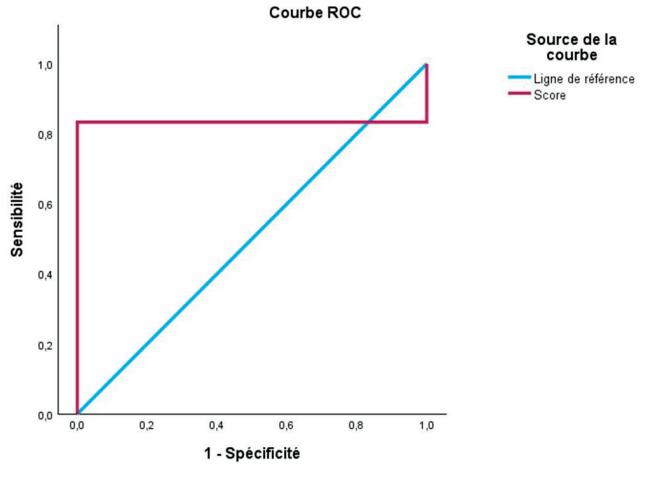


Table 1: PIFN scores in negative control groups, patients symptomatic for type 1 interferonopathy and positive controls obtained by RTqPCR and for of 9 samples from the negative control group and 6 positive controls obtained by ddPCR. Threshold set at 3.2 for RTqPCR and at 3.3 for ddPCR.

Samples	IFN scores (RTqPCR)	IFN scores
Healthy control 1	1,3	NA
Healthy control 2	1,8	NA
Healthy control 3	0,3	0,5
Healthy control 4	2,3	3,3
Healthy control 5	2,4	NA
Healthy control 6	3,8	NA
Healthy control 7	0,4	NA
Healthy control 8	0,6	NA
Healthy control 9	0,7	NA
Healthy control 10	0,5	NA
Healthy control 11	0,2	NA
Healthy control 12	1	0,8
Healthy control 13	0,3	NA
Healthy control 14	0,9	1,0
Healthy control 15	1,1	0,9
Healthy control 16	1,4	NA
Healthy control 17	0,8	0,7
Healthy control 18	0,9	1,0
Healthy control 19	1	0,9
Healthy control 20	2,3	NA
Healthy control 21	2,1	2,8
Healthy control 22	6,1	NA
Healthy control 23	0,6	NA
Patient with interferonopathy 1	3,6	NA
Patient with interferonopathy 2	0,8	NA
Positive controls 1	148,2	152,7
Positive controls 2	11,3	NA
Positive controls 3	0,1	NA
Positive controls 4	23,8	NA
Positive controls 5	46,8	39,9
Positive controls 6	23,1	NA
Positive controls 7	4,2	3,8
Positive controls 8	0,2	0,1
Positive controls 9	86,7	89,5
Positive controls 10	8,8	NA
Positive controls 11	7,8	NA
Positive controls 12	11	9,7
Positive controls 13	26,9	NA

a known IFN score to compare the results obtained by RTqPCR with the results obtained at the CHU de Lyon using the Nanostring method. The correlation between Nanostring results and RTqPCR results was calculated using the determination coefficient (R2). The scores obtained by RTqPCR were close to the scores given by CHU de Lyon, giving an R2 of 0.976.

ddPCR test and Comparison with RT-qPCR

The ddPCR was performed on the same transcripts as those used for RTqPCR, as described above. It was performed on 9 control samples and 6 positive samples previously tested by RTqPCR.

The performance of RTqPCR and ddPCR for measuring IFN scores was compared. The ROC curve (Figure 3) obtained from the ddPCR data gave an AUC of 0.833, and the threshold was established at 3.3 (comparable to the threshold calculated from the mean of the scores of healthy controls + 2SD), giving a sensitivity of 83.3%, a specificity of 100%, an NPV of 90% and a PPV of 100%. The ddPCR model performed almost identically to RTqPCR, both of which showed high sensitivity and specificity for the diagnosis of type 1 interferonopathies.

Results of the validation

Analysis of results

In the healthy control group, only 2 samples showed a positive and weakly positive IFN signature of 6.1 and 3.8 (8.7%).

