WORLD JOURNAL OF ADVANCE HEALTHCARE RESEARCH

SJIF Impact Factor: 5.464

ISSN: 2457-0400
Volume: 4.
Issue: 4.
Page N. 84-90
Year: 2020

Original Article www.wjahr.com

CIRCULATING CD34-POSITIVE STEM CELLS IN PERIPHERAL BLOOD OF PATIENTS WITH HODGKIN LYMPHOMA

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Received date: 22 May 2020 **Revised date:** 12 June 2020 **Accepted date:** 02 July 2020

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ABSTRACT

Hematopoietic stem cells are characterized by the properties of self-renewal and multi-lineage differentiation. Bone marrow residing stem cells are mobilized from their BM niches into peripheral blood in many pathological situations including tissue injury and systemic inflammation. Although the role of non-mutated normal stem cells in cancer progression is still under debate. Circulating normal peripheral blood stem cells may play an important role in the vascularization of the growing tumor. We assessed $CD34^+$ cells from the peripheral blood of 15 patients with *de novo* Hodgkin Lymphoma using flowcytometry. We found that $CD34^+$ cell counts and $CD34^+$ cell percentages were significantly higher in Lymphoma patients at diagnosis than in controls. After the induction of chemotherapy, $CD34^+$ cell count and $CD34^+$ cell percentage levels were significantly decreased in Lymphoma patients compared with their levels at diagnosis. The mean baseline levels of $CD34^+$ cell count and $CD34^+$ cell percentage in Lymphoma patients who achieved complete response were significantly lower than in those who did not achieve it in response to induction treatment. We concluded that the number and percentage of circulating $CD34^+$ cells in peripheral blood in cancer patients depend on the tumor type, and can be used as a prognostic value for complete response.

KEYWORDS: Hematopoietic stem cells, CD34-positive, Hodgkin Lymphoma, Flowcytometry.

INTRODUCTION

Lymphoma is defined as a group of hematopoietic malignancies of lymphocytes with more than 90 subtypes.[1] It is traditionally classified as non-Hodgkin or Hodgkin lymphoma. Lymphoma typically presents as painless adenopathy, with some systemic symptoms such as fever, unexplained weight loss, and night sweats occurring in more advanced stages of the disease.[2] There are no clearly defined risk factors for the development of Lymphoma. Some of the contributing factors include familial factors, viral exposure and immune suppression, but the definite cause of Lymphoma remains not fully known.[3] Like other hematopoietic cells, lymphocytes derive from hematopoietic stem cells (HSCs) in the bone marrow (BM) through a multistep process of differentiation into mature cells.[4] HSCs are characterized by the properties of self-renewal and multi-lineage differentiation.[5] HSCs can be harvested from peripheral blood, bone marrow, and umbilical cord blood.[6]

Studies of both common and uncommon lymphoma subtypes indicate that at least some alterations within mature lymphoid neoplasms can develop in uncommitted hematopoietic progenitors. HSCs are long-lived, which affords the opportunity to acquire mutations over time. It has also been shown that different acute myeloid leukemia (AML) subgroups were derived from a common leukemia stem cell (LSC) that shares a $CD34^+CD38^-$ phenotype with normal HSCs,[7] because CD34 is the standard surface marker for HSCs.

It is already known that very rare putative cancer stem cells (SCs) may circulate in the peripheral blood.[8] On

the other hand, the role of non-mutated normal stem cells in cancer progression is still under debate. BM residing stem cells are mobilized from their BM niches into peripheral blood in many pathological situations including tissue injury and systemic inflammation. For example, it was reported that the number of BM-derived stem cells (SCs) increases in patients with pancreatic and stomach cancer.[9] It has also been shown that circulating normal peripheral blood (PB) stem cells may play an important role in the vascularization of the growing tumor.[10]

Different studies have proved the effect of tissue organ defects and injuries in mobilizing hematopoietic stem/progenitor cells (HSPCs) into the PB and its role in providing several soluble trophic factors for growing tumor cells.[11,12] All types of stem cells are also a source of extracellular microvesicles that directly affect the proliferation and survival of cancer cells.

Many studies demonstrated that tumor growth is accompanied by tissue organ hypoxia and damage,[13] we aimed in our study to test whether normal HSCs could be mobilized into the PB during tumor growth and expansion.

