

Evaluation of flow cytometric expressions of CD96 and CD123 on leukemic stem cells in patients with adult acute myeloid leukemia and their utility as prognostic markers

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Background Acute myeloid leukemia (AML) is a disease associated with a risk of relapse or refractoriness to the frontline agents. This is attributable to the quiescent leukemic stem cells (LSC). We aimed to determine the expression status of CD96 and CD123 on the surface of LSC in adult patients with AML and their relationship to prognosis.

Patients and methods A total of 40 adult patients with de novo AML and 40 age-matched and sex-matched controls were recruited from Center 'X,' City 'Y,' Country 'Y' from June 2017 to February 2018, with 1-year follow-up. Bone marrow samples were collected for flow cytometric analysis using CD34, CD38, CD96, and CD123 monoclonal antibodies. For cases, samples were obtained at diagnosis and on day 28 after chemotherapy, whereas for controls, samples were taken once.

Results CD96 and CD123 expressions are significantly higher in patients with AML as compared with controls. CD96 expression is associated with higher initial bone marrow and peripheral blood blast percentages. Day28 CD96 expression is positively correlated with its expression on day 0 and with CD123 expression at diagnosis, with

P values of less than 0.001 and 0.034, respectively. Both markers were much more frequently expressed on LSCs in differentiated AML as compared with ill-differentiated subtypes (*P*<0.05). Both markers are linked to poor therapy outcome, with inferior progression-free survival among CD96-positive and CD123-positive cases at day 28 (*P*=0.035 and 0.041, respectively).

Conclusions CD96 and CD123 represent potential targetable markers for the future development of therapeutic armamentarium for AML.

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Background

Acute myeloid leukemia (AML) is a disease that poses a therapeutic challenge to the clinicians with high mortality ascribable to either therapy-related complications or disease refractoriness [1]. Resistance of AML is attributable to the presence of a subset of malignant cells known as leukemic stem cells (LSC) [2]. These cells are capable of self-renewal and proliferation. Moreover, they remain in quiescent state (G0), evading the effect of the current chemotherapeutic regimens [3].

CD96 is a transmembrane glycoprotein receptor, which is a member of the immunoglobulin superfamily. Its expression is restricted to the cells of hematopoietic origin. It interacts with its cognate receptor, CD 155, suppressing NK-cell activation and killing [4].

Interleukin-3 is a cytokine that is fundamentally secreted by the activated T cells to regulate the production and function of many hematopoietic, immune, and endothelial cells. Its receptor is a heterodimer formed of a cytokine specific alpha subunit (CD123) and a common beta subunit (CD131) [5]. When interleukin-3 binds to CD123, this leads to the subsequent heterodimerization of this subunit

with CD131 [6]. CD123 is overexpressed by LSC, influencing several signaling pathways and causing unchecked cellular proliferation as well as repression of apoptosis [7].

The present study aims at identifying the surface expression status of CD96 and CD123 on LSC (i.e. CD34+ and CD38- population) in adult patients with AML and to establish a relationship between their expressions on one side and disease variables as well as therapy outcomes on the other side. This may shed light on the utility of targeting these two molecules to amplify the success of chemotherapeutic regimens in those patients expressing them on the surface of their LSCs.

Aim

The present study aims at demonstrating the flow cytometric expression of CD96 and CD123 on LSC

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and to examine their associations with other prognostic variables and disease outlook.

Patients and methods

The study setting and participants

The current study was conducted on 80 participants, including 40 adult patients with newly diagnosed AML and 40 age-matched and sex-matched controls free of any hematologic disorder. Patients were enrolled from Center 'X,' City 'Y,' and Country 'Z.' Recruitment took place in the period extending between June 2017 and February 2018. Controls were retrieved from the cases referred to the hospital for the purpose of undergoing open heart surgery. A written informed consent has been obtained from all the study participants along with the approval of the study by the Ethics Committee Board, Center 'X.' The study conformed to the stipulations of Declaration of Helsinki of 1975, as revised in 2008. Study participants were divided into two groups: group I contained adult patients with de novo AML, age between 18 and 65 years. De novo AML is defined as AML occurring with no association to prior chemotherapy or radiotherapy and with no antecedent hematologic disorder.

