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Short communication

Detection of circulating immunosuppressive cytokines in malignant pleural mesothelioma patients for prognostic stratification

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ABSTRACT

Background: No data on circulating biomarkers for the prognostic stratification of Malignant Pleural Mesothelioma (MPM) patients are available. We prospectively explored the prognostic role of circulating monocyte and cytokine levels and their dynamic change during chemotherapy.

Patients and Methods: MPM patients receiving a first line treatment based on a platinum compound plus pemetrexed were eligible. Blood samples were collected at the baseline and at the end of induction chemotherapy. CCL-2, IL-10 and TGF-β levels in plasma were quantified by Enzyme-Linked Immunosorbent Assay (ELISA); white blood cells, monocytes and platelets were evaluated by blood count test.

Results: Thirty-one patients were included in the study. Median overall survival (OS) was 12.13 months *versus* 9.6 months in patients with lower and higher monocytes count, respectively (p value = 0.02). We further stratified patients according to a combined score based on the association of IL-10, TGF-β levels and monocytes count. High combined score was associated with shorter OS and PFS in univariate and multivariate analysis. Chemotherapy induced an increase in monocytes, IL-10, but not TGF-β levels.

Conclusion: The prognostic value of circulating levels of multiple immunosuppressive cytokines and inflammatory cells should be confirmed in a wider validation set of MPM patients.

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive asbestos-related neoplasm with increasing incidence and dismal prognosis, due to the complex biology of the disease and unsuccessful treatment strategies over the last two decades [1]. Since 2003, the standard first-line chemotherapy with platinum-pemetrexed has been the only treatment supported by prospective and randomized data, achieving a median overall survival (OS) of about 12 months [2].

More recently, standard systemic treatment of MPM was bolstered by the innovation of immune checkpoint inhibitors as single or combination agents, in previously treated [3] or chemonaïve patients [4].

In the recent phase III randomized Checkmate 743 trial, Ipilimumab plus nivolumab improved survival compared with standard chemotherapy in the first-line treatment of PD-L1 unselected MPM patients. Survival benefit was more evident for the non-epithelioid subtype (median OS 18.1 *versus* 8.8 months; HR 0·46, 95% CI 0·31-0·68), compared to epithelioid tumors (median OS 18.6 vs. 16.5 months; HR 0·86, 95% CI 0·69–1·08) [4].

This clinical study confirmed previous evidence for the biological complexity and heterogeneity of different MPM histological subtypes [5].

The MPM immune microenvironment involves several actors, among them chronically activated macrophages that promote the local immune infiltrate and eventually to the malignant transformation of mesothelial cells [6].

Increased levels of monocytes and immunosuppressive cytokines in pleural effusions of MPM patients and their prognostic role have been reported [7]; particularly, transforming growth factor beta (TGF-b), interleukin-10 (IL-10), and chemokine ligand 2 (CCL-2) (also called

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monocyte chemoattractant protein-1) were shown to drive immune evasion through different mechanisms [8,9].

Serum mesothelin and osteopontin have been proposed as circulating prognostic biomarkers, although their reliability for patient stratification is still controversial [10].

The primary aim of this study was to explore the prognostic role of circulating immune cells and selected cytokine levels and their dynamic changes during cytotoxic chemotherapy.

We also investigated how baseline circulating cytokine levels were correlated with objective response and progression-free survival (PFS).

2. Patients and methods

This was a single-centre prospective study including a series of consecutive MPM patients referred to the Istituto Oncologico Veneto (IOV) between April 2018 and November 2020.

Eligibility criteria were histological diagnosis of MPM (epithelioid, biphasic, or sarcomatoid histologies); eligibility for standard first-line platinum-pemetrexed chemotherapy. Patients who were candidate for surgery were considered eligible for the study; patients who received any other treatment than standard induction chemotherapy before surgery were excluded.

