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## EFFICACY OF RUXOLITINIB THERAPY IN A PATIENT WITH SEVERE ENTEROCOLITIS ASSOCIATED WITH A STAT3 GAIN OF FUNCTION MUTATION

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**Short title: JAK inhibitor for severe enteropathy**

### **Abbreviations:**

JAK: Janus Kinase; STAT3: Signal transducer and activator of transduction 3; Gain-of-function: GoF

**The authors indicate no conflict of interest**

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**Contributors:** M. Parlato, F. Charbit-Henrion, N. Cerf-Bensussan and G. Malamut, conceived the study, reviewed data and wrote the manuscript. GM performed the retrospective analysis of medical files. F. Charbit-Henrion analysed WES data. M. Parlato, E. Abi Nader, B. Begue N. Guegan performed functional analyses. J. Bruneau performed histological studies and E. Macintyre studied T cell clonality. M. Allez and L. Le Bourhis provided RNA from colonic tissue samples. F. Rieux-Laucat provided the EBV cell line carrying the known *STAT3* mutation. C. Picard performed Sanger analysis. S. Khater, O. Goulet and C. Cellier provided clinical data and O. Hermine initiated treatment with Ruxolitinib. All the authors reviewed the paper.

**Key Words:** immune regulation, autoimmune enteropathy, monogenic disorders, JAK1/2 inhibitor

## **INTRODUCTION**

Severe enterocolitis raises diagnostic and therapeutic challenges. Empirical treatment by steroids and immunosuppressive drugs is commonly used but, to date, little attempt has been made to specifically target the mechanism underlying inflammation. Possible causes of severe enterocolitis include refractory celiac disease <sup>1</sup>, autoimmune enteropathy <sup>2</sup> and common variable immunodeficiency <sup>3</sup>, the two latter being overlapping entities of possible monogenic inheritance. Besides *FOXP3* mutations that cause immune dysregulation polyendocrinopathy X-linked (IPEX) syndrome<sup>4</sup>, additional genetic causes have recently been reported <sup>5-7</sup>. Herein, using whole exome sequencing (WES), we identified a novel *STAT3* gain of function (GoF) mutation in an adult patient displaying chronic diarrhea and autoimmune manifestations refractory to several lines of immunosuppressive drugs and biotherapies. Identification of the mutation enabled successful treatment with the JAK1/2 inhibitor ruxolitinib.

## **METHODS**

### **Ethics Statement**

The study was approved by the Ile-de -France II ethical committee (Paris, France).

### **Characterization of enteropathy**

Histological analysis, flow cytometry analysis of isolated lymphocytes and molecular analysis of gastrointestinal specimens were performed as described <sup>1</sup>.

### **DNA preparation**

Genomic DNA was extracted from PBMC isolated by Ficoll HyPaque Plus (GE Healthcare, Velizy-Villacoublay, France) using the QIAamp<sup>®</sup> DNA Blood Mini Kit (Qiagen, Courtaboeuf, France).

### **Whole Exome Sequencing**

Whole exome sequencing was performed as previously described<sup>8</sup>. Genomic DNA libraries were generated from DNA (3µg) sheared with a Covaris S2 Ultrasonicator using SureSelect<sup>XT</sup> Library PrepKit (Agilent, Garches, France) on the Genomic Platform at the Imagine Institute. Capture by hybridization was performed using Agilent Sure Select All Exon V5 (Agilent, Les Ulis, France). Targeted exons were pulled out with magnetic streptavidin beads, PCR-amplified using indexing primers and sequenced on an Illumina HiSeq2500 HT system (Illumina, San Diego, USA). Data analysis was performed with Paris Descartes University / Imagine Institute's Bioinformatics core facilities. Paired-end sequences were mapped on the human genome reference (NCBI build37/hg19 version) using the Burrows-Wheeler Aligner. Downstream processing was carried out with the Genome Analysis Toolkit (GATK), SAMtools, and Picard, according to documented best practices (<http://www.broadinstitute.org/gatk/guide/topic?name=best-practices>). Variant calls were made with the GATK Unified Genotyper based on the 72<sup>nd</sup> version of ENSEMBL database. Genome variations were defined using the in-house software PolyQuery which filters out irrelevant and common polymorphisms based on frequencies extracted from public databases: US National Center for Biotechnology Information database of SNP (dbSNP), 1000 genomes, Exome Variant Server (EVS, <http://evs.gs.washington.edu/EVS/>), and Exome Aggregation Consortium (ExAC, <http://exac.broadinstitute.org>). Consequences of mutations on protein function were predicted using three algorithms: Polyphen2 (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (Sorting Intolerant From Tolerant, J. Craig Venter Institute) and Mutation Taster ([www.mutationtaster.org](http://www.mutationtaster.org)). Mutations were then ranked on the basis of the predicted impact of each variant by combined annotation-dependent depletion (CADD), and compared with the mutation significance cut-off (MSC), a gene-level specific cut-off for CADD scores (<http://pec630.rockefeller.edu:8080/MSC/>)<sup>9</sup>.

