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*Original Article*

# Proinflammatory cytokines as potential risk factors of acute graft-versus-host disease and infectious complications after allogeneic hematopoietic stem cell transplantation

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**Abstract:** Objective: Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is associated with a risk of graft-versus-host disease (GvHD) and infections. The pathogenesis of acute GvHD is related to T-lymphocytes, which identify alloantigens on host antigen-presenting cells, induce production of interferon (IFN) gamma and interleukin (IL)-2, recruit immune effector cells and destroy tissues and organs. Material and methods: The study involved 62 patients, 30 (48%) men and 32 (52%) women [median age 49.5; (19-68) years] after myeloablative conditioning (MAC) n = 26 (42%) or reduced intensity conditioning (RIC) n = 36 (58%) therapy before allo-HSCT from a sibling (n = 12) or unrelated (n = 50) donor due to acute myeloid leukemia (AML). All patients received standard immunosuppressive therapy with cyclosporine A and methotrexate plus pre-transplant anti-thymocyte globulin in the unrelated transplant setting. Blood samples were collected pre-transplant before the start of and after conditioning therapy (1 day pre-transplant) and 2, 4, 6, 10, 20, 30 days following allo-HSCT. The analysis of potential risk factors included IL-2 and IFN-gamma concentrations, patients' age, the use of MAC/RIC and CR/non-CR status before transplantation. Results: The statistical analysis revealed that independent risk factors for aGvHD included non-CR status before allo-HSCT [odds ratio (OR) = 10.52, P = 0.040], the use of MAC [hazard ratio (HR) = 4.80, P = 0.007] and a high level of IFN-gamma on day 6 post-transplant (HR = 1.03, P = 0.032). MAC was also the independent risk factor for infectious complications (OR = 4.04, P = 0.024). Conclusion: A high level of IFN-gamma on day 6 post-transplant, non-CR status before allo-HSCT and the use of MAC are independent risk factors for aGvHD. MAC is also the independent risk factor of infectious complications.

**Keywords:** IFN-gamma, myeloablative conditioning therapy, allogeneic stem cell transplantation, acute graft-versus-host disease, infectious complications

## Introduction

The allogeneic hematopoietic stem cell transplantation (allo-HSCT) is associated with a risk of complications such as graft-versus-host disease (GvHD) and infections. Graft-versus-host disease is one of the most important and potentially fatal complications following allo-HSCT. It is observed after transplants from related and unrelated donors as acute (aGvHD) or chronic (cGvHD) form of the disease. Donor T-lymphocytes responsible for GvHD induction are activated by classical human leukocyte antigens (HLA) in the case of transplants from partially matched donors or by poor HLAs in the case of transplants from fully matched donors [1-4]. According to the pathophysiological concept of aGvHD proposed by Ferrara et al. [5] the

etiology of aGvHD is associated with three consecutive stages, i.e. in the first phase, pre-transplant conditioning treatment leads to damage and activation of host tissues with the induction of proinflammatory cytokines [tumor necrosis factor (TNF)-α and interleukin (IL)-1], followed by activation of antigen-presenting cells (APCs). In the second phase, T-lymphocytes recognizing alloantigens on host cells initiate the so-called "cytokine storm", including secretion of interferon (IFN)-gamma and IL-2 and recruit immune effector cells, followed by tissue and organ damage in the third phase due to an inflammatory process induced by cytokines produced by activated cytotoxic T lymphocytes, natural killer (NK) cells and macrophages that inhabit organs and tissues. Both IFN-gamma and IL-2 are key cytokines that trigger graft-versus-host response by multiple activation of immune cells in response to alloantigens [6-10].