Group II comprises controls that were selected according to the aforementioned criteria.

Cases were diagnosed as AML as per the last WHO revision of classification of myeloid neoplasms [8]. They were classified according to the cytogenetics using G-banding and fluorescence in-situ hybridization techniques into good, poor, or intermediate risk categories as per the National Cancer Comprehensive Network guidelines [9].

Plan of treatment

(1) Induction therapy:

All patients with AML were induced with the standard induction protocol 3+7 mentioned elsewhere [10].

(2) Consolidation (postremission) therapy:

Cases received high-dose cytarabine when attaining complete remission (CR) as described elsewhere [11].

Materials

Reagents

All the reagents (anti-human CD34, anti-human CD38, anti-human CD123 antibodies) were purchased from Beckman Coulter Company (Brea, California, USA), except anti-human CD96 (TACTILE)

purchased from Affymetrix eBioscience (San Diego, California, USA).

Sample collection procedure

One milliliter of bone marrow has been collected from patients with AML, at the initial diagnosis and on day 28 after induction, and from the control groups through bone marrow aspiration under complete aseptic conditions. Samples have been delivered to a vacutainer tube containing EDTA for the flow cytometry analysis.

Multicolor flow cytometry analysis

In the current study, the analysis was performed using direct staining method by the following monoclonal antibodies: anti-CD34, -CD38, -CD123, and -CD96 antibodies. Samples were measured using EPICS XL-MCL Beckman Coulter. A logarithmic scale was implemented for forward scatter signal, side scatter signal, and for each fluorescent channel. Data analysis was performed as follows: for each specimen, a minimum of 10 000 events were studied. Then, the primary gate was constructed on CD34+ CD38- cells. After that, the measurement of CD123+ and CD96+ percent within the primary gate has been performed using an appropriate isotypic control. Finally, the data have been recorded as percentages.

Statistical analysis

Data were analyzed using IBM SPSS advanced statistics, version 21 (SPSS Inc., Armonk, NY: IBM Corp.). The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were expressed as mean±SD when parametric and median and IQR when nonparametric. Qualitative data were expressed as frequencies and percentages. To compare parametric quantitative variables between the two groups, Student *t* test was applied. For comparison of nonparametric quantitative variables between two groups, Mann-Whitney test was used. The comparison between two groups with qualitative data was done using χ^2 test and/or Fisher exact test instead of χ^2 when the expected count in any cell was found less than 5. Level of confidence was set to 95%, and margin of error was accepted at 5%.

Results

Table 1 demonstrates different clinical and laboratory parameters of the study population. This prospective study included 40 adult newly diagnosed patients with de novo AML. There were 19 (47.5%) males and 21 (52.5%) females. Their ages ranged between 18 and 65 years, with a median of 36.5 years. Moreover, 40 age-matched and sex-matched controls have been

Table 1 Different clinical and laboratory parameters of the study population

Variables	n (%)
Extramedullary disease (N=40)	
Negative	33 (82.5)
Positive	7 (17.5)
Day 28 status (N=40)	
CR	20 (50)
CRi	2 (5)
PR	7 (17.5)
Refractory disease	2 (5)
Died	9 (22.5)
Outcome at day 28 (N=40)	
Favorable	20 (50)
Unfavorable (PR/refractory disease/death)	20 (50)
Survival (N=40)	
Survivors	22 (55.0)
Dead	18 (45.0)
BM blast%	
Range	20–97
Mean±SD	61.1±20.2
Median (IQR)	62 (45–77)
PB blast%	
Range	7–98
Mean±SD	51.4±23
Median (IQR)	51.5 (32.5–71.5)
WBC's (×10 ⁹ /l)	
Range	1.7–109
Mean±SD	32.4±31.2
Median (IQR)	17.9 (10–47)
HGB (g/dl)	
Range	3.3–13.9
Mean±SD	7.5±2.5
Median (IQR)	7.1 (6.1–8.9)
PLT (×10 ⁹ /l)	
Range	6–303
Mean±SD	50.4±52.7
Median (IQR)	30 (19.9–70)
AML FAB subtypes	
M0	1 (2.5)
M1	13 (32.5)
M2	11 (27.5)
M4	9 (22.5)
M5	6 (15)
Cytogenetics	
Good	16 (40.0)
Intermediate risk	14 (35.0)
Poor risk	10 (25.0)
D0 CD96 (N=40)	
Negative	20 (50.0)
Positive	20 (50.0)
D0 CD123 (N=40)	
Negative	21 (52.5)
Positive	19 (47.5)
Day 28 CD96 (N=31)	
Negative	10 (32.3)
Positive	21 (67.7)
Day 28 CD123(N=31)	
Negative	12 (38.7)
Positive	19 (61.3)

