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BRIEF REPORT

Fluorescent energy transfer causing misleading signal in multicolor flow cytometry

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Abstract

Multiple immunolabeling introduces high risks of interferences between fluorochromes. In an intend to analyze T cell clonality using CD3-APC Alexa750, CD4-Pac Blue, CD8-Krome Orange, CD56-PE-Cy7 and Vbeta clonotypes FITC and PE, we repeatedly observed a clear, unexpected signal on B770 (PE-Cy7) detector on the Vb subset mimicking a lymphoproliferative disorder. The aim of this study was to identify and prevent this source of artifact. The study was performed on a seven color panel performed on fresh whole blood, labeled, fixed, lyzed and analyzed on Navios Cytometer Beckman Coulter. Data were reanalyzed using Kaluza. Eleven tubes tested two clonotypes each with the same T cell backbone. Only one representative combination is presented. Using this panel, we observed repeatedly a strong CD56 PE-Cy7 (B755 LP) on all Vbeta1 T cell subsets but not on Vbeta 2-FITC T cells. The effect was still observed after removing CD56-PE-Cy7 (Full Minus One). Changing anti-CD3 APC-Alexa 750 with CD3APC, the B755 LP signal disappeared but a B695/30 signal appeared. Shifting to CD3-FITC abolished any unexpected red signal. This demonstrates a fluorescent energy transfer (FRET) between PE excited by the blue laser and Alexa750 to be excited by the red laser. Accordingly, the Vbeta PE fluorescence intensity was reduced when FRET happened and clearly increased when CD3-FITC was used instead. This observation clearly reminds that FRET can give misleading results in case of labeling of very close markers with complementary fluorochromes. This risk has to be considered in panel design.

KEYWORDS

APC-Alexa 750, flow cytometry, FRET, multicolor panel, PE-Cy7

1 | INTRODUCTION

Simultaneous detection of multiple immunolabelings is an exceptional property of flow cytometers. However, mixing multiple fluorochromes on a very small surface is not without risks of interferences. The Förster (fluorescence) resonance energy transfer (FRET) is well known from physicists for several years specially [1] and is commonly used for immunoglobulin conjugates by coupling two fluorochromes in “tandems” [2].

The use of tandems is necessary to detect more conjugates excited by each laser. New flow cytometers commonly use 3–6 lasers to excite fluorochromes but the number of fluorochromes directly excitable by each laser is limited. As an example, two lasers are frequently used for immunodiagnostic panels. The blue laser (488 nm) excites fluorescein (FITC) or Alexa Fluor 488 detected by B525/40 photomultiplier (PMT); Phycoerythrin (PE, emission peak at 560–575 nm) detected by B575/30 PMT and Peridinin-Chlorophyll-Protein (PerCP) detected on B695/30 PMT. The Red laser

(630 nm) can only excite allophycocyanin and Alexa Fluor 647 (emission peak at 660–675 nm) detected by R660/20 PMT.

Tandems use FRET: Briefly, PE, PerCP or APC named “donor,” produce strong fluorescence that is used to excite another fluorochrome named “acceptor” [3, 4]. Part of donor energy is transferred to acceptor. The most frequently used acceptors in flow cytometry are Texas red (emission peak at 610–625), Cyanine 5 (emission peak at 660–675) both excitable by PE; Alexa Fluor 700 (emission peak at 710–720) excitable by APC. Cyanine 7 or Alexa Fluor 750 or H7 (emission peak at 780–790) are excitable by PE or APC. FRET is only efficient if the donor and acceptor are in very short distance (below 100 nm) which is easily the case when the two fluorochromes are conjugated on the same molecule. In most instruments, lasers are separated and fluorescences excited by different lasers are treated separately in space and time and should not interfere too much.

Adding more immunolabelings gives the possibility to analyze more parameters, up to five colors, per laser but also introduces more risks of interferences between fluorochromes. As an example in a 10 colors, 3 lasers system, we designed a panel to analyze the clonality of the T cell receptor on CD3+ CD4+ or CD8+ T Cells in a 11 tubes panel. We observed a clear immunolabeling on B770 detector (in our case detecting CD56-PE-Cy7) that was not expected on some T cell subsets. The aberrant phenotype was observed on all clonotypes labeled with PE and not the ones labeled with FITC.