## Material and methods

The study involved 62 patients with acute myeloid leukemia (AML) who underwent allo-HSCT from 2012 to 2014 in the Department of Hematology and Bone Marrow Transplantation of the SPSKM Hospital, Katowice, Poland. Approval No. KNW/0022/KB1/71/I/12 was obtained from the Bioethics Committee of the Medical University of Silesia in Katowice, Poland on 3rd July 2012. All patients have singed the informed consent before entering the study. The study group comprised 30 (48%) men and 32 (52%) women, aged 19 to 68 (median age 49.5). The time from diagnosis to transplantation ranged from 4 months to 10 years (median 11 months). Fifty four (87%) patients achieved complete remission (CR), 3 (5%) partial remission (PR) and other patients did not achieve remission at the time of transplantation. Conditioning treatment was performed according to the following regimens: TreoFluATG (n = 26; 42%), BuCyATG (n = 14; 23%), BuCy (n = 6; 10%), TreoFlu (n = 5; 8%), TBICyATG (n = 5; 8%), BuFluATG (n = 3; 5%) and TreoFluThymo, BuFlu, BuCyThymo in individual cases, of which myeloablative conditioning (MAC) accounted for 42% (n = 26) while reduced intensity conditioning (RIC) for 58% (n = 36). Fifty (81%) patients underwent unrelated donor hematopoietic stem cell transplantation (URDHSCT), while others (n = 12, 19%) underwent matched related donor hematopoietic stem cell transplantation (MRDHSCT). All patients underwent standard immunosuppressive therapy based on cyclosporine A (CsA) and methotrexate (MTX) in 95% of cases plus anti-thymocyte globulin (ATG) in URDHSCT. aGvHD was diagnosed based on the clinical criteria and graded according to the Glucksberg scale. Infectious complications were diagnosed on the basis of clinical symptoms and microbiological results of the collected samples. Four patients (6%) died during the hospital stay within 30 days after allo-HSCT. Peripheral blood samples (5 ml) were collected from each patient at the following time points: prior to conditioning treatment, after its completion (day 1 pre-transplant) and on days 2, 4, 6, 10, 20 and 30 post-transplant, unless death occurred earlier. The collected blood was immediately centrifuged and the obtained serum was stored frozen at -80°C until analysis. Concentrations of IFN-gamma and IL-2 were determined by ELISA.

### *Statistical analysis*

Patients characteristics were presented as the percentage distribution of qualitative variables, while the median and range were used for quantitative variables. Concentrations of the cytokines underwent preliminary assessment at all measurement points by calculating the average value, the median, standard deviation (SD), standard error of the mean (SEM) and by determining the minimum and maximum values. The hypothesis on the normality of their distribution verified by the Shapiro-Wilk test was rejected due to the strong right-sided asymmetry of cytokine distribution. In further analyses, the median was used as a measure of central tendency, and the interquartile range was used as a measure of dispersion. In addition, nonparametric procedures were used to test statistical hypotheses. Variables determining the values of cytokine concentrations at the measurement points were categorized not only in relation to the cut-off points specified by the kit manufacturer (7 pg/ml for IL-2 and 5 pg/ml for IFN-gamma), but also in relation to the 0 value.

To determine risk factors, we used the assessment of the impact of selected explanatory variables on the probability of the occurrence of a

**Table 1.** Post-allo-HSCT complications

| | | |
|---|---|---|
| Infection, n (%) | Yes | 38 (61) |
| | No | 24 (39) |
| Onset of the first symptoms of infection [day], median (range) | | 9 (0-27) |
| Mucositis, n (%) | Yes | 26 (42) |
| | No | 36 (58) |
| Onset of the first symptoms of mucositis [day], median (range) | | 1 (0-20) |
| aGvHD, n (%) | 0 | 32 (52) |
| | I | 26 (42) |
| | II | 3 (5) |
| | III | 1 (2) |
| | IV | 0 (0) |
| aGvHD, n (%) | Skin | 28 (45) |
| | Intestine | 3 (5) |
| | Liver | 0 (0) |
| Time of the manifestation of aGvHD [day], median (range) | | 17 (8-29) |

specific event using the logistic regression analysis and the assessment of the impact of selected explanatory variables on the risk at the time of the occurrence of a specific event based on Cox proportional hazards regression analysis. Statistical analysis was performed with significance level at 0.05. The value of $P < 0.05$ was considered statistically significant with the confidence interval (CI) of 95%.

## Results

Hematological restoration after HSCT was achieved in 61 (98%) patients with following median post-transplant recovery times: white blood count (WBC) > 1.0 G/l - 15 (11-25) days, absolute neutrophil count (ANC) > 0.5 G/l - 17 (11-27) days.

