



Letter to the Editor

Practical challenges for functional validation of *STAT1* gain of function genetic variants

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Abbreviations: CMC: chronic mucocutaneous candidiasis; CVID: common variable immunodeficiency; CXCL-10: C-X-C motif chemokine ligand 10; GOF: gain of function; HC: healthy control; IEI: inborn errors of immunity; IFN- α : interferon-alpha; MFI: mean fluorescence intensity; NGS: next generation genetic sequencing; PBMC: peripheral blood mononuclear cell; PID: primary Immunodeficiency; PMA: phorbol 12-myristate 13-acetate; SD: standard deviation; pSTAT1: phosphorylation of signal transducer and activator of transcription 1; *STAT1*: signal transducer and activator of transcription 1; VUS: variant of unknown significance.

Wider application of next generation genetic sequencing (NGS) has significantly improved diagnosis for patients with inborn errors of immunity (IEI) and is increasingly a routine part of clinical practice [1, 2]. However, functional validation of genetic variants of unknown significance (VUS) remains a significant challenge for confirming definitive diagnoses, both due to inconsistent access to specialized testing and poor standardization of currently available assays [3]. The challenges are greater for conditions where clinical phenotype is not confirmatory due to heterogeneity or incomplete penetrance.

STAT1 gain-of-function (GOF) immunodeficiency typically presents with chronic mucocutaneous candidiasis (CMC), with or without a combination of bacterial, viral or mycobacterial infections, along with other complications including bronchiectasis, autoimmunity and vascular abnormalities [4, 5]. Over 75 different individual genetic mutations have been described to confer gain of function in the *STAT1* gene [6] and classically result in increased phosphorylation of *STAT1* (pSTAT1) in immune cells, either at baseline or following cytokine stimulation [7]. Therefore, most immunology laboratories that offer validation assays for *STAT1* VUS rely on comparing pSTAT1 levels between a potential index case and one or a group of healthy controls. However, levels of pSTAT1 at baseline and upregulation after cytokine stimulation vary considerably in the healthy population and overlap with levels observed in *STAT1* GOF patients.

In this study, we performed technically standardized flow cytometry assays using peripheral blood obtained from six patients with five different *STAT1* VUS, identified through whole genome or targeted chip panel sequencing for suspected IEI (see [Supplementary Table 1 and Methods](#)). We compared these against 4 patients with known pathogenic GOF mutations in *STAT1* and 16 healthy controls. A group of six patients with Common Variable Immunodeficiency (CVID) who had no pathogenic mutations identified in currently known IEI genes by NGS and no rare VUS in *STAT1* were used as disease controls. Healthy control samples were taken on the same day as patient or disease control samples and assays performed in parallel. *STAT1* VUS tested here were rare (allele frequency $<10^{-4}$ in reference databases) with variable prediction of deleterious impact using *in silico* prediction tools ([Table 1 and Supplementary Fig. 1a and b](#)).

Significant variability was seen in *STAT1* levels and in pSTAT1 upregulation in response to interferon-alpha (IFN- α) stimulation for all groups ([Supplementary Figs 1c-e and 2a](#)). When considered by group, both *STAT1* GOF mutations and *STAT1* VUS showed a significant increase in upregulation of pSTAT1 compared with healthy control ([Supplementary Fig. 1d](#)), although there was an overlap in the range observed for each group. We then considered each genetic variant individually to determine whether or not VUS could be assigned as GOF. When normalized against their matched healthy control sample, all patients with GOF mutations and 4/6

Table 1. Predicted functional impact of *STAT1* variants evaluated by *in silico* analysis and functional assays

Group	SIFT raw (<0.05)	PolyPhen2 Raw	CADD PHRED	Patient/ mutation	pSTAT1	pSTAT1	IL-17
	<0.05	>0.85	>20		Fold change $> 2^*$ (normalized for HC)	$>Mean +$ SD of HC	$<HC$ of the day
VUS	Yes	Yes	Yes	1/G416R	Yes	No	Yes
	Yes	Yes	Yes	2/F404V	No	Yes	Yes
	No	No	No	3/E284K	No	Yes	Yes
	No	No	No	4/E284K	Yes	Yes	Yes
	Yes	Yes	Yes	5/K344Q	Yes	Yes	Yes
	No	No	No	6/T419K	Yes	Yes	Yes
GOF	Yes	Yes	Yes	7/K388E	Yes	Yes	Yes
	No	No	No	8/T720I	Yes	Yes	Yes
	Yes	Yes	Yes	9/T385M	Yes	Yes	Yes
	Yes	Yes	No	10/P293S	Yes	Yes	Yes