All eligible patients signed a specific informed consent for the study, approved by the Ethical Committee (cod. CESC IOV: 2017/77/PU). Clinical and biological data were collected in a password-protected database.

All patients received first-line treatment based on a platinum compound (cisplatin 75 mg/m^2 day 1 every 3 weeks or carboplatin AUC 5 day 1 every 3 weeks) plus pemetrexed (500 mg/m2 day 1 every3 weeks) for 3 to 6 cycles, followed by surgery and/or radiation therapy according to the decision of the multidisciplinary team.

CT scans were performed at baseline and after systemic treatment. Tumor were evaluated through modified response evaluation criteria in solid tumors (mRECIST) for mesothelioma and overall response rate (ORR) was calculated accordingly.

Blood samples were collected at baseline (T0) and at the end of induction chemotherapy (T1).

3. Collection of plasma samples

Blood samples were collected in K2-EDTA-anticoagulated Vacutainer tubes (BD Bioscience) and processed within 30 min. Briefly, 5 ml of plasma was separated by centrifugation at 2000 × g for 10 min at 4 °C. Plasma was aliquoted to avoid multiple freeze–thaw cycles and stored at − 80 °C until analysis.

4. Circulating biomarkers measurement

CCL-2 level in plasma was measured by Diaclone ELISA assay (cat n° 873030192) following the manufacturer's instructions. The results were expressed in pg/ml.

Total TGF-β1 in plasma samples was measured by Diaclone ELISA assay (cat n°650010192)

after converting latent TGF-β1 to active TGF-β1 by acidification (20-minute incubation at RT with 0.1 vol of HCl 1 N followed by neutralization with 0.1 vol of NaOH 1 N). The results were expressed in ng/ml.

IL-10 level was quantified using the High Sensitivity Diaclone ELISA assay (cat n° 850880192) following the manufacturer's instructions. The results were expressed in pg/ml.

Optical density at 450 nm was determined using a Victor microplate reader (PerkinElmer).

White blood cell (WBC) (monocyte, lymphocytes, neutrophil) and platelet counts have been obtained from blood exams performed for routine clinical practice and expressed as number of cells/ml.

5. Statistical analysis

Statistical analyses were performed using OriginPro Software (version 2020). Values greater than and lower than the median were defined as high and low, respectively. Correlations between plasma levels of cytokines, WBC and platelet blood counts were investigated through Spearman linear correlation analysis. OS and PFS curves were constructed according to the Kaplan-Meier method. Univariate (log rank Mantel Cox test) and multivariate (Cox Regression Proportional Hazards Model) analyses were performed to assess the impact of considered markers on PFS and OS.

The Wilcoxon Signed Rank Test was carried out to evaluate differences in plasma TGF-β, IL-10 and CCL-2 levels and monocyte counts between paired treatment-naive and post-chemotherapy samples.

6. Results

We report data from thirty-one patients affected by MPM. The median age at diagnosis was 74 years (range 44–83). All but 4 patients had epithelioid histology (N = 27, 87%). All patients received first-line chemotherapy with cisplatin (N = 3) or carboplatin (N = 28) plus pemetrexed for a maximum of 6 cycles.

Seven patients received a multimodality treatment with chemotherapy followed by surgery (pleurectomy/decortication) and/or radiation therapy.

Ten patients received at least one subsequent systemic treatment for disease progression or relapse. Supplementary Table 1 summarizes the main clinical features of the study population.

Blood samples were collected from all patients at T0. Twenty-three patients underwent a second blood sampling at T1 for dynamic assessment of circulating biomarkers before and after chemotherapy.