*Analysis of risk factors in patients with aGVHD*

*Clinical parameters*: Manifestation of aGvHD after allo-HSCT was reported in 30 (48%) patients-median time of manifestation was day 17 post-transplant (range 8-29). Acute GvHD grade I was diagnosed in 26 (42%) patients, grade II in 3 (5%) patients, grade III in 1 (2%) patient and grade IV was not reported. The disease involved the skin in 28 (45%) patients and the intestines in 3 (5%) patients. Liver involvement was not found. In the remaining 32 (52%) patients, symptoms of aGvHD were not observed (**Table 1**).

Patients with aGvHD (grades I-III) were younger than patients without (median 44 years vs. 53 years, P = 0.012) (**Table 2**; **Figure 1**) and this group simultaneously included more patients who underwent transplantation without CR (23% vs. 3%, P = 0.046) (**Table 2**) and those who underwent MAC (60% vs. 25%, P = 0.005) (**Table 2**).

Additionally, 21 (70%) patients with aGvHD reported complications in the form of bacterial and/or fungal and/or viral infection(s) - the median time of onset of the first infectious episode was day 10 post-transplant (range 1-27). Mucositis was diagnosed in 15 (50%) patients - the median time of onset was day 2 post-transplant (range: 0-8), which was, however, not reported as significantly different if compared to patients without aGVHD (**Table 2**).

*Statistical analysis:* To assess the influence of cytokine concentration on the risk of aGvHD (grades I-III) at a given time, the cumulative incidence method, considering events constituting "competing risk"-death within 100 days post-transplant without aGvHD, was used. The analysis showed that high IFN-gamma concentration on day 6 post-transplant was a significant risk factor for aGvHD [hazard ratio (HR) = 1.04, P = 0.008] (**Figure 2**). IL-2 level had no effect on the risk of aGvHD at any of the time points analyzed.

The logistic regression model included the following variables that significantly influenced the probability of aGvHD (grades I-III) in the univariate analysis: age (continuous variable), disease status before allo-HSCT (CR/non-CR status), and the type of conditioning treatment (MAC/RIC). Backward stepwise regression sh-

**Table 2.** Comparison of clinical parameters in patients with aGvHD and without aGvHD

| | | aGvHD I-III (n = 30) | aGvHD 0 (n = 32) | p |
|---|---|---|---|---|
| Recipient age [years], median (range) | | 44 (19-61) | 53 (19-68) | 0.012 |
| Recipient gender; n (%) | Male | 13 (43) | 17 (53) | 0.441 |
| | Female | 17 (57) | 15 (47) | |
| Time from diagnosis to transplant [years], median (range) | | 0.8 (0.4-7.1) | 0.9 (0.4-9.8) | 0.410 |
| Disease status prior to allo-HSCT; n (%) | CR | 23 (77) | 31 (97) | 0.046 |
| | Other | 7 (23) | 1 (3) | |
| Conditioning treatment; n (%) | MAC | 18 (60) | 8 (25) | 0.005 |
| | RIC | 12 (40) | 24 (75) | |
| Anti-thymocyte globulin; n (%) | Yes | 21 (70) | 27 (84) | 0.180 |
| | No | 9 (30) | 5 (16) | |
| Type of allo-HSCT; n (%) | MRD | 8 (27) | 4 (13) | 0.162 |
| | URD | 22 (73) | 28 (87) | |
| Immunosuppressive treatment; n (%) | (CsA+MTX) | 28 (93) | 31 (97) | 0.954 |
| | Other | 2 (7) | 1 (3) | |
| Infection; n (%) | Yes | 21 (70) | 17 (53) | 0.173 |
| | No | 9 (30) | 15 (47) | |
| The onset of the first symptoms of infection [day], median (range) | | 10 (1-27) | 8 (0-19) | 0.162 |
| Mucositis; n (%) | Yes | 15 (50) | 11 (34) | 0.213 |
| | No | 15 (50) | 21 (66) | |
| The onset of the first symptoms of mucositis [day], median (range) | | 2 (0-8) | 1 (0-20) | 0.807 |