The clinical importance of increased $CD34^+$ cells concentration in PB of patients with Hodgkin Lymphoma is still not fully understood. Therefore, our aim was the assessment of $CD34^+$ cell concentration in Lymphoma patients and to test whether $CD34^+$ cell concentration might be used as a predictive parameter in disease development and response to treatment.

MATERIALS AND METHODS

This is a prospective case-control study including 15 patients with *de novo* Hodgkin Lymphoma, who were presented to the hematology/Oncology clinic of Al-Biruni hospital (Damascus, Syria), and 5 healthy controls. All patients and controls were subjected to a thorough history evaluation, no other medical conditions were known at the time of enrollment.

All subjects of this study provided written informed consent. Results of experiments are reported as mean ± standard error.

1. Analysis of Peripheral blood

Five milliliters of peripheral blood collected on EDTA (Vacutainer, Becton Dickinson, USA) were obtained from the controls and the patients who had been recently diagnosed with Hodgkin lymphoma disease and did not receive any treatment.

Patients who already started treatment were excluded. A complete blood test was performed on the samples using fully automated blood counters.

2. Flowcytometry $CD34^+$ cells analysis

Whole blood samples were stained with (10µl) phycoerythrin (PE-) conjugated anti-CD34 monoclonal antibody (MoAB), (10µl) fluorescein isothiocyanate (FITC)-conjugated anti-CD45 MoAB (Miltinyi Biotic, Bergisch Gladbach, USA), and propidium iodide (Sigma-Aldrich, USA) for 20 min at 4°C.

Mouse IgG1-FITC/IgG1-PE was used as the isotype control (Sigma-Aldrich, USA). Samples were analyzed on FACSCalibur (488 and 635 nm; Becton Dickinson, USA). The morphological forward and side scatter parameters allowed the exclusion of debris from the analysis. For each sample, 10000 events were acquired as list mode data using CellQuest software (Becton Dickinson, USA).

Our method for analyzing HSCs was based on the International Society of Hemototherapy and Graft Engineering (ISHAGE) guidelines: four-parameter flowcytometry method (CD45FITC/CD34PE staining, side and forward angle light scatter).

Percent of $CD34^+$ cells was determined by analyzing the entire CD45-positive (dim and bright) population on a CD34-PE FL2-height (X) vs SSC height (Y) dot plot. Background fluorescence activity was determined by isotype antibody and subtracted from each measurement. The total number of $CD34^+$ cells was calculated by multiplying the percentage of $CD34^+$ cells by the total number of nucleated cells in the blood sample obtained on white blood cell (WBC) count.