*Normalized for the healthy control of the day; VUS = variant of unknown significance; GOF = gain of function; HC = healthy control; SD = standard deviation.

with VUS demonstrated at least 2-fold higher upregulation in pSTAT1 levels after stimulation (Fig. 1a). Two patients with VUS did not achieve this arbitrary cut off and one of these had a matched healthy control at the upper limit of the healthy control range (Fig. 1b). To reduce the impact of individual healthy control variation, we compared each VUS and GOF mutation against the whole healthy control range and identified that all patients with GOF and 5/6 with VUS upregulated pSTAT1 above a cut off of mean+1SD of the healthy control range (Fig. 1c). Variant location did not appear to impact degree of upregulation (Fig. 1d). Upregulation of total STAT1 was more variable; although all GOF and VUS showed upregulation to at least an arbitrary cut-off of 1.4x healthy control (Fig. 1e), this was considered to be not sufficiently robust to assist diagnosis (Supplementary Fig. 1e). CVID disease controls as a group behaved like healthy controls, with comparable upregulation of pSTAT1 and STAT1 (Fig. 1a, c–e, and Supplementary Fig. 1c–e). To test inclusion of other parameters that could improve diagnosis of GOF mutations, we quantified the frequency of CD4+ T cells producing IL-17 (Fig. 1f, Supplementary Figs 1f and 2b) and upregulation of the expression of STAT1-inducible CXCL-10 in monocytes (Supplementary Figs 3 and 4). In keeping with the known suppression of STAT3 signalling in *STAT1* GOF [8], all known GOF mutations supported reduced frequency of Th17 cells (Fig. 1f; Supplementary Figs 1f and 2b). All patients with *STAT1* VUS also had lower levels of Th17+ cells than the matched healthy control samples analysed on the same day. In contrast, CXCL-10 expression by monocytes at baseline and fold-change in levels following stimulation were variable between known GOF mutations (Supplementary Fig. 3), reducing the utility of this assay for functional validation of *STAT1* VUS.

To support diagnostic reporting, we considered a combination of pSTAT1 and IL-17 production results for each patient (Table 1). All GOF mutations demonstrated elevated levels of pSTAT1 in response to IFN- α stimulation (at least 2-fold upregulation compared with the matched healthy control and above the mean+1SD of the healthy control group range) plus had a frequency of Th17 cells

below the matched healthy control. All six patients with VUS also demonstrated increased upregulation of pSTAT1, either when considered against the matched healthy control of the day or against the healthy control group range (Fig. 1a–d) and all 6 had low levels of Th17 compared with the control (Fig. 1f). Thus, using this combination of pSTAT1 and IL-17 measurements, we concluded that all 5 VUS in 6 patients were GOF.