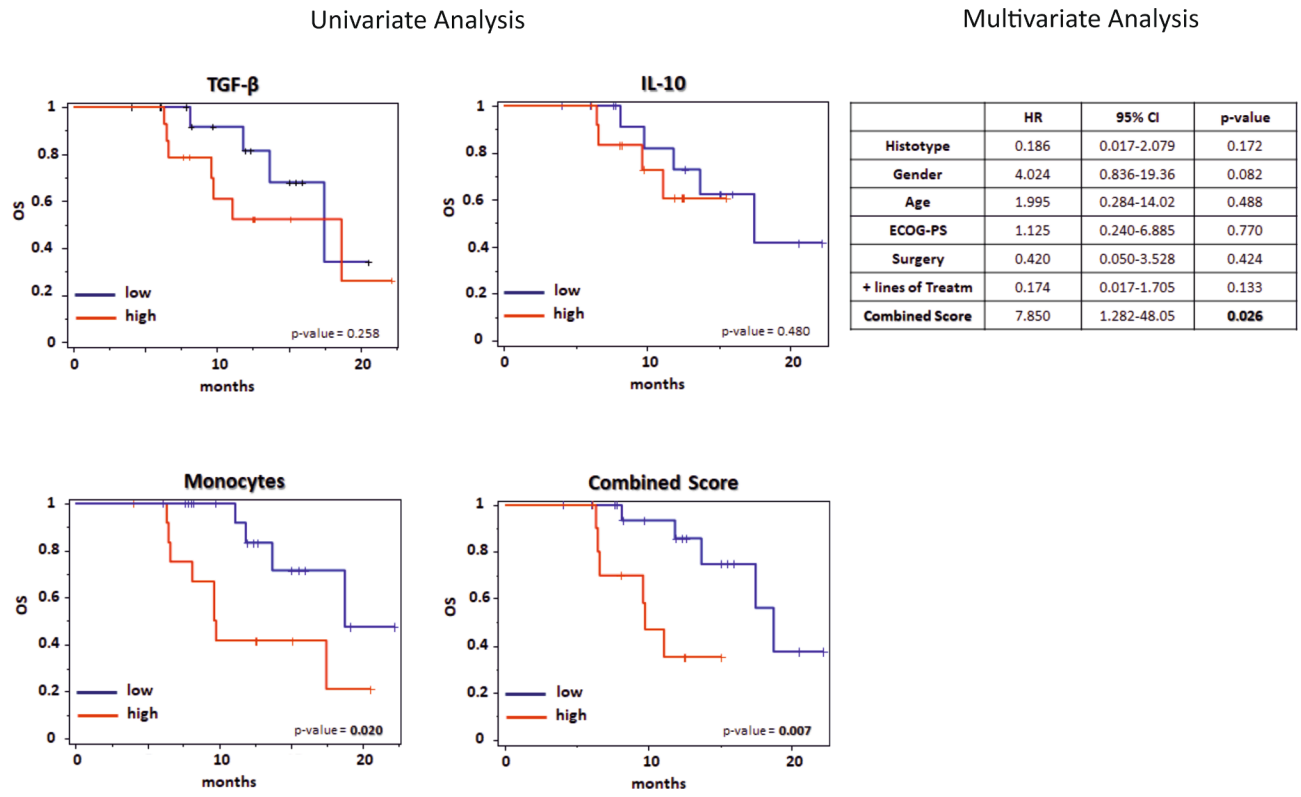
7. Correlation between levels of circulating blood cells monocytes and cytokines.

We first examined the baseline expression of CCL-2, IL-10 and TGF-β in plasma samples of MPM patients. The mean level of CCL-2 was 106.12 ± 127.2 pg/ml (min 0, median 83, max 773.1), the mean level of IL-10 was 4.22 ± 3.07 pg/ml (min 0.86, median 3.5, max 14.3 pg/ ml), and the mean level of TGF-β was 2.36 ± 1.34 ng/ml (min 0.82, median 1.84, max 6.35 ng/ ml).