## **Sanger Sequencing**

The mutations were confirmed by Sanger sequencing using specific primers targeting exon 13: for: TCCGGCTACTTGGTCACCTA-3; rev: 5-CCCCCATTCACATCTCTG -3.

## **STAT3 luciferase reporter assay of STAT3 N401D allele**

Mutations into human WT STAT3 sequence (NM\_139276; Origene) were introduced using the GENEART® Site-Directed Mutagenesis System (Invitrogen, Thermo Fisher Scientific) and the following primers: c.1201A>G for: ATGGAAGAATCCAACGACGGCAGCCTCTC TG; c.1201A>G rev: CAGAGAGGCTGCCGTCGTTGGATTCTTCCAT; c.1175A>G for: TGGGCACAAACACAAGAGTGATGAACATGGA; c.1175A>G\_rev: TCCATGTTTCATC ACTCTTGTGTTTGTGCCCA according to manufacturer's instructions. Each mutation was confirmed by direct sequencing of the STAT3 insert (Eurofins). STAT3 inserts were, then, subcloned into pLVX-EF1alpha-IRES-mCherry lentiviral expression vector (transfer vector) (Clontech, Saint-Germain-en-Laye, France) via the restriction site Not1 (New England Biolabs, Evry, France) and the correct orientation of the insert was confirmed by sequencing. Lentiviral particles encoding the different mutants were generated by transfecting HEK293T cells with transfer plasmid, packaging expressing plasmid psPAX2 (Addgene, Cambridge, USA), VSV-G envelope expressing plasmid PMD2.G (Addgene) using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific). 6 h after transfection, cells were washed and new medium without antibiotics was added for 60 h. The recombinant virus-containing medium was filtered and used to transduce HEK293T cells, stably expressing STAT3-responsive firefly luciferase reporter (Qiagen, Courtaboeuf, France) in presence of polybrene (4µg/mL). M-cherry-positive cells were sorted by FACS. Comparable expression of STAT3 protein following transduction of each lentiviral construct was assessed by western blot using mouse anti-STAT3 antibody (124H6, 1:1000, Cell Signaling, Saint Quentin Yvelines Cedex,

France), and rabbit anti-gapdh (14C10, 1:1000, Cell signaling). STAT3 reporter activity was evaluated upon 10 ng/ml IL-6 stimulation for 48 h using the Dual-luciferase reporter assay system (E1910, Promega, Charbonnières-les-Bains, France) according to the manufacturer's recommendations.

### **Gene expression analysis**

For analysis of SOCS3 transcription, Epstein Barr virus (EBV) immortalized B cell lines derived from the patient, from two healthy donors, and from one patient carrying the previously described GoF p.T716M mutation in STAT3 DNA binding domain, were stimulated for 20 hours with 10 ng/mL IL-21 (R&D systems, Lille France) or with 5 ng/mL IL-6 (R&D systems). Cells ( $1 \times 10^6$ ) were lysed in RLT plus buffer (Qiagen) and RNA extracted using RNAeasy kit (Qiagen). For cytokines mRNA expression studies, intestinal tissues were immediately placed in RNA later (ThermoFisher Scientific) and stored at  $-80^\circ\text{C}$ . RNA was extracted using RNAeasy kit (Qiagen). QRT-PCR was performed on Applied Biosystems 7300 Real-Time PCR apparatus as described using TaqMan universal PCR Master Mix and Taqman probe assays (ThermoFisher Scientific, Montigny-Le-Bretonneaux France): *RPLP0* (Hs99999902\_m1); *SOCS3* (Hs02330328\_s1); *GzmB* (Hs01554355\_m1); *TNFalpha* (Hs99999043\_ml); *IFNG* (Hs99999041\_m1); *IL6* (Hs00985639\_m1); *IL10* (Hs00961622\_m1); *IL12A* (Hs009673447\_m1); *IL17A* (Hs00174383\_m1); *IL21* (Hs00222327\_m1); *IL22* (Hs01574154\_m1); *IL23* (Hs00372324\_m1). Results were expressed as relative expression  $2^{-\Delta\text{Ct}}$  normalized to the house-keeping ribosomal Protein Large PO (*RPLP0*) gene.