In the positive control group, out of 13 samples analysed, 2 were negatives (13.3%). These 2 negatives results actually involved two patients for whom a negative result might have been expected. One had a residual viral load, but had been clinically infected 15 days previously, and the other was an incidental finding of an elevated viral load in the setting of sepsis following digestive perforation. It would therefore appear that these patients are simply asymptomatic or have a hyperstimulated inflammatory system in other inflammatory pathways, which could interfere with the IFN response. So, these are two cases where we would a priori have expected a negative or disturbed signature.

Finally, in patients with symptoms of interferonopathy, 1 sample was negative and the other positive (50%) (Table 1).

Comparison of RTqPCR and ddPCR results

The correlation between the results obtained by RT-qPCR and ddPCR was also analysed using the R2 for IFN scores and the relative expressions of each ISG. The R2 was 0.88 for *IFI27*, 0.9761 for *IFI44L*, 0.9816 for IFIT1, 0.9892 for *ISG15*, 0.9973 for *RSAD2* and 0.9975 for IFN scores.

All samples assessed in ddPCR showed identical results to those obtained in RTqPCR with close scores (Table 1). Once again, this shows that RT-qPCR and ddPCR have similar performance.

Advantages and disadvantages of the two interferon signature techniques

Both RTqPCR and ddPCR offer some advantages and disadvantages for interferon signature analysis. Indeed, ddPCR requires only a small amount of starting material, which can be advantageous when sample quantities are limited. However, ddPCR may require more time to perform the analysis due to the additional sample emulsification step compared to RTqPCR.

Limitations of diagnostic tests based on the interferon signature

Corticosteroids can partially suppress the interferon signature, as high-dose steroids can inhibit IFN responses (6, 12). This can lead to a reduction in ISG expression, which in turn can lower the IFN score and lead to a false-negative result. Therefore, it is usually recommended that the IFN signature be performed prior to the administration of this type of treatment and throughout an acute phase of the disease (6). JAK inhibitors, due to their specific action on JAK/STAT pathway, also modulate the signature.

It should also be noted that by the primary role of interferon system, interferon signature can be influenced by infections, mainly viral ones (6). Indeed, the interferon signature refers to a set of genes that are regulated by type 1 interferon, which is produced by the immune system in response to viral infections or other inflammatory stimuli (4). We observe as predicted that in our group of patients infected with the influenza virus, the IFN score can be remarkably high. Therefore, it is important that the patient could be assessed in the absence clinical signs of infection, or after eradication of the virus. In doubtful cases, diagnosis of type 1 interferonopathies can also be made at an early stage using a combination of clinical and genetic criteria (13).

Conclusion and clinical perspectives

In conclusion, RTqPCR and ddPCR are two comparable methods and, like the Nanostring method used by Pescarmona et al., both can be used routinely to study the IFN signature. The diagnosis of type 1 interferonopathies using the interferon signature has opened new perspectives in the management of these rare diseases. By continuing research and integrating technological, clinical and genetic advances, we can look forward to a more accurate, earlier and individualized diagnosis of type 1 interferonopathies, leading to better patient management. Furthermore, the interferon signature could also be used to assess the efficacy of treatments in patients with type 1 interferonopathies. Indeed, treatment of patients with type I interferonopathy, particularly with drugs targeting the type I IFN pathway, is associated with a decrease in the IFN signature. Another way of development could be treatment monitoring. By following ISG expression levels before and after treatment, it would be possible to determine whether treatment has been successful in reducing excessive interferon expression. The decrease in IFN score could then be used as an objective criterion of therapeutic efficacy, although the clinical correlation remains to be clarified (4, 14). On the other hand, many of the more common autoimmune pathologies ("non-monogenic" systemic lupus erythematosus, Sjögren's syndrome, dermatomyositis and other inflammatory myositis, ...) demonstrate a positive interferon signature in some subgroups of patients, which would make it possible to discuss a more targeted therapeutic orientation. Some autosomopathies (mainly trisomy 21, with a probable gene dosage effect on interferon pathway receptors) or mitochondrial diseases are also concerned, and the role of the type 1 interferon pathway in the long-term manifestations of these pathologies, as well as possible therapeutic perspectives, opens up a field of research to be explored (15). Finally, the development of the interferon signature is one of the possible transcriptomic analyses, but the development of knowledge of other pathways specific to autoinflammation, which may have diagnostic and therapeutic implications in other mono or polygenic diseases, may suggest other applications, possibly in combination with precision cytokine assays such as the Single MOlecular Assay (SiMOA).

None of the authors has a conflict of interest related to this article.

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