AML, acute myeloid leukemia; BM, bone marrow; CR, complete remission; CRi, complete remission with incomplete hematologic recovery; DIC, disseminated intravascular coagulopathy; FAB, French American British; HGB, hemoglobin; PB, peripheral blood; PLT, platelets; PR, partial remission; TLS, tumor lysis syndrome; WBC's, white blood cells.

recruited. There were 25 (62.5%) males and 15 (37.5%) females. Their ages spanned from 24 to 56 years with a median of 37 years.

The majority of cases had M1 and M2 French American British (FAB) subtype, making up 32.5 and 27.5%, respectively. AML with monocytic differentiation was there in 15 patients. Only one case had M0 phenotype. G-banding and fluorescence in-situ hybridization revealed that 16 cases were allocated in the good risk stratum as per the National Cancer Comprehensive Network guidelines [9], 11 patient had t (8;21), and the remaining five patients had derangements of the gene controlling the beta subunit of core binding factor, with one having t (16;16) and four exhibiting inversion 16. A total of 14 patients fitted into the intermediate-risk category, making up 35% of the entire cohort, and 10 cases had cytogenetically normal AML. The remaining four patients were divided into three patients having trisomy 8 and one having t (9;11). Ten patients had poor risk cytogenetics, with the same number of patients having lysine methyltransferase 2 A (KMT2A) gene rearrangements, 5q deletion, and t (6;9) (three cases each), and only one patient had inversion 3.

On day 28, the response to chemotherapy has been evaluated, and all cases have been followed up for 1 year. Of 40 patients recruited, 18 (45%) cases succumbed to their illness. Of 18 cases, nine of them died owing to infections and complications during induction, and the rest died after induction, where seven cases had refractory bleeding, one had renal failure, and another had hepatic failure. Disease recurrence occurred in 15 patients, representing 37.5% of the entire cohort.

Comparison between cases and controls regarding the percentages of CD34+ CD38– LSCs versus hematopoietic stem cells as well as CD96 and CD123 expressions was done. At diagnosis and after induction, our cases have significantly higher mean LSC than controls ($P<0.001$). Regarding CD96 and CD123, their expressions on the surface of stem cell population (i.e. CD34+, CD38– cell population) at diagnosis and after induction showed a statistically significant difference between cases and controls, being higher in AML cases ($P<0.001$). This is well illustrated in Table 2.

Expression of the marker on the surface of more than or equal to 20% of the cells was a prerequisite to consider it positive [12]. Based on that, of 40 patients examined, it is obvious that CD96 and CD123 were positively expressed on the surface of LSC in 20 and 19 patients, respectively, at the time of the diagnosis. Follow-up of their expressions on survivors (31 patients) on day 28

demonstrated that they were expressed in 21 and 19 cases, respectively, by that time.

Using the data aforementioned, the patients' cohort has been segregated based on their levels of expression of either marker into positive and negative expressers at either day 0 or 28 and a comparison has been held between both groups regarding disease-related factors and therapy outcome. This is depicted in Tables 3 and 4.

There is a remarkable association between CD96 positivity in either day 0 or day 28 and significantly higher initial bone marrow blast percentage ($P=0.027$

and 0.017, respectively). It is also confirmed that CD96 exhibited positive correlation to both peripheral blood as well as bone marrow blast percentages, either at diagnosis or after induction ($P=0.036$, 0.018, 0.012, and 0.036, respectively). In addition, CD96 expression on day 28 was positively correlated with its expression on day 0 as well as to CD123 expression at diagnosis ($P<0.001$ and 0.034, respectively). This is shown in Table 5. CD96 was more frequently expressed among well-differentiated AML subcategories as compared with less-differentiated subtypes ($P=0.037$ and 0.043 for days 0 and 28, respectively). As for CD123 expressions on days 0 and 28, it is obvious that it bears no association to any of the patient- or disease-related parameters except for a strong association with differentiated AML subtypes, with P values of 0.016 and less than 0.001, respectively. Neither of the two markers has been linked to extramedullary infiltration or to cytogenetic risk group, in either time points, with P values more than 0.05.