Here we explored the causes of this misleading detection that appeared to be due to incidental FRET in multiple labelings of components from the same molecular complex.

2 | METHODS

T cell clonality was analyzed using 22 monoclonal antibodies directed to Vbeta clonotypes of the T cell receptor (TCR), conjugated with FITC or PE. The panel included CD3 ϵ APC-Alexa 750 (cloneUCHT1), CD4 Pacific Blue (clone13B8.2), CD8 Krome Orange (clone B9.11), CD56-PE-Cy7 (clone N901) as a backbone. Eleven combinations of two antibodies to Vbeta were used with the same backbone. For clarity, only one representative example is reported here. Clonotypes Vbeta 1 PE (clone BL37.2) and Vbeta 17 FITC (clone E17.5F3.15.13) were selected as they were frequently represented in this patient T cell repertoire. All monoclonal antibodies were provided by Beckman-Coulter; Fullerton, CA (BC). Alternative anti-CD3 labeling used CD3 ϵ FITC or CD3 ϵ APC (clone SK7) from BD Biosciences San Jose, CA (BD).

The labeling was performed on whole blood anti-coagulated by EDTA. Fixation and erythrolysis was performed with Immunoprep™ using the TQ.prep™ Workstation (Beckman-Coulter). Cells were washed with 4 ml Phosphate Buffer Saline (PBS, Eurobio, France), centrifuged at 300g and the pellet resuspended in PBS supplemented with Bovine Serum Albumin 10G/L (BSA, Eurobio). The analysis was performed within 4 hours on Navios™ using Navios™ software (Beckman-Coulter). Instrument settings and calculation of compensation were performed using single labeling on capture beads

Versacomp™ beads (Beckman-Coulter). Instrument settings were checked every working day using Flow-check Pro™ and Flow-set Pro™ beads (Beckman-Coulter) according to the manufacturer instruction and to our Iso 15189 procedures. Data were analyzed on Kaluza™ software (Beckman-Coulter).

The sample quality was first checked on forward (FSC)/side scatter (SSC) plot (Figure 1A) and acquisition stability on a time plot (Figure 1B). The triggering threshold was set up on CD3 labeling. Singlets were selected on the plot of the highest intensity versus the area under curve FSC correlation (Figure 1C). Then T cells were selected on CD3 ϵ expression (Figure 1D), dichotomized according to their expression of either CD4 Pacific Blue or CD8 Krome Orange (Figure 1E). Vbeta FITC or PE (Figure 1F) and CD56 PE-Cy7 (Figure 1G) expression was analyzed on each T cell subset. <http://flowrepository.org/id/FR-FCM-Z3H7>.

3 | RESULTS

Results showed an expression of CD56PE-Cy7 on all Vbeta 1 PECD8 + T cells but also on Vbeta 1 PECD4+ T cells (Figure 1G) which is unusual. All CD56 + CD4 + T cells expressed the Vbeta PE chain (Figure 1H) suggesting a monoclonal disorder. Despite the spectral overlap was properly compensated on single labelings, the Vbeta PE signal was correlated with the CD56PE-Cy signal (Figure 1J) but not with unlabeled B695/30 (PE-Cy5) signal (Figure 1K). This was never observed with Vbeta FITC labeled T cells.

By removing CD56-PE-Cy7 from the full panel as Full Minus One (FMO) strategy, we could still see a strong aberrant signal on B755 LP (PE-Cy7) detector (Figure 2A). This “PE-Cy7” signal was still directly correlated with the PE signal signing an artifact (Figure 2A second column). No signal was detected on B695/30 (PE-Cy5) detector (Figure 2 row A columns 3 and 4). By replacing CD3-APC-Alex750 with CD3-APC, the aberrant signal on B755 LP disappeared (Figure 2 row B) but a signal was detected on B695/30 detector despite there was no labeling of this color. The B695 signal was correlated with the Vbeta PE signal. Using CD3 conjugated with FITC (instead of using Vbeta FITC), we did not show any unexpected red signal.