## RESULTS

### Case report

A Caucasian woman followed up for diarrhea since the age of 5 months was referred to our adult center in 2011 at the age of 19. Severe intestinal villous atrophy refractory to gluten free diet was diagnosed at 3 years. At 6 years, she developed neutropenia, thrombopenia with anti-platelet antibodies and Hashimoto thyroiditis with anti-thyroglobulin antibodies. At 8 years, severe diarrhea relapsed with subtotal duodenal atrophy and colonic apoptotic lesions. No antibodies against gliadin or enterocytes were detected but serum IgG were low ( $4\text{g/L} < 5.5\text{g/L}$ ) with normal IgM, IgA and IgE. Between 3 and 18 years, she was successively treated by steroids, tacrolimus and azathioprine but had frequent *Clostridium difficile* infections. Growth hormone treatment failed to improve the severe growth retardation ( $-2.5$  SD). In 2011 she had moderate diarrhea on azathioprine with normal duodenal architecture and mild colonic inflammation. CD4, B and NK lymphopenia in peripheral blood was ascribed to prolonged treatment by azathioprine. Frequency of CD25<sup>high</sup>Foxp3<sup>+</sup> cells among peripheral CD4<sup>+</sup> T cells was 4% (normal: 4-20%). Phenotyping of isolated duodenal lymphocytes showed predominance of CD3<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> cells among intraepithelial lymphocytes and comparable frequency of CD4<sup>+</sup> and CD8<sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> cells in the *lamina propria*. Expression of the activating Natural Killer receptor NKG2C on 10% and 5% of lymphocytes in epithelium and *lamina propria* respectively suggested moderate activation. Molecular analysis of gastric, duodenal and colonic biopsies revealed polyclonal TCR $\gamma$ , IgH and Ig Kappa repertoires. No anti-AIE75 KD antibodies were detected and serum IgG1 ( $2.4\text{g/L} < 4\text{g/L}$ ) and IgG3 ( $0.04\text{g/L} < 0.17\text{g/L}$ ) were low. Between 2012 and 2014, the patient had severe viral infections, including primary parvovirus B19, cytomegalovirus infections and Epstein Barr virus (EBV) reactivation. She received high doses of intravenous immunoglobulins and azathioprine was switched to budesonide. Adalimumab, introduced in

2014 because of increased serum concentrations of TNF- $\alpha$ , decreased diarrhea and allowed 8kg weight gain. However, clinical relapse occurred and was not controlled by golimumab, a distinct TNF- $\alpha$  antibody.

### **Diagnosis of *STAT3* GoF mutation**

Disease severity and very early onset suggested a monogenic disease. Accordingly, WES identified a *de novo* heterozygous missense variant, c.1201A>G, in exon 13 of *STAT3* (Figure 1A, Supplementary Table 1), which was confirmed by Sanger sequencing (Figure 1B-C). Both germline loss-of-function mutations and germline and somatic GoF *STAT3* mutations have been described<sup>5,6,10-12</sup>. Notably, activating germline *STAT3* mutations can cause multi-organ autoimmunity and enteritis<sup>5,6</sup>. The c.1201A>G variant was absent in all public databases and in our in-house database (12,925 exomes). This variant replaces the highly conserved asparagine in position 401 to aspartic acid (p.N401D, Figure 1D-E) within the DNA binding domain. This change was predicted to be damaging by all algorithms (Polyphen2 (0.949), Sift (0.02), MutationTaster). CADD score was very high (29.7). As for other previously reported Gof *STAT3* mutations within the DNA binding domain<sup>5,6</sup>, we predicted that the mutated protein will retain increased AGG-element binding activity following activation and therefore increased transcriptional activity. Expression of the N401D mutant, in HEK293T cells expressing *STAT3*-responsive element, resulted in increased luciferase reporter activity compared to wild-type *STAT3* upon IL-6 stimulation and comparable to the known *STAT3* GoF allele K392R<sup>5,6</sup> (Figure 1F). Accordingly, transcription of Suppressor of cytokine signaling 3 (*SOCS3*), a major target of *STAT3*, was significantly increased upon stimulation by IL-21 or by IL-6 in the EBV cell line from the patient compared to EBV lines derived from two controls and comparable to the EBV line from the know *STAT3* GoF T716M carrier patient (Figure 1G). Importantly, IL-6- or IL21-induced

transcription was significantly reduced upon inhibition of JAK1 signaling with ruxolitinib (Figure 1G).