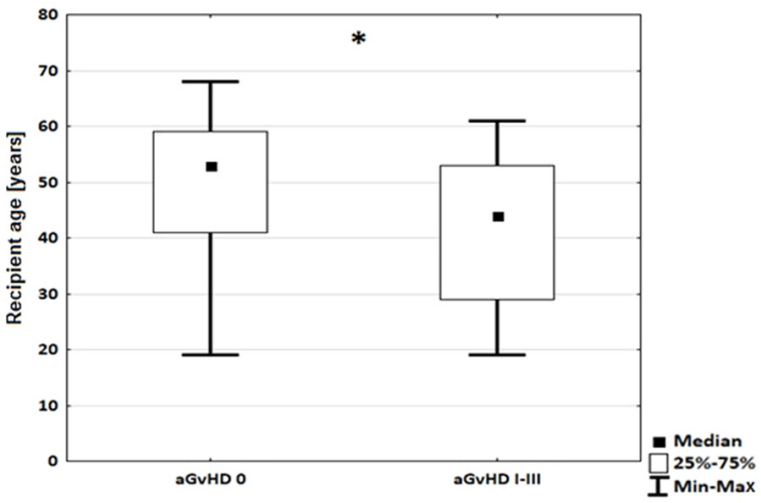


**Figure 1.** The prevalence of aGvHD depending on the age of the recipient *P < 0.05.

owed that independent parameters significantly increasing the risk of aGvHD included non-CR status before allo-HSCT [odds ratio (OR) = 10.52, P = 0.040] and MAC (HR = 4.80, P = 0.007) (**Table 3**).

The Cox proportional hazards regression model included the following variables that significantly influenced the risk of aGvHD (grades I-III) in the univariate analysis at a given time: age, disease status before allo-HSCT (CR/non-CR status), the type of conditioning treatment (MAC/RIC) and IFN-gamma concentration on day 6 post-transplant. Based on the backward stepwise regression, independent clinical parameters significantly increasing the risk of aGvHD included non-CR status before allo-HSCT (HR = 3.38, P = 0.006), MAC (HR = 2.30, P = 0.032) and high IFN-gamma concentration on day 6 post-transplant (HR = 1.03, P = 0.032) (**Table 4**).

*Analysis of risk factors in patients with infection*

*Clinical parameters:* The post-allo-HSCT period was complicated by bacterial and/or fungal and/or viral infection(s). Complications in the form of infection regardless of the etiology occurred in 38 (61%) patients (median time of occurrence of the first episode on day 9 post-transplant; range 0-27). Mucositis occurred in

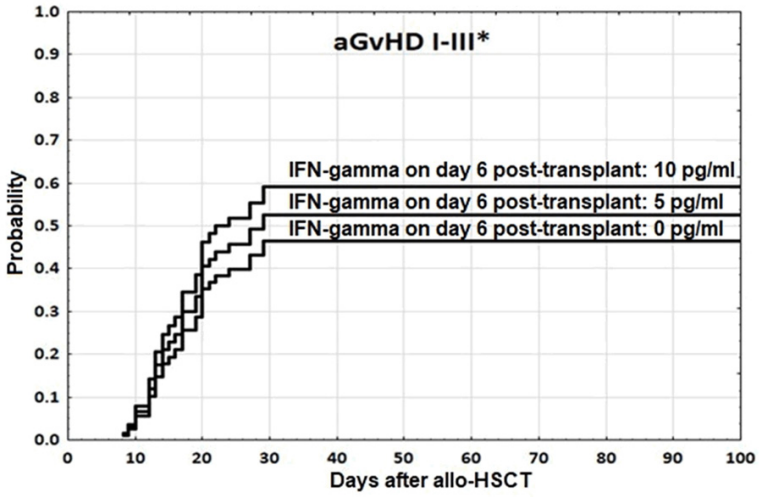


**Figure 2.** Cumulative prevalence curves for aGvHD depending on IFN-gamma serum concentrations on day 6 post-transplant *P < 0.05.

26 (42%) patients (median time day 1 post-transplant; range 0-20).

Patients with infection were younger than patients who did not present with infectious complication (median 44.5 vs. 53.5 years, P = 0.024) (**Table 5**; **Figure 3**). In addition, MAC was more frequently used in those patients (55% vs. 21%, P = 0.007) (**Table 5**).

*Statistical analysis:* The logistic regression model included the following variables that significantly influenced the probability of infection in the univariate analysis: age, type of conditioning treatment (MAC/RIC), IFN-gamma concentration on day 6 post-transplant (variable categorized in relation to the 0 value). Backward stepwise regression showed that the parameter significantly increasing the risk of infection was MAC (OR = 4.04, P = 0.024) (**Table 6**).