Interestingly, Vbeta PE Median Fluorescence Intensity (MdfI) was low when CD56-PE-Cy7 was in the panel (263; Figure 2A) or removed (272; Figure 2B) but rose when CD3 was conjugated with APC (328; Figure 2C) or FITC (424; Figure 2D) showing a partial adsorption of the PE signal when FRET happened.

4 | DISCUSSION

So, in this panel, we observed repeatedly aberrant labeling of CD56-PE-Cy7 on (1) a large (all) proportion of CD8+ T cells of VB1 clonotype but (2) also on CD4+ T cells with the same clonotype that was much unexpected (Figure 3). The signal was only observed with PE clonotypes and was still there despite CD56 was removed. The only 755 signal we had in the panel was CD3 ϵ APC-Alexa 750 supposed to be excited by the red laser only. But, due to the very close proximity of TCR and CD3, we suspected that the unexpected

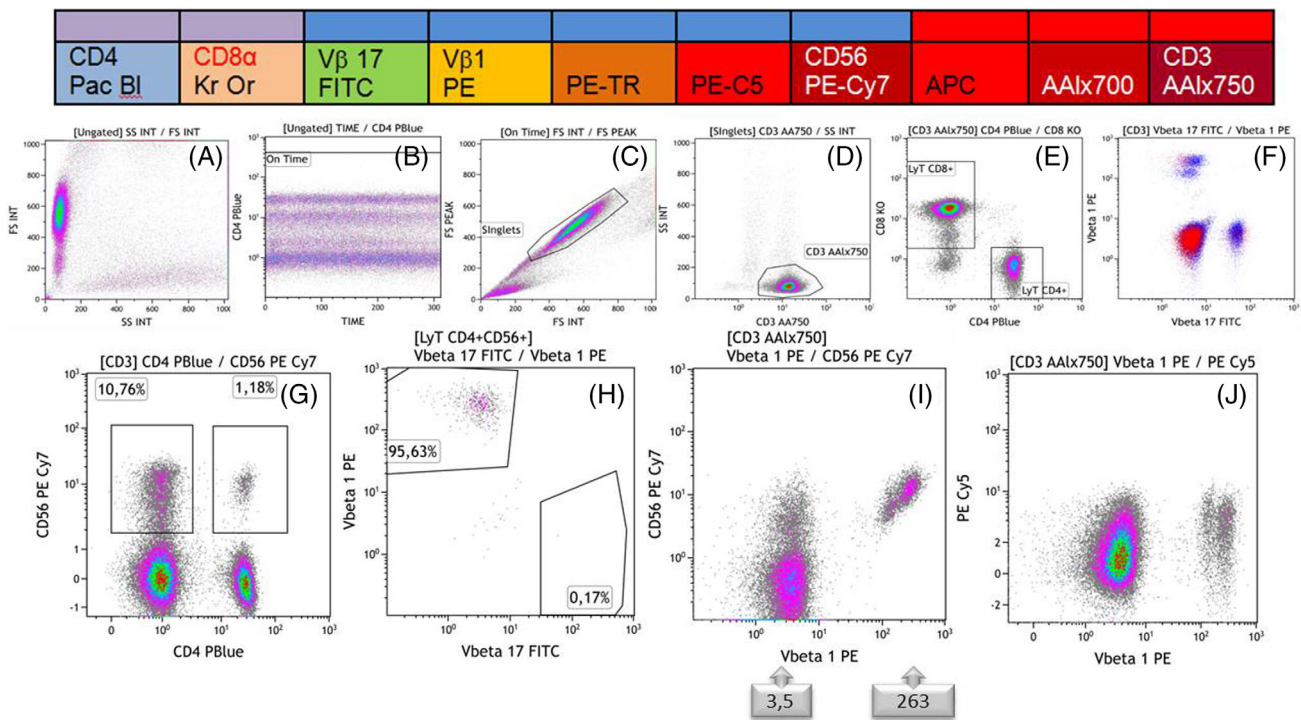


FIGURE 1 Lymphocytes were identified on forward (FSC)/side scatter (SS) plot (A) and CD4 Pacific blue /time plot followed by doublets exclusion on FSC-H/FSC-A. T cells were selected on CD3-APC-Alexa 750 and CD4-Pac blue or CD8 Krome Orange expressions. One representative example of Vbeta labeling showing Vbeta2-FITC and Vbeta1-PE that gave unexpected CD56 PE-Cy7 expression on both CD8+ and CD4+ T cell subsets. All CD56+ CD4+ T cells apparently expressed the Vbeta1 PE [Color figure can be viewed at wileyonlinelibrary.com]