The mean level of neutrophil-to-lymphocyte ratio (NLR), an indicator of systemic inflammation, was 3.08 (min 1.09, median 2.45, max 12.76), and the mean monocyte counts was 0.61 ± 0.25 x106/ml (min 0.11, median 0.60, max 1.09 x106/ml). We found a positive correlation between TGF-β and IL-10 levels (Spearman correlation coefficient = 0.41, p-value = 0.02), and between TGF-β level and monocytes count (Spearman correlation coefficient = 0.37, p-value = 0.045), and no correlation between the other factors.

8. Correlation between baseline circulating biomarkers and patients outcome

The median follow-up of the patients cohort was 11.5 months. We performed a univariate Log-Rank analysis to explore the impact of analysed biomarkers on OS. Patients with higher plasma levels of TGF-β and IL-10 showed a trend toward worse prognosis compared to patients with lower levels of these cytokines, although statistical significance was not reached (Fig. 1). High monocytes count showed a statistically significant negative prognostic value (Fig. 1). The median OS in patients with lower monocyte counts were 12.13 months *versus* 9.6 months in patients with higher monocyte counts (p-value = 0.02). CCL-2 levels and NLR did not show any correlation with survival (data not shown). We further stratified patients according to a combined score based on the association of IL-10 and TGF-β levels and monocyte counts. The score definition was as follows: high combined score if two of three markers



	HR	95% CI	p-value
Histotype	0.186	0.017-2.079	0.172
Gender	4.024	0.836-19.36	0.082
Age	1.995	0.284-14.02	0.488
ECOG-PS	1.125	0.240-6.885	0.770
Surgery	0.420	0.050-3.528	0.424
+ lines of Treatm	0.174	0.017-1.705	0.133
Combined Score	7.850	1.282-48.05	**0.026**

Fig. 1. Correlation between baseline circulating biomarkers and OS. Kaplan-Mayer curves show OS of MPM patients based on TGF-β and IL-10 plasma level, monocyte counts and combined score. The table shows multivariate (cox regression proportional hazards model) analysis assessing the impact of the combined score on OS.

were high, and low combined score if two of three markers were low. Results showed that a high combined score was associated with shorter OS (median OS 12.13 *versus* 9.6 in low and high score, respectively; p-value = 0.007). Importantly, the impact of the combined score was confirmed in multivariate analysis (p-value = 0.026) (Fig. 1). Similarly, shorter PFS was correlated with both high baseline monocyte count (median PFS: 11.2 *versus* 5.8 months, for low and high monocyte counts, respectively; p = 0.015) and high combined score (median PFS: 9.4 *versus* 4.5 months, for low and high combined score, respectively; p < 0.001) (Supplementary Fig. 1). Multivariate analysis for PFS confirmed histology (p-value = 0.024), surgery (p-value = 0.020), and combined score (p-value = 0.028) as independent prognostic factors (Supplementary Fig. 1).

No correlation was found between baseline cytokine and monocyte levels and ORR nor disease load assessed by modified RECIST criteria (data not shown).

9. Circulating monocyte counts and cytokine levels change after chemotherapy

We compared circulating CCL-2, IL-10, TGF-β and monocytes in paired samples collected pre- and post-chemotherapy treatment. As shown in Fig. 2 chemotherapy induced an increase in circulating CCL-2, IL-10, but not TGF-β (p-value = 0.03 for IL-10 and 0.43 for TGF-β). Although the total monocyte count did not significantly increase (data not shown), post-chemotherapy samples had a higher percentage of monocytes in the total WBC count (p-value = 0.007) (Fig. 2).

10. Discussion

The tumor immune microenvironment of malignant pleural mesothelioma is characterized by the infiltration of tumor-associated macrophages (TAMs) and by secretion of cytokines which may contribute to an immunosuppressive loop, with subsequent tumor proliferation and spreading of cancer cells [6].