## Discussion

This paper presents potential risk factors that may be associated with the occurrence of aGvHD and infectious complications after allo-HSCT.

Statistical analysis of risk factors for post-allo-HSCT complications was performed separately for the group of patients with aGvHD symptoms and the group of patients with infectious complications. It was demonstrated that statistically significant risk factors for the occurrence of aGvHD include high IFN-gamma concentration on day 6 post-transplant, non-CR status before allo-HSCT and the use of MAC. In the group of patients with infectious complications, the use of MAC was the risk factor for infection, while the influence of IFN-gamma concentration > 0 on day 6 post-transplant did not exceed the trend (P = 0.065). Due to a small number of analyzed groups, the results of the analyses should be verified on larger and more homogeneous groups.

Risk factors that have a decisive impact on the occurrence of aGvHD include donor-recipient HLA incompatibility, age and gender of the donor and the recipient, prior recipient alloimmunization, source and amount of transplanted material, a small percentage of FOXP3-positive regulatory T lymphocytes in the recipient's body and in the transplant material, type of conditioning treatment [MAC with total body irradiation (TBI)] and the prophylaxis of aGvHD [11-16]. However, risk factors for post-allo-HSCT infection include hematological status of the underlying disease at the time of allo-HSCT, MAC, especially based on TBI, comorbidities, disruption of natural protective anatomical barriers (mucositis, catheters and peripheral/central vascular access), immunosuppressive treatment and the occurrence of aGvHD and its treatment [17].

According to the above reports and the results of this study, the MAC regimen is both a risk factor for aGvHD and infectious complications. In turn, the hematological status of the underlying disease in this study was considered a risk factor for aGvHD, as opposed to literature data that recognized it as a risk factor for infectious complications. In this study, however, the recipient's age was not found to be a risk factor for aGvHD, which is contrary to previously published reports. IFN-gamma was indicated as a potential risk factor and a biomarker of aGvHD that is useful in early disease detection. The diagnostic usefulness of IL-2α receptor (IL-2Rα), TNF receptor-1 (TNFR-1), hepatocyte growth factor (HGF), IL-8, elafin (skin-specific marker),

**Table 3.** Logistic regression of aGvHD (grades I-III) risk factors

| | Assessment of β parameter | Standard error | OR | 95% CI | P |
|---|---|---|---|---|---|
| Intercept | -0.97 | 0.39 | 0.38 | 0.17-0.83 | 0.013 |
| Type of conditioning treatment (RIC/MAC) | 1.57 | 0.58 | 4.80 | 1.50-15.35 | 0.007 |
| Disease status prior to allo-HSCT (CR/non-CR status) | 2.35 | 1.14 | 10.52 | 1.07-103.78 | 0.040 |

**Table 4.** Assessment results of the Cox proportional hazards regression model of risk factors for aGvHD (grades I-III)

| | Assessment of β parameter | Standard error | HR | 95% CI | P |
|---|---|---|---|---|---|
| Type of conditioning treatment (RIC/MAC) | 0.83 | 0.39 | 2.30 | 1.07-4.94 | 0.032 |
| Disease status prior to allo-HSCT (CR/non-CR status) | 1.22 | 0.45 | 3.38 | 1.41-8.10 | 0.006 |
| IFN-gamma concentration on day 6 post-transplant (continuous variable) | 0.03 | 0.01 | 1.03 | 1.00-1.06 | 0.032 |

**Table 5.** Comparison of clinical parameters of patients with and without infection

| | | Infection (n = 38) | No infection (n = 24) | P |
|---|---|---|---|---|
| Recipient age [years], median (range) | | 44.5 (19-66) | 53.5 (21-68) | 0.024 |
| Recipient gender; n (%) | Male | 20 (53) | 10 (42) | 0.400 |
| | Female | 18 (47) | 14 (58) | |
| Time from diagnosis to transplant [years], median (range) | | 0.9 (0.4-9.8) | 0.9 (0.4-7.1) | 0.618 |
| Disease status prior to allo-HSCT; n (%) | CR | 32 (84) | 22 (92) | 0.643 |
| | non-CR status | 6 (16) | 2 (8) | |
| Type of conditioning treatment; n (%) | MAC | 21 (55) | 5 (21) | 0.007 |
| | RIC | 17 (45) | 19 (79) | |
| Anti-thymocyte globulin (ATG); n (%) | Yes | 29 (76) | 19 (79) | 0.795 |
| | No | 9 (24) | 5 (21) | |
| Type of allo-HSCT; n (%) | MRD | 7 (18) | 5 (21) | 0.924 |
| | URD | 31 (82) | 19 (79) | |
| Type of immunosuppression; n (%) | CsA+MTX | 35 (92) | 24 (100) | 0.422 |
| | Other | 3 (8) | 0 (0) | |