B755LP (PE-Cy7) signal was due to a FRET. Accordingly, referring to Spectra viewer shows that PE (B570) excited by the blue laser, produces light that partially fit with the PE7 or Alexa 750 excitability spectrum. As a matter of fact, PE-Cy7 tandem is commonly used. This explain that PE bound to the TCR can excite Alexa 750 bound to the very close CD3 ϵ chain and can be detected on the B755 LP detector (as if it was a PE-Alexa750 tandem) while APC-Alexa 750 is excited again, by the red lasers and detected on the R755 LP detector.

FRET only happened because (1) two labeled antibodies were in very close proximity. This is the case in labeling components of membrane complexes like TCR, B cell receptor or receptor heterodimers; (2) PE emission could excite Alexa 750 excitation. It would be the same with Cyanine 7 or H7 that have similar excitability and emission spectra. Accordingly, the PE signal was partially reduced when FRET happened as compared to other combinations. Fortunately, the PE signal is not fully adsorbed by Alexa750 and Vbeta1 was still detectable. Similarly some PE signal is detected in labeling with PE-Cy7 tandem due to what is commonly named "leakage" of signal when not fully transferred to the acceptor. Comparatively, PE-Cy5 tandems have low leakage of PE signal.

The labeling was misleading because we used the PE-Cy7 for CD56 labeling that is occasionally expressed by CD8+ T Cells. Fortunately, we were alerted because of the unusual expression of CD56 by CD4 T cells and the repeat of the effect on each clonotype labeled with PE but not on the ones labeled with FITC. This might be more difficult to detect with another labeling.

This risk of misinterpretation due to FRET has already been presented as communication by Jean Luc D'Hautcourt and collaborators several years ago, oral communication in a French Association of Cytometry (AFC) meeting. They reported FRET between PE and APC on B695/30 (PE-Cy5) detector in a labeling CD3 ϵ -PE; CD4-PE-Cy5 and TCR gamma chain-APC. In this case, all gamma-delta T cells unexpectedly expressed CD4. But we think it is the first time incidental FRET is shown with Cy7 or Alexa 750 as well. Other risks are possible when labeling heterodimers such as CD8, cytokines receptors or Integrins (alpha and beta chains) or complexes such as B cell receptor or co-stimulation complex like CD21, CD19 and CD81. As unexpected signal (like APC-Cy-7 excited by PE and detected on B755 LP) is exactly the same as the one expected from PE-Cy7 tandem, the emission spectrum is not distinct and changing the filters should not really help in preventing these risks.

The FRET effect is different from another cross-excitation effect that explains why PE-Cy5 is strongly detected on APC channel (R660/20 in our case). Indeed, this effect is due to the excitability of Cy-5. Using PE-Cy5 tandem, Cy5 is not only excited by the blue laser through PE emission (FRET) but also by the red laser (633–635 nm) (cross-excitation). Cy5 fully accepts PE signal by FRET and produces signal on the B695/30 but also on R660/20 that need strong mathematical compensation. This does not happen with Cy5.5 provided the instrument can efficiently detect Cy5.5 spectrum.