The immunosuppressive 'secretome' of MPM includes TGF-β, CCL-2 and IL-10, which have been described also in pleural effusions of MPM patients [11]. CCL-2 is one of the major cytokines responsible for monocyte recruitment and TAMs polarization into a M2 immunosuppressive phenotype, while TGF-β and IL-10 are very active in T-cell suppression [9]. TGF-β also plays a role in the epithelial-to-mesenchymal transition, a biological trigger of cancer progression and metastasis, and exerts autocrine and paracrine effect on myeloid cell survival and immunosuppressive commitment [7].

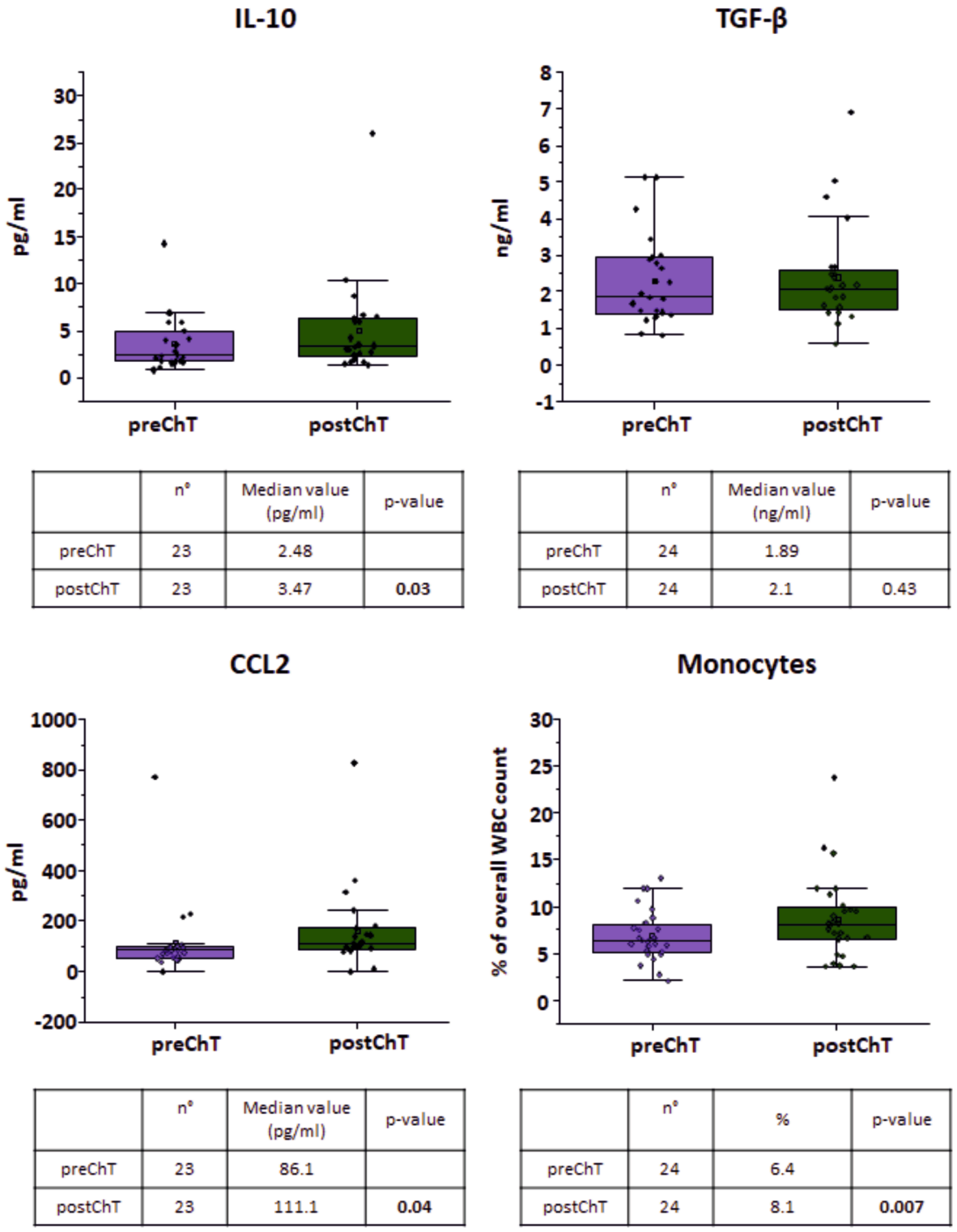
In our exploratory investigation, we observed a negative prognostic impact of high circulating levels of monocytes, TGF-β and IL-10 at the time of diagnosis; the combined score of these three components further stratified MPM patients in different survival subgroups. Moreover, high baseline cytokine and monocyte levels correlated with shorter PFS.