This study highlights the difficulty of definitively assigning GOF status to *STAT1* VUS using the single assessment of pSTAT1 and the benefit of including Th17 quantification as an additional parameter. For laboratories performing pSTAT1 assays, highly standardized phosflow protocols and flow cytometer settings together with a local healthy control range are important to reduce variation and improve assessment of index cases. In our hands, pSTAT1 assays only perform reproducibly on fresh blood (taken same day) and older samples are not reliably interpretable. To mitigate against healthy control variation, we suggest that assessment of pSTAT1 is made both against a same-day matched control and the local healthy control range. Future refinement of functional assessment for *STAT1* VUS requires development of additional robust assays that can be translated from research to diagnostic laboratories.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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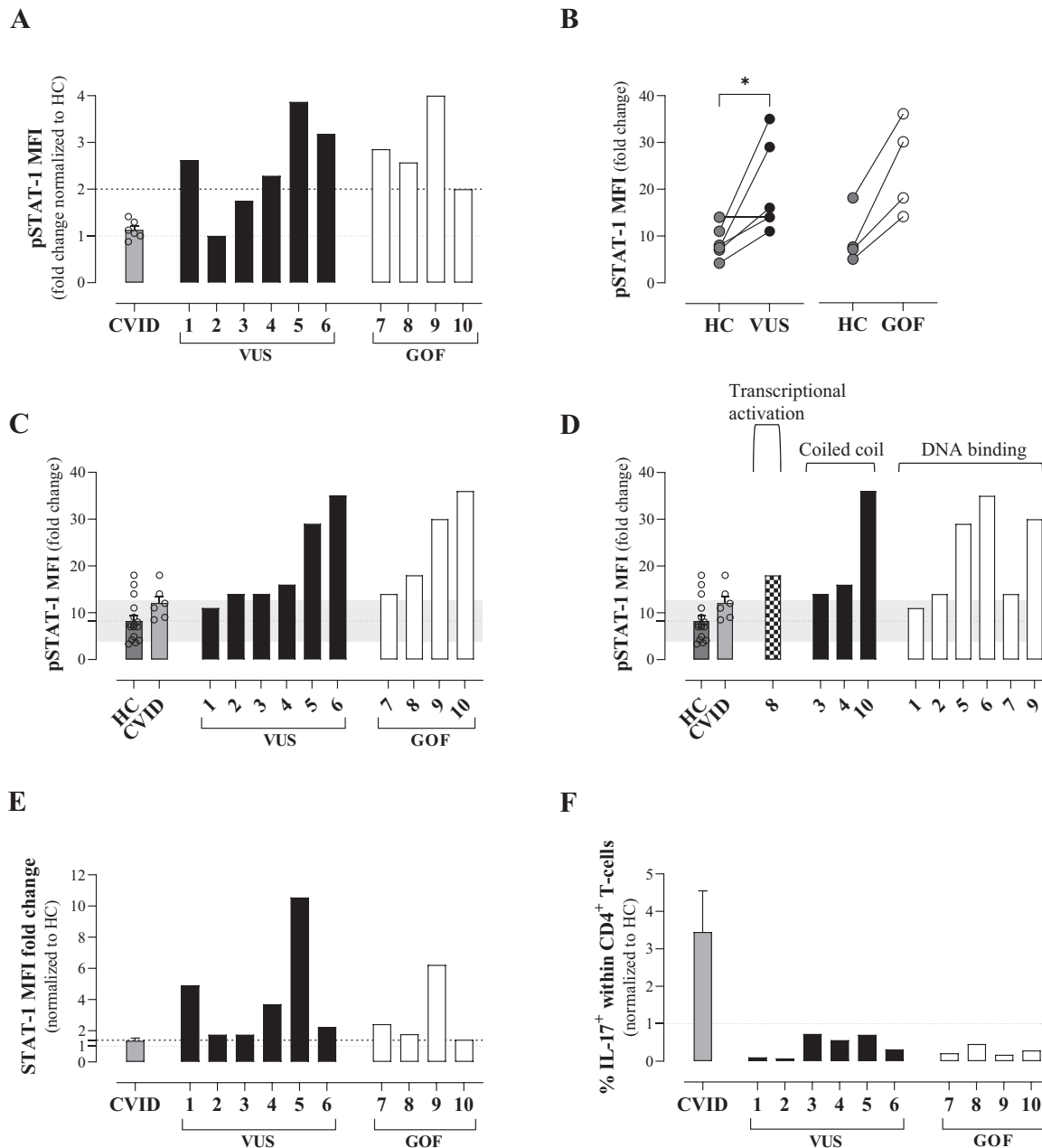


Figure 1. STAT1 phosphorylation and Th17 cells. Levels of expression of pSTAT1 (a–d) or total STAT1 (e) on gated CD3⁺ T cells after stimulation with IFN- α . (f) Frequency of IL-17⁺ cells within CD4⁺ lymphocytes. Fold change was calculated as the MFI ratio between IFN- α -stimulated and non-stimulated cells. Each dot or number represents one individual. For (b and c), the mean of the HC group is shown as a dotted line and the standard deviation of the HC group as grey shading.

Consortia

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Ethics approval and consent to participate

The local ethics committees have approved our study (reference numbers 04/Q0501/119 and 06/Q0508/16) and patients and healthy controls gave written informed consent.

Conflict of interests

S.O.B. has received grant support from CSL Behring and personal fees or travel expenses from Immunodeficiency Canada/IAACI, CSL Behring, Baxalta US Inc. and Biotest. E.C.M. has received honoraria from GlaxoSmithKline, AstraZenica, and Orchard Therapeutics. The rest of the authors declare that they have no conflicts of interest.

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Data availability

The datasets used and analysed in the current study are available from the corresponding author on reasonable request.

Author contributions

A.S.A. performed experiments and analysed data. J.M. and A.J.M. analysed data. E.C.M. and S.O.B. designed the project and supervised the work. A.S.A., J.M., A.J.M., E.C.M., and S.O.B. wrote and edited the manuscript. J.M., J.L., E.C.M., and S.O.B. collected clinical data. F.M. and S.W. consented the patients and healthy controls and collected blood. J.M., K.M., I.S., H.L.A., and NIHR BioResource-Rare Disease Consortium facilitated and identified the genetic variants. J.M. did the bioinformatics analysis. All authors contributed to the article and approved the submitted version.

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