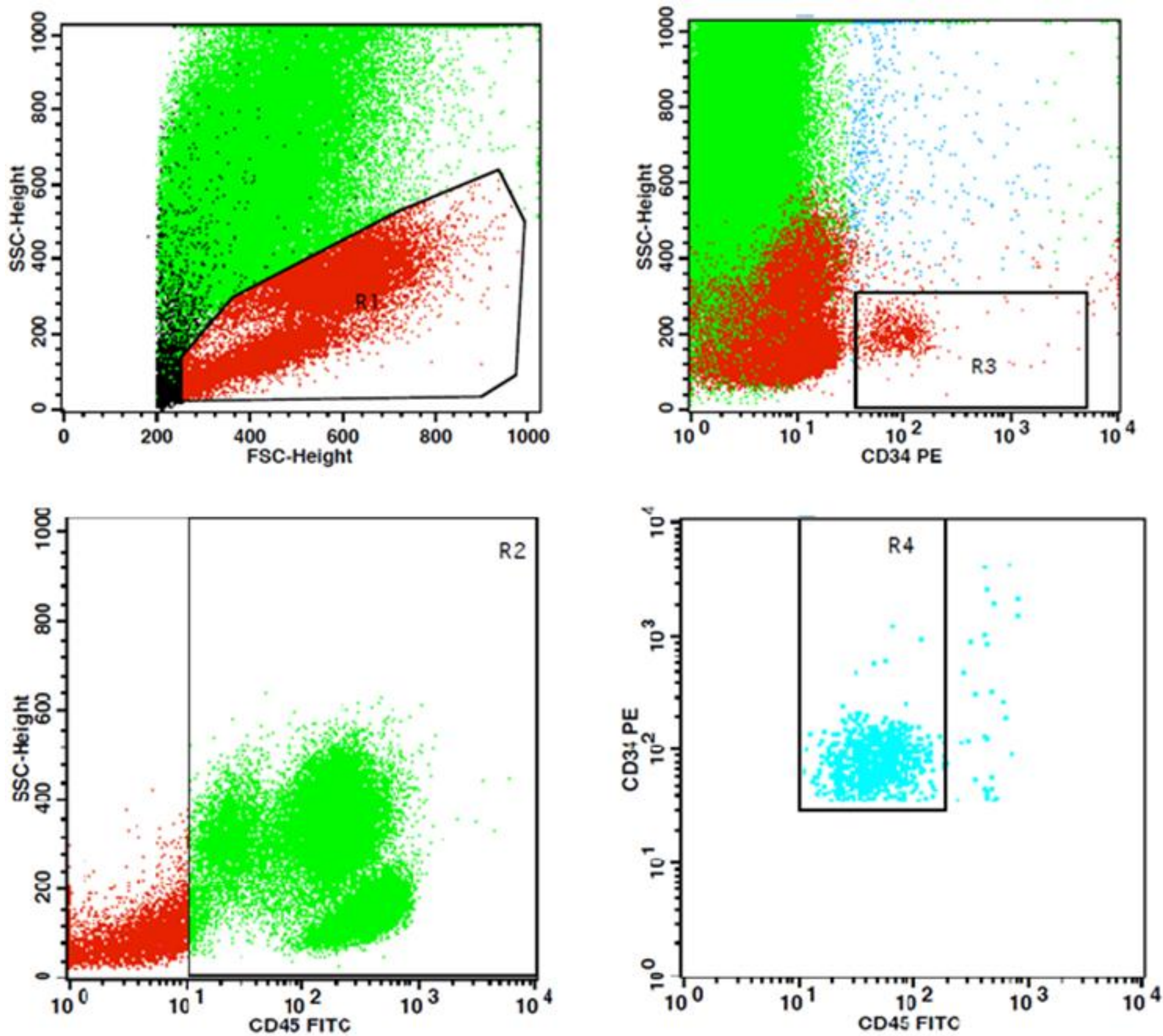

Fig 1: Flowcytometry analysis of CD34+ cells in peripheral blood of patients with Lymphoma. The analysis gate (R1) included mononuclear cells including lymphocytes, monocytes and hematopoietic stem cells. (R2) included CD45+ cells. (R3) included CD34+ cells. (R4) included the target population of CD34+ CD45dim cells.

3. Statistical analysis

Data analysis was performed using SPSS software (V25) (SPSS, Inc., Chicago, IL, USA). Statistical differences between groups were evaluated using the Mann‑Whitney U test and the Wilcoxon signed‑rank test, while the t‑test and the $\chi 2$ test were used for the analysis of continuous and categorical parameters. The Spearman's rank correlation coefficient was used to examine the correlations among the different studied parameters. $P \leq 0.05$ was considered to indicate a statistically significant difference.

RESULTS

A total of 15 Lymphoma patients were included in the present study. The clinical findings and laboratory investigations of both Lymphoma patients and controls are shown in Table 1. There was no significant difference between the two groups in the mean age or gender percentages.

Table 1: Comparative analysis between patients and controls regarding their age and sex.

Parameter	Controls (n=5)	Patients (n=15)	P value
Age (years)	29±4.28	34±3.6	P. value = 0.426[a]
Sex (male/female)	3/2	9/6	P. value = 0.410[b]

[a]: Using Chi square test. [b]: Using Independent t Test.

Regarding white blood cell count and hemoglobin, we noticed a significant difference between the groups as shown in Table 2.

Table 2: Comparative analysis between patients and controls regarding some laboratory parameters.

Parameter	Controls (n=5)	Patients (n=15)	P value[a]
WBC (cell/ml)	5.9±0.52 x 10^6	11.2±1.12 x 10^6	P. value < 0.000
Hemoglobin (g/dl)	13.65±0.38	8.82±0.27	P. value < 0.000

[a]: Using Mann-Whitney U test.