Relating CD96 and CD123 expressions to therapy outcome after induction failed to demonstrate associations either on day 0 or 28. However, CD96-positive and CD123-positive expressors at day 28 exhibited significantly shorter progression-free survival (PFS) ($P=0.035$ and 0.041, respectively) compared

Table 2 Comparison between cases and controls in terms of CD96 and CD123 expressions at different time-points

Variables	Case (mean±SD) (N=40)	Control (mean±SD) (N=40)	P value
CD34+, CD38- cells at diagnosis	43.2±15.4	7.3±1.1	<0.001
CD34+, CD38- cells at day 28	35.4±11.1	7.3±1.1	<0.001
CD123 diagnosis (N=40)	20.7±14.7	3.6±2.4	<0.001
CD 123 day 28 (N=31)	20.28±15.4	3.6±2.4	<0.001
CD 96 diagnosis (N=40)	22.5±12.6	2.5±1.6	<0.001
CD 96 day 28 (N=31)	26.6±13.1	2.5±1.6	<0.001

Table 3 Comparison of the study cohort in terms of CD123 and CD96 expression status at D0 and their relationship to different disease variables

Disease variables	D0 (N=40) [n (%)]					
	CD123			CD96		
	Positive (N=19)	Negative (N=21)	P value	Positive (N=20)	Negative (N=20)	P value
Extramedullary disease						
Present (N=7)	4 (21.1)	3 (14.3)	0.689	4 (20.0)	3 (15.0)	1.000
Absent (N=33)	15 (78.9)	18 (85.7)		16 (80.0)	17 (85.0)	
BM blasts						
IQR	20–90	25–97	0.764	30–97	20–90	0.027
Median	63	60		70.5	51	
Cytogenetics						
Good (N=16)	9 (47.4)	7 (33.3)	0.396	8 (40.0)	8 (40.0)	0.693
Intermediate risk (N=14)	6 (31.6)	8 (38.1)		8 (40.0)	6 (30.0)	
Poor (N=10)	4 (21.1)	6 (28.6)		4 (20.0)	6 (30.0)	
FAB subtypes						
M0,M1 (N=14)	5 (26)	9 (38)	0.016	8 (40)	6 (30)	0.037
M2,4,5 (N=26)	14 (74)	12 (62)		12 (60)	14 (70)	
PFS (days)						
IQR	206.6–340.2	136.6–367.0	0.678	136.6–355.4	211.1–346.5	0.770
Median	265.7	307.0		271.2	298.3	
Treatment outcome at day 28						
Favorable (N=20)	9 (47.4)	11 (52.4)	0.796	9 (45)	11 (55)	0.523
Unfavorable (N=20)	10 (52.6)	10 (47.6)		11 (55)	9 (45)	
Median OS						
IQR	200–320	222–350	0.689	210–330.6	250–345	0.345
Median	270.3	310.4		290.5	320	

BM, bone marrow; FAB, French American British; IQR, interquartile range; OS, overall survival; PFS, progression-free survival.

Table 4 Comparison of the study cohort in terms of CD123 and CD96 expression status at day 28 and their relationship to different disease variables