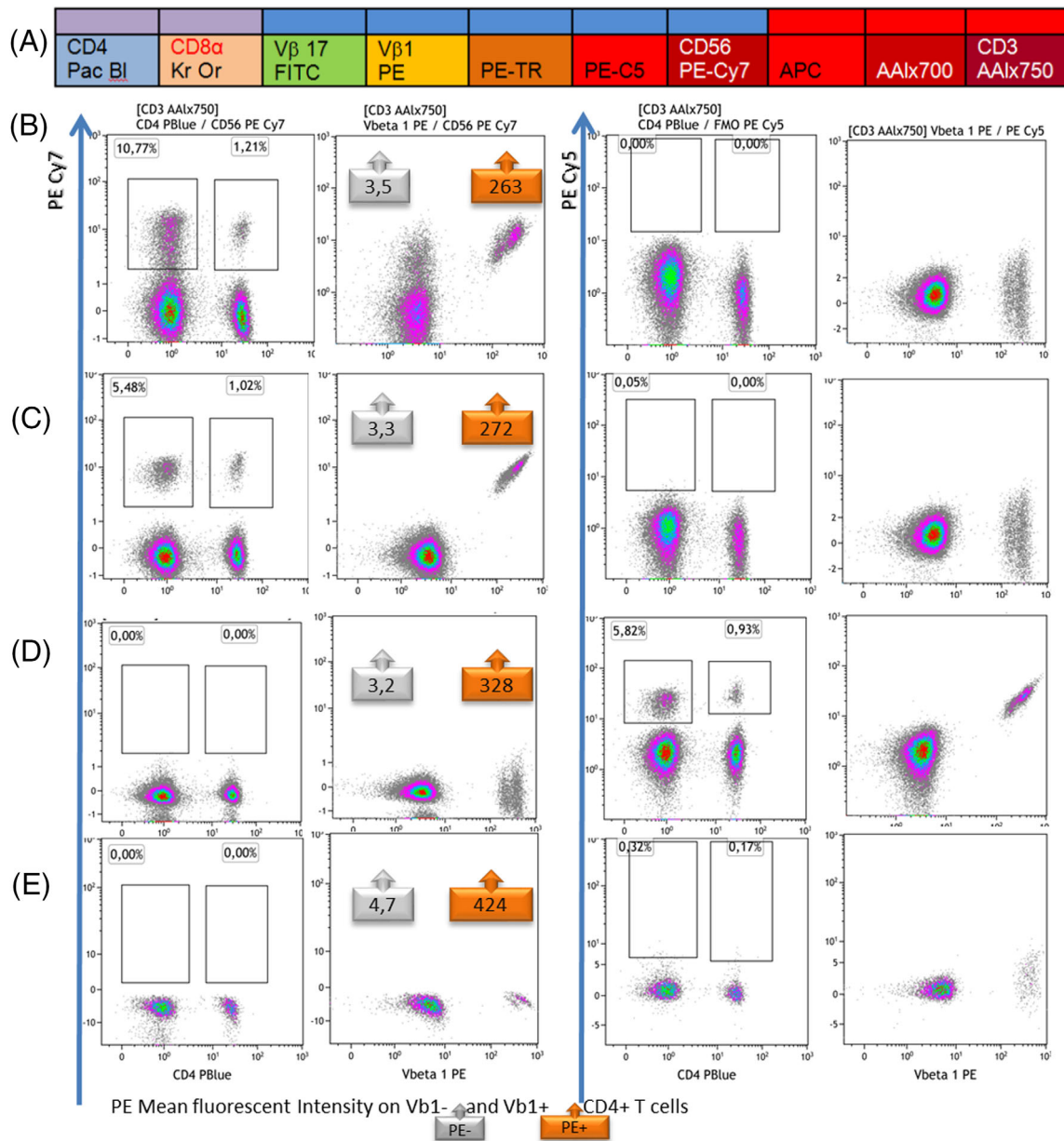


FIGURE 2 Detection of PE-Cy7 (B755 LP on blue laser optical bench) on CD4+ T cells (first column) and compared to PE signal (second column). PE-Cy5 (B695/30) signal on CD4+ T cells (third column) and compared to PE signal (fourth column). Row (A) the 6 color panel is presented. Row (B) full panel including CD3 APC-AA750 and CD56-PE-Cy7 shows a fraction of CD4+ and CD4- T cells that apparently expresses CD56-PE-Cy7. This signal is correlated to Vbeta1-PE expression. Row (C) CD56-PE-Cy7 labeling is excluded from the panel (FMO): The PE-Cy7 signal is still observed and correlated to V beta1-PE expression. Row (D) CD3 APC-AA750 is replaced with CD3-APC: The PE-Cy7 signal has disappeared but a PE-Cy5 signal appeared, that was correlated to Vbeta1-PE expression. Row (E) CD3 APC is replaced with CD3-FITC (instead of Vb17): The PE-Cy7 and PE-Cy5 signals have disappeared. Accordingly, PE median fluorescence intensities on V β 1 PE+ CD4 + T cells were reduced in case of FRET (row B, C, D) as compared to the panel using CD3-FITC (row E) while the negative signal MFI on V β 1 PE-CD4 + T cells remained stable [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.com)]

FRET has also been used in flow cytometry or microscopy to analyze the proximity between two molecules and is used in several applications in cell and molecular biology [3, 5–12].