At diagnosis (baseline levels), $CD34^+$ cell counts and $CD34^+$ cell percentages were significantly higher in Lymphoma patients than in controls. However, after the induction of chemotherapy, $CD34^+$ cell count and $CD34^+$ cell percentage levels were significantly decreased in Lymphoma patients compared with their levels at diagnosis, and the levels were also significantly lower than those in the controls (Table 3).

Table 3: $CD34^+$ cell count and percentage in Lymphoma patients at presentation and after chemotherapy vs. controls.

Parameter	Control (n=5)	Patients at diagnosis (n=15)	Patients after chemotherapy (n=15)	P.value[a]	P.value[b]	P.value[c]
$CD34^+$ cell count (cell/µl)	10.64 ± 2.69	131.85 ± 32.19	3.7 ± 0.84	0.004	0.018	<0.000
$CD34^+$ cell percentage (%)	0.56 ± 0.06	2.31 ± 0.67	0.24 ± 0.2	0.009	<0.000	<0.000

[a]P-value of Lymphoma patients at presentation vs. controls (Mann-Whitney U test). [b]P-value of Lymphoma patients after induction chemotherapy vs. controls (Mann-Whitney U test). [c]P-value of Lymphoma patients at presentation vs. Lymphoma patients after induction chemotherapy (Wilcoxon signed-rank test).

After induction chemotherapy, 9 patients (60%) achieved complete response (CR) to treatment, while 6 (40%) did not achieve it. The mean baseline levels of $CD34^+$ cell count and $CD34^+$ cell percentage in Lymphoma patients who achieved CR were significantly lower than in those who did not achieve it in response to induction treatment (Table 4).

Table 4: $CD34^+$ cell count and percentage in Lymphoma patients at presentation and their correlation with treatment response.

Parameter	Patients who achieved CR (n=9)	Patients who did not achieve CR (n=6)	P value
$CD34^+$ cell count (cell/µl)	1.02 ± 0.25	4.58 ± 1.34	0.004
$CD34^+$ cell percentage (%)	67.56 ± 14.33	258 ± 49.27	0.001

Correlation has been assessed between CD34+ cell count and CD34+ cell percentage and some of the tested parameters. No correlation of CD34+ cell count or CD34+ cell percentage with patients' age or gender was observed.

There were significant positive correlations between total leukocyte count with both $CD34^+$ cell count and $CD34^+$ cell percentage (Fig. 2). There was no significant correlation between CD34+ cell count or CD34+ cell percentage levels and hemoglobin levels (Table 5).