Even though a multimodality strategy usually achieves the best outcome in MPM patients, we did not observe any impact of stage, surgery, radiation therapy and further systemic treatments on survival, and this may be due to the small subset of patients assessed and the heterogeneity of treatment pathways among them.

This underlines the importance of a prognostic stratification based on circulating levels of multiple immunosuppressive cytokines, which is currently lacking to our knowledge.

Stockhammer et al. recently reported that high TGF-β levels in pleural effusions of 48 MPM patients were associated with shorter survival, while a prognostic stratification on the basis of serum levels was not confirmed [7]. Other studies indicated a correlation between high circulating levels of a single cytokine (e.g. CCL-2) and higher tumor stage of MPM, suggesting an impact on prognosis [12].

The importance of a composite immunoscore to stratify MPM patients with different prognosis was also brought to light in studies of tumor tissue-based biomarkers [5].



	n°	Median value (pg/ml)	p-value
preChT	23	2.48	
postChT	23	3.47	**0.03**

	n°	Median value (ng/ml)	p-value
preChT	24	1.89	
postChT	24	2.1	0.43

	n°	Median value (pg/ml)	p-value
preChT	23	86.1	
postChT	23	111.1	**0.04**

	n°	%	p-value
preChT	24	6.4	
postChT	24	8.1	**0.007**

Fig. 2. Dynamic changes in circulating biomarkers after chemotherapy. The figure shows the expression levels of TGF-β, IL-10, CCL-2 and monocytes in paired samples of MPM patients before and after chemotherapy (preChT and postChT, respectively).

The availability of reliable circulating prognostic biomarkers instead of inflammatory markers in tumor tissue and pleural effusion has a relevant clinical application, considering the lower risk and limited invasiveness of blood sample collection.

We also observed that monocytes and cytokine levels may be increased by chemotherapy itself.

Previous studies showed that cisplatin as well as other cytotoxic agents such as taxanes and fluoropyrimidines may increase inflammatory and protumorigenic cytokines, thus leading to chemo-refractoriness of different tumor types [13]. These mechanisms are likely to acquire a particular relevance in tumors with high levels of oxidative stress, such as MPM [14].

Recently, maintenance treatment with gemcitabine in MPM patients has been shown to affect circulating immune cells, thus stratifying responders and non-responder patients [15].

The main weakness of our study is the small sample size, which limits

the strength of our findings. However, this is the first study showing a significant stratification of MPM patients, both in terms of OS and PFS, on the basis of a circulating composite inflammatory score in blood samples of MPM patients, collected at different time points during first line systemic treatment.

Our results represent preliminary findings to be incorporated in the new treatment algorithm of MPM patients which has been recently changing, with the introduction of nivolumab plus ipilimumab in the first line setting, and the upcoming results from phase III clinical trials investigating the combination of chemotherapy and immune checkpoint inhibitors with or without antiangiogenic agents. A wider validation set of patients receiving chemotherapy and/or immune checkpoint inhibitors is currently under investigation.

The inflammatory status of the disease at diagnosis may affect treatment choice and radiological follow-up timings, according to PFS and OS stratification; dynamic monitoring of circulating cytokines and monocytes during treatment may identify patients with early disease progression rather than longer benefit, thus avoiding ineffective and detrimental treatments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2021.155622.

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