Disease variables	Day 28 (N=31) [n (%)]					
	CD123			CD96		
	Positive (N=19)	Negative (N=12)	P value	Positive (N=21)	Negative (N=10)	P value
Extramedullary disease						
Present (N=3)	2 (10.5)	1 (8)	0.226	2 (9.5)	1 (10)	0.681
Absent (N=28)	17 (89.5)	11 (92)		19 (90.5)	9 (90)	
BM blasts						
IQR	20–70	33–97	0.498	33–97	20–90	0.017
Median	61	62		76	55	
Cytogenetics						
Good (N=16)	8 (42)	8 (67)	0.953	10 (48)	6 (60)	0.520
Intermediate risk (N=10)	7 (37)	3 (25)		8 (38)	2 (20)	
Poor (N=5)	4 (21)	1 (8)		3 (14)	2 (20)	
FAB subtypes						
M0,M1 (N=8)	6 (31.5)	2 (16.5)	<0.001	5 (24)	3 (30)	0.043
M2,4,5 (N=23)	13 (68.5)	10 (83.5)		16 (76)	7 (75)	
PFS (days)						
IQR	81.8–325.0	194.9–361.2	0.041	215.8–355.4	165.0–326.0	0.035
Median	210.8	313.5		200	283.6	
Treatment outcome at day 28						
Favorable (N=20)	12 (63)	8 (66)	0.443	14 (67)	6 (60)	0.720
Unfavorable (N=11)	7 (37)	4 (34)		7 (33)	4 (40)	
OS (days)						
IQR	195.5–315.6	260–340.7	0.111	287.6–330.6	260–355.8	0.367
Median	300	320.3		300.5	340.3	

BM, bone marrow; FAB, French American British; OS, overall survival; PFS, progression-free survival.

Table 5 Correlation of CD123 and CD96 with other quantitative patients' and disease-related variables

	CD123 diagnosis	CD123 day 28	CD96 diagnosis	CD96 day 28
CD96 diagnosis				
<i>r</i>	0.264	0.214		
<i>P</i>	0.100	0.185	–	
CD96 day 28				
<i>r</i>	0.336	0.268	0.914	
<i>P</i>	0.034	0.095	<0.001	
Age				
<i>r</i>	0.130	0.097	–0.018	0.052
<i>P</i>	0.422	0.552	0.913	0.752
BM blast%				
<i>r</i>	0.053	0.167	0.397	0.336
<i>P</i>	0.750	0.311	0.012	0.036
PB blast%				
<i>r</i>	–0.061	0.010	0.333	0.372
<i>P</i>	0.710	0.952	0.036	0.018
WBCs				
<i>r</i>	–0.192	–0.104	0.036	0.066
<i>P</i>	0.234	0.524	0.825	0.686
Hb				
<i>r</i>	–0.014	–0.068	0.073	0.039
<i>P</i>	0.930	0.678	0.654	0.809
Platelets				
<i>r</i>	0.209	0.206	0.156	0.146
<i>P</i>	0.196	0.201	0.336	0.369

BM, bone marrow; Hb, hemoglobin; PB, peripheral blood; WBC, white blood cell.

with negative expressors. Moreover, positive expressors of both markers at diagnosis and after induction exhibited shorter median overall survival (OS), but these differences did not culminate into statistical significance.

Discussion

AML represents a challenging clinical entity, with significant heterogeneity at the molecular level. This disease has witnessed discernible alterations in the recent years, thanks to the better understanding of the molecular pathogenesis with the subsequent development of novel targeted agents [13].

CD96 and CD123 are two among several markers observed to be expressed on the surface of LSC [14].

Upon testing these markers in our study, it is noticeable that CD96 and CD123 expressions among CD34+ CD38- LSCs were significantly higher in AML cases either at diagnosis or after treatment when compared with their expressions on the surface of normal bone marrow hematopoietic stem cells in the control group. This result is reproducible in a study conducted by Chávez-González *et al.* [15] that had assessed expressions of four markers, that is, CD90, CD117, CD96, and CD123 on the surface of LSC and hematopoietic progenitor cells, which are CD34+ and CD38+.

Again, these findings were corroborated by the work done by Hussein *et al.* [16].

In our study, we concluded no correlation or association between the expressions of CD96 or CD123 on the surface of LSCs from one side and the patients' age or sex from the other side were encountered. This is in agreement with Zhao *et al.* [17].

It is observable that CD96 expression was correlated to bone marrow as well as peripheral blast percentages. These findings are accordant with the study done by Al-Fatlawi and Musa [18]. They observed a higher initial total leukocytic count as well as bone marrow blast percentage in CD96-positive cases in comparison with CD96-negative ones. Moreover, they noticed a higher median total leukocytic count in CD123-positive expressors than negative expressors [18]. This may be reflected on prognosis of CD96-positive and CD123-positive patients.