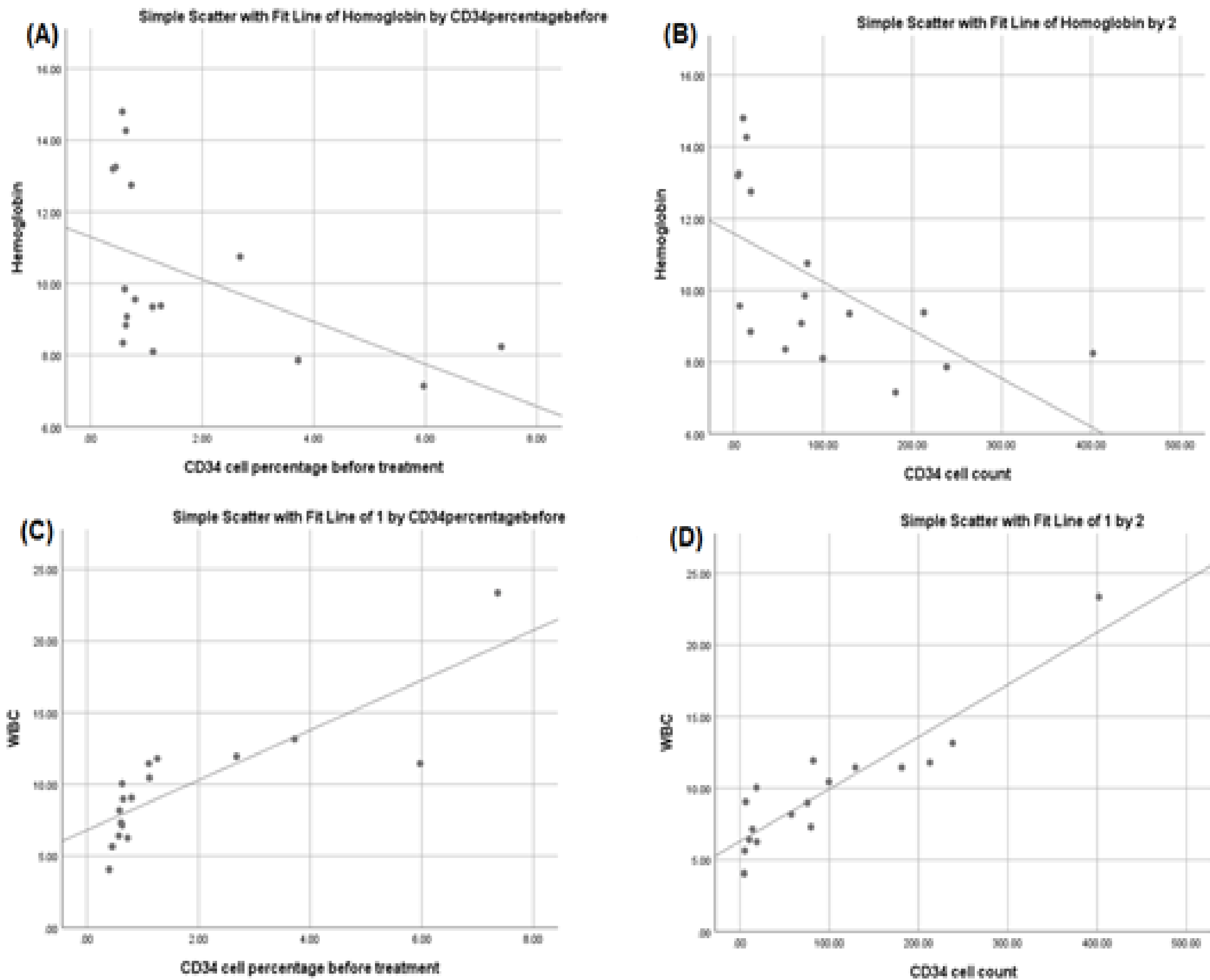

Fig. 2: Spearman's correlation between (A and C) CD34$^+$ cell percentage and (B and D) CD34$^+$ cell count in lymphoma patients at presentation with (A and B) hemoglobin and (C and D) white blood cell count.

Table 5: Spearman's correlation between CD34$^+$ cell count and percentage in patients at presentation and the investigated parameters.

Parameter	WBC (cell/ml)	Hemoglobin (g/dl)
CD34$^+$ cell count		
R (correlation coefficient)	0.869	-0.427
P-value	0.000	0.1
CD34$^+$ cell percentage		
R (correlation coefficient)	0.901	-0.39
P-value	0.000	0.208

DISCUSSION

Lymphoma is a heterogeneous group of malignant tumors of the hematopoietic system and is characterized by the aberrant proliferation of mature lymphoid cells or their precursors.[14] Studies on HSCs depend on different definitions and phenotypic characterization of HSCs in each study, due to the lacking of a universal definition of these cell populations.[15] It is widely accepted that HSCs express the surface marker CD34.[16] Also, HSCs do not express a variety of surface markers that are associated with the terminal maturation of specific blood cell types .CD34 is expressed on less than 0.1% of the nucleated cells in steady-state human PB.[16] There is no definitive hematopiotic stem cell phenotype, only combinations of cell surface markers. In this study, we used the ISHAGE guidelines for HSCs cell determination, that is based on the cell ability to express CD34$^+$ and CD45^{dim}.[17]

The exact mechanism underlying the release of stem cells from BM to the PB is not yet fully understood, but many mechanisms had been described.[12] Some of these mechanisms include the activation of a complement cascade and/or an increase of chemotactic factors in the PB.[9] It is not yet clear why some patients mobilize CD34$^+$ cells better than others do even after the induction of immobilization agents. Some studies suggest that prior

therapy could be a contributing factor.[18] Bensinger, W *et al*. suggested that disease status and prior treatment influence the ability to mobilize PB stem cells.[19] Many patients are described as "poor mobilizers," as they fail to mobilize high levels of HSCs in response to granulocyte colony stimulating factor and other mobilization factors. This mobilization failure rate can be as high as 40%.[20]