### **Therapeutic efficacy of ruxolitinib**

Following validation of the *STAT3* GoF mutation, and because the patient progressively developed uncontrolled enterocolitis (Figure 2 A-B: subtotal duodenal villous atrophy and severe colitis with massive lymphocytic infiltrate and glandular apoptotic bodies), ustekinumab and tocilizumab were tested but without efficacy. In contrast, diarrhea stopped rapidly after introduction of ruxolitinib 10mg twice a day in April 2017. Ruxolitinib was increased to 15mg twice a day in July 2017 and budesonide discontinued after two months. In November 2017, the patient had gained 5 kg and completed mucosal healing (Figure 2C-D); *SOCS3* transcription was markedly reduced in duodenum and colon, indicating efficient blockade of STAT3 signaling; *IFN- $\gamma$*  and *Granzyme B* transcripts were decreased in duodenum and colon and *IL-12* and *TNF- $\alpha$*  transcripts in colon (Figure 2E-F, Supplementary Figure1). One year later the patient is still in remission.

## **DISCUSSION**

Herein, WES identified a novel *STAT3* GoF mutation as the cause of a severe enterocolitis. In keeping with previous reports of *STAT3* GoF mutations<sup>5, 6, 11</sup>, the patient displayed other autoimmune symptoms, primary hypogammaglobulinemia and short stature. Following functional validation of the mutation, the patient was successively treated with ustekinumab and tocilizumab in order to prevent activation of STAT3 downstream IL-23 and IL-6 receptor respectively. Both treatments were inefficient despite one case report of enterocolitis with *STAT3* GoF mutation clinically improved by tocilizumab<sup>13</sup>. In contrast, ruxolitinib induced rapid and complete remission, indicating that blocking STAT3 activation

simultaneously downstream different cytokines may be necessary to control intestinal inflammation. This case is reminiscent of the previous success of this inhibitor in patients with GoF mutation in *JAK1*, which encodes the kinase upstream *STAT3*<sup>14</sup>. Importantly, somatic *STAT3* GoF mutations are observed in many lymphoproliferative disorders and one patient with germline *STAT3* GoF mutation developed large lymphocytic leukemia at 14 years<sup>11</sup>. Ruxolitinib treatment may help to prevent such complication in our patient.

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## FIGURE LEGENDS

**Figure 1: Novel STAT3 GoF mutation.** (A) WES analysis. (B, C) Sanger sequencing confirming *de novo* mutation. (D) Schematic representation of STAT3 domains. (E) Conservation of N401 among orthologs. (F) STAT3 transcriptional activity following 48-hour activation with IL-6 (10 ng/mL) of HEK293T cells containing STAT3-responsive luciferase and stably transduced with empty vector (EV), WT and mutants STAT3. Results represent the mean of 5 independent experiments (\*\*p < .01, \*\*\*p < .001, one-way ANOVA). (G) RT-PCR of STAT3-target SOCS3 in IL-21 or IL-6 activated EBV cell lines treated or not with ruxolitinib (RX). Results are shown as relative expression normalized to RPLPO housekeeping gene and represent the mean of 4 independent experiments. IL-6 vs IL-6 + RX (\*p < 0.01, Mann Whitney), control vs STAT3 mutants (\*\*p < 0.01, \*\*\*\*p < 0.0001, one-way ANOVA).

**Figure 2: Induction of mucosal healing by ruxolitinib.** (A-B) HES staining (x100) and immunohistochemistry staining (x200) of CD3<sup>+</sup>, CD8<sup>+</sup>, GzmB<sup>+</sup> lymphocytes before and after 6 months of ruxolitinib (RX). (C-D) RT-PCR quantification of indicated cytokines in patient's duodenum (C) and colon (D) before (BRu) and after ruxolitinib (ARu). Results are compared with histologically normal duodenal (n=4) or colonic biopsies (n=4) (CT), and with duodenal biopsies from 5 active celiac disease (ACeD) (C) and colonic biopsies from 5 active Crohn's disease (CrD) (D).