According to our study, AML with differentiation (M2,4,5) was the predominant FAB type in CD96-positive and CD123-positive cases on day 0. This

finding is replicated on day 28. Hosen *et al.* [19] reported that CD96 expression in the AML-LSC population was more frequent in M2 compared with M0/M1 or M4/M5 samples, whereas Zhao *et al.* [17] stated that among the different subtypes of acute leukemia, much difference has been observed regarding their expressions of both CD96 and CD123. The expressions of CD96/CD123 were much lower in AML-M3 subtype but higher in AML-M4, M5, M6, and T-acute lymphoblastic leukemia [17].

Al-Fatlawi and Musa [18] demonstrated that the highest percentages of CD123 expression had been observed in M5 subtype, whereas Testa *et al.* [7], and Hussein *et al.* [16], denied any association between CD123 expression and the different AML FAB categories.

Most of our positive cases for both markers had no extramedullary disease and had unfavorable cytogenetics; however, this exhibits no statistical significance, with *P* value exceeding 0.05. Hwang *et al.* [1], reported that no certain cytogenetic findings have been associated with the proportions or immunophenotypes of LSCs.

Trial to find correlations between our two studied markers revealed that CD96 expression on day 28 was positively correlated with its expression on day 0 as well as to CD123 expression at diagnosis. This may reflect that cells expressing CD96 exhibit poor response to the conventional induction regimen and may need specific target therapy to avoid relapse or progression by the later activation of these quiescent cells. Al-Fatlawi and Musa [18] addressed that all CD96-positive LSCs were also CD123 positive.

We found that there is a statistically significant association between CD96-positive cases at day 28 and short PFS. Furthermore, they have shorter median OS as compared with negative expressors, even though this is statistically insignificant. This may reflect the need of studying the two markers on a more ample sample size so as to address an association of markers expression with survival.

This is in contrast to Zhao *et al.* [17], observing that patients with the expression of LSCs immunophenotype, especially those expressing CD96, had decreased survival rate and exhibited poorer prognosis.

Concerning CD123, there is no statistically significant relationship between this marker expression at diagnosis or day 28 from one side and therapy outcome

after induction from the other side, but longer PFS among CD123-negative cases in comparison with CD123-positive ones on day 28 is notice. Moreover, they showed longer median OS despite lack of statistical significance. This is compatible with the observation of Hwang *et al.* [1]. They observed higher level of CD123 expression on LSCs in refractory/relapsed patients, but the difference was extremely mild, yielding no statistical significance. Conversely, Al-Fatlawi and Musa [18] reported that among the 12 CD123-positive expressors, only four (33.3%) cases acquired CR, whereas 16 (88.9%) of 18 negative expressor patients acquired CR, implying worse initial response to induction protocols among cases expressing that marker. These results were similar to those results noted by Zhao *et al.* [17], Farweez *et al.* [20], and Ge *et al.* [21] who found that CD123 expression was associated with a reduced response to induction chemotherapy. Moreover, in study done on 80 patients with AML, multiple LSC marker expressions (CD25, CD96, and CD123) were significantly associated with shorter 3-year OS, compared with those patients with single or no LSC marker expression (18.2 vs. 65.0%, $P < 0.001$) [22]. The cause of inability to yield statistically significant difference of OS might be the need to study this marker on a wider scale.

Given the above, CD96 and CD123 may be considered as poor prognostic surface markers of LSCs, predicting more aggressive disease with higher risk of recurrence and lower survival probabilities.

Conclusion

CD96 and CD123 may be used as a marker for speculating the risk of recurrence in AML. Moreover, they may serve as potential targetable markers for the future therapy for AML. Further studies with the repeated assessment of CD96 and CD123 expression throughout the course of the treatment on a larger number of patients would help better evaluation of their influence on the clinical outcome and disease progression.

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author. All authors have read and approved the manuscript. Moreover, the requirements for authorship have been met, and each author believes that the manuscript represents honest work.

Prior presentations: this research has been presented in poster session of Seventh Annual Society of Haematologic Oncology (SOHO) Convention held in Houston in 2019. Moreover, an abstract titled 'Prognostic significance of CD96 and CD123 in adult acute myeloid leukemia in Egypt' has been published in *Clinical Lymphoma Leukaemia and Myeloma Journal*; the official Journal of SOHO.

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Conflicts of interest

There are no conflicts of interest.

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