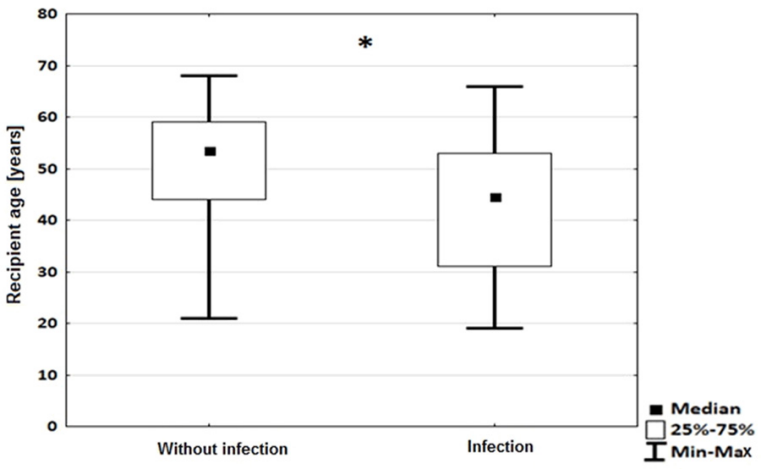


**Figure 3.** The prevalence of infection depending on the age of the recipient *P < 0.05.

Reg3α (gastrointestinal tract-specific marker) and cytokeratin-18 fragment as the biomarkers of aGvHD have been confirmed by others [18-21]. All the above substances (except for cytokeratin-18 fragment) seem to have a prognostic value for the occurrence of a particular form of aGvHD, response to treatment and treatment-related mortality (TRM) [20, 21]. Another study showed that a 6-proteins composite aGvHD biomarker panel (IL-2Rα, TNFR-1, HGF, IL-8, elafin, Reg3α) is

**Table 6.** Assessment results of the logistic regression model of risk factors for infection

| | Assessment of β parameter | Standard error | OR | 95% CI | P |
|---|---|---|---|---|---|
| Intercept | -0.35 | 0.38 | 0.70 | 0.33-1.50 | 0.355 |
| Type of conditioning treatment (RIC/MAC) | 1.40 | 0.62 | 4.04 | 1.17-13.94 | 0.024 |
| IFN-gamma concentration on day 6 post-transplant (0/> 0) | 1.34 | 0.73 | 3.84 | 0.89-16.48 | 0.065 |

useful in predicting treatment response and determining aGvHD-related mortality. It was found that the higher the concentration of the above biomarkers, the worse the response to treatment and the higher mortality in aGvHD [21].

Published reports of others did not include IFN-gamma or IL-2 as potential biomarkers of aGvHD. The results of this study indicate that IFN-gamma can be considered a potential biomarker of this disease. However, a small sample group in this study and the coexistence of infectious complications that overlap the symptoms of aGvHD seem to be factors interfering the analysis. Further studies are warranted to confirm the association between IFN-gamma and aGvHD. Such studies should be based on larger and more homogeneous cohorts of patients.

## Conclusions

Non-CR status before allo-HSCT, use of myeloablative conditioning and high IFN-gamma concentration on day 6 post-transplant are independent risk factors of aGvHD. The use of myeloablative conditioning also impacts the incidence of infectious complications.

## Acknowledgements

Approval No. KNW/0022/KB1/71/I/12 was obtained from the Bioethics Committee of the Medical University of Silesia in Katowice, Poland on 3rd July 2012. All patients have singed the informed consent before entering the study. To finance research work, the contract was concluded with the Medical University of Silesia in Katowice (No. KNW-2-047/D/3/N) on 30th September 2013.

## Disclosure of conflict of interest

None.

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