Increased circulating CD34$^+$ cell levels have been reported in patients with Myelodysplastic syndrome and chronic myeloid leukemia. Previous studies reported that circulating CD34$^+$ cell level is correlated with disease grade-related and leukemia-free survival, and have found its usefulness as a clinical marker of disease prognosis.[21]

In our study, both CD34$^+$ cell count and percentage were higher in Lymphoma patients than in control group at diagnosis which indicates that CD34$^+$ cells are increased in Lymphoma patients and that may present another helpful parameter in Lymphoma diagnosis. Our results showed that the frequency of CD34$^+$ cells in the PB of lymphoma patients was three to seven fold higher than that of healthy donors which is in accordance with Wu, Sun *et al.* findings.[22]

After induction chemotherapy, CD34$^+$ cell count and percentage dropped lower than their initial levels, this may indicates that chemotherapy affects the cells even in the BM. Even though the percentage of CD34$^+$ cells that are left are small, they are capable of reconstitution the BM.. These findings suggest that the altered circulating HSCs may serve as an important evidence of dysregulated hematopoiesis in patients with cancer.

There was no correlation between CD34$^+$ cell count and percentage and patients' age, gender or hemoglobin, which may indicates that increase in their levels is a common feature in all subtypes of Lymphoma.

The positive correlations between CD34$^+$ cell count and percentage with white blood cell count may indicate that the levels of CD34$^+$ cell counts and percentages are correlated with the tumor mass.

These findings are in accordance with those by Wu, Sun *et al*., who observed that circulating HSCs were highly increased in tumor tissues and positively correlated with disease progression.[22]

Data from Marlicz, W *et al*. indicated that in contrast to pancreatic and stomach cancer patients from previous studies, patients suffering from colorectal cancer did not demonstrate an increase in the number of circulating SCs [9]. Based on this, we conclude that the mobilization of SCs in cancer patients depends on the type of malignancy and other factors as well.

In our study, the CD34$^+$ cell count and percentage levels decreased after induction chemotherapy compared with pre-chemotherapy levels in Lymphoma patients. This reduction in CD34$^+$ cell count and percentage levels may support the clinical relevance of these cells in reflecting the tumor mass. The lower levels of CD34$^+$ cell count and percentage in Lymphoma patients after induction chemotherapy compared with those in controls may be due to the fact that these cells were measured precisely on the time of low active bone marrow recovery, as they were counted on the same day of the chemotherapy dose.

The lower CD34$^+$ cell count and percentage levels in patients who achieved CR compared with patients who did not achieve it at presentation treatment indicate that those levels may be used to detect the treatment response, and they could be used to reflect the level of minimal residual disease in Lymphoma.

There is evidence that EPCs are mobilized from the bone marrow simultaneously with the hematopoietic progenitor,[23] and this may indicates a role in tumor angiogenesis.

In conclusion, the number of circulating CD34$^+$ cells in PB in cancer patients depends on the tumor type, the clinical status of the patient and prior treatment. In contrast to pancreatic and stomach cancer patients and patients with lymphoma from our study, the number of circulating SCs in colorectal cancer was similar to their number that was noted in healthy controls. This could be explained by the lack of activation of pro-mobilizing pathways in those patients. However, we are aware that the number of circulating CD34$^+$ cells may change due to other additional complications, that will lead to increasing their level in PB.[9]

CONCLUSION

CD34$^+$ cells circulate in the peripheral blood of patients with Hodgkin Lymphoma at higher levels than healthy individuals which indicates that these cells contribute with tumor growth. Also, CD34$^+$ cell percentage is reduced after the induction of chemotherapy which indicates that therapy affects cells inside BM. Patients who achieved complete response had lower CD34$^+$ cell levels at diagnosis than patients who had higher CD34$^+$ cell levels. Circulating CD34$^+$ cells may have prognostic significance in Hodgkin lymphoma.

ACKNOWLEDGEMENTS

The authors are grateful to the authorities of Atomic Energy Commission of Syria for the facilities.

Conflict Of Interest
We declare that we have no conflict of interest.

Author's Funding
This work was financed by the Faculty of Pharmacy in Tishreen University.

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