In conclusion, multicolor labeling exposes to several risks of artifact that could mislead the results like spectral overlapping, cross excitation, fluorescence spreading, fluorescence leaking, tandem

dissociation, bleaching. FRET is another risk that should be considered in panel designing, specially the panel aims to label proteins in complex or in close contact. Donor/acceptor fluorochromes should be avoided for markers in close contact. Special attention must focus on the use of tandems. The issue is even more critical on 4–8 laser panels.

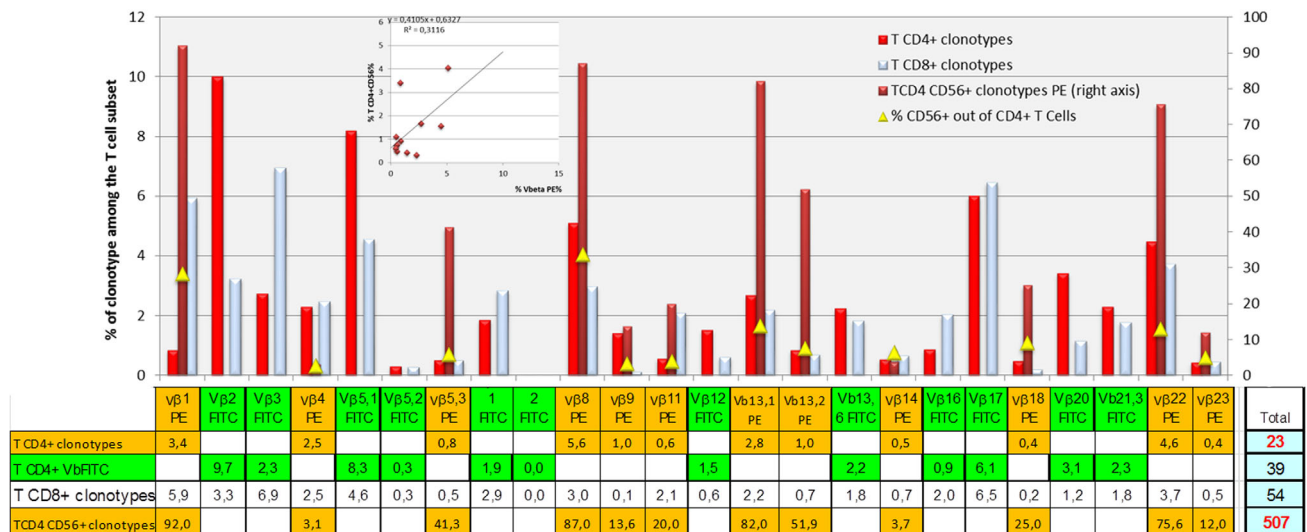


FIGURE 3 (suppl). Analysis of T cell receptor using monoclonal antibodies specific for 22 Vbeta chains: Up to 62% of the CD4+ T cell repertoire is identified (red bars), 23% with PE and 39% FITC labeled antibodies. Each clonotype represent between 0.4% and 10% of total CD4+ T cells as compared to 0.1%–12% of CD8+ T cells (light blue bars). The triangle marks shows the respective number of CD4+ T cells expressing CD56. These numbers were directly correlated to the expression of a Vbeta PE chain as shown in the dot plot. Comparatively, from 3.1% to 92% (in total 507%) of CD56 + CD4+ T cells expressed a clonotype labeled with PE (brown bars; right scale) [Color figure can be viewed at wileyonlinelibrary.com]

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AUTHOR CONTRIBUTIONS

Hana KHENINE: Formal analysis; writing-review & editing. **Louis WAECKEL:** Investigation; writing-review & editing. **Fouad SEGHROUCHNI:** Conceptualization; methodology; writing-review & editing.

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