Original paper

Assessment of RANTES, MIP4A, MMP7, MMP9, MMP14, TIMP 1, TIMP 2 and TIMP 3 concentration in the follicular fluid of patients undergoing *in vitro* fertilization/embryo transfer procedure

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Abstract

Introduction: Matrix metalloproteinases (MMP) degrade extracellular matrix. Some studies show that MMP9 concentration in follicular fluid (FF) may play a role in oocyte maturation and *in vitro* fertilization (IVF) success. The immunology of follicular fluid is still not fully understood.
Aim: Assessment of RANTES, MIP4A, MMP7, MMP9, MMP14, TIMP 1, TIMP 2 and TIMP 3 concentration in the follicular fluid of patients undergoing *in vitro* fertilization/embryo transfer procedure.
Material and methods: This case-control study included 20 randomly selected patients with a positive pregnancy (PPG) test and 20 patients with a negative pregnancy (NPG) test after IVF/ET. In FF obtained during oocyte retrieval, the concentrations of MIP4A, MMP7, MMP14, TIMP 1, TIMP 2, TIMP 3, RANTES, IL-12p40, and IL-17A were measured. Their effect on the characteristics of follicles, embryos, and the efficiency of IVF and ET were analysed.
Results: There was no statistically significant relationship between a positive pregnancy test and the results of the immunoassay performed. The number of COC-1 correlates significantly and positively with RANTES ($r = 0.34$; $p = 0.038$) and IP-10 ($r = 0.329$; $p = 0.038$). MII correlates significantly and positively with RANTES ($r = 0.341$, $p = 0.031$). The number of top-quality embryos correlates significantly and positively with IL-17A ($r = 0.451$, $p = 0.004$) and TIMP 1 ($r = 0.44$, $p = 0.005$).
Conclusions: The concentration of IL-17A and TIMP 1 may predict IVF/ET success. Further studies are required on the influence of the follicular fluid immunological environment on oocyte maturation and quality and, subsequently, embryo development.

Key words: follicular fluid, TIMP, matrix metalloproteinases, *in vitro* fertilization/embryo transfer, pregnancy, cytokine.

Introduction

The human ovary has functions both in obtaining hormonal balance and in the production of fertilizable oocytes. Follicular development is a complex process, during which crucial immunological balance between the different lines of cytokines is obtained. Successful follicular development requires extensive tissue and extracellular matrix remodelling [1].

Matrix metalloproteinases (MMP) degrade extracellular matrix. Horka *et al*. found that in women with successful *in vitro* fertilization (IVF), MMP9 concentration both in blood serum and follicular fluid (FF) is higher than in women, who did not conceive [2].

Yang *et al*. found that the human follicular fluid MMP-2 level in his study group was significantly associated with the rate of maturity of oocytes ($p < 0.001$). What is more, the MMP-2 was significantly associated with a higher fertilization rate ($p < 0.01$) [3].

Our previously published research did not find such a correlation between cytokine and MMP9 concentration [4].

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Aim

Due to the difference in results, we decided to analyse additionally the concentration of MIP4A, MMP7, MMP14, TIMP 1, TIMP 2, TIMP 3, RANTES, IL-12p40, and IL-17A and compare it with different aspects of *in vitro* fertilization/embryo transfer efficiency.

Material and methods

The study group consisted of patients that underwent IVF in the fertility clinic in Bydgoszcz, Poland, between May 2021 and September 2021. A total of 40 randomly selected patients were included in the study, 20 with a positive pregnancy test after the IVF procedure and 20 patients with a negative pregnancy test. All patients suffered from infertility defined as the inability to achieve pregnancy after 1 year of regular intercourse. The size of the study population was established prior to the study and was based on previous studies on the cytokine profile of FF in women who underwent IVF. Another factor taken into account was the number of patients who underwent fertility treatment during the COVID-19 pandemic as well as the limited financial support for the research.

Detailed inclusion and exclusion criteria, gynaecological assessment regime, patients' hormonal stimulation and the procedure leading to IVF, ovarian puncture procedure, and embryo assessment strategies were described in detail in our published research in 2022 [4].

The results of ovarian stimulation were analysed on the basis of the total number of good-quality COCs (cumulus–oocyte complexes) retrieved, the number of oocytes in metaphase (M) II and MI, and the number of germinal vesicles. On the third and fifth days after fertilization embryos were assessed (on the basis of the Gardner and Schoolcraft criteria) and divided into three subcategories using the Istanbul consensus workshop on embryo assessment (2011) and standards for the assessment of oocytes and embryos – Polish Society of Reproductive Medicine and Embryology recommendations as follows: top-quality embryos, non-top-quality embryos, and non-viable embryos [5, 6].

Clinical confirmation of pregnancy was based on blood serum B-hCG concentration between the 10th and the 15th day after transfer.

Laboratory analysis

FF obtained during ovarian puncture in stimulated cycles was stored at a temperature of –80°C until assessment.

RANTES, TGF β2, MIP4a, MMP7, MMP9, MM14, TIMP 1, TIMP 2, TIMP 3

The concentration of RANTES, TGFβ2, MIP4a, MMP7, MMP9, MM14, TIMP 1, TIMP 2, and TIMP 3 was measured using commercially available ELISA kits (Cloud-Clone Corp.: Human RANTES ELISA Kit SEA116Hu; Human TGFβ2 ELISA Kit SEA218Hu; Human MIP4a ELISA Kit SEC091Hu; Human MMP7 ELISA Kit SEA102Hu; Human MMP9 ELISA Kit SEA553Hu, Human MMP14 ELISA Kit SEC056Hu, Human TIMP 1 ELISA Kit SEA552Hu, Human TIMP 2 ELISA Kit SEA128Hu, Human TIMP 3 ELISA Kit SEA129Hu).

All test kits used are intended for the *in-vitro* quantitative determination of the specific target antigen in serum, plasma, cell culture supernatants, cell lysates, tissue lysates, and other biological fluids of human origin.

This assay employs the quantitative sandwich enzyme immunoassay (ELISA) technique using horseradish peroxidase (HRP) as the enzyme and tetramethylbenzidine (TMB) as the substrate. The colour change is measured spectrophotometrically at a wavelength of 450 ±10 nm (Infinite® F50; Tecan, Switzerland). The Magellan™ reader control and data analysis software is used to plot the calibration curve and read the concentrations of the test samples.

The sensitivity or minimum detectable dose of human RANTES was found to be 0.059 ng/ml. The maximum measurable dose of human RANTES was found to be 10 ng/ml. No significant cross-reactivity or interference between human RANTES analogues was observed.

The sensitivity or minimum detectable dose of human TGF-β2 was found to be 12.2 pg/ml. The maximum measurable dose of human TGF-β2 was found to be 2000 pg/ml. No significant cross-reactivity or interference between human TGF-β2 and analogues was observed.

The sensitivity or minimum detectable dose of human MIP4a was found to be 6.1 pg/ml. The maximum measurable dose of human MIP4a was found to be 1000 pg/ml. No significant cross-reactivity or interference between human MIP4a and analogues was observed.

The sensitivity or minimum detectable dose of human MMP7 was found to be 0.063 ng/ml. The maximum measurable dose of human MMP7 was found to be 10 ng/ml. No significant cross-reactivity or interference between human MMP7 and analogues was observed.

The sensitivity or minimum detectable dose of human MMP9 was found to be 0.055 ng/ml. The maximum measurable dose of human MMP9 was found to be 10 ng/ml. No significant cross-reactivity or interference between human MMP9 and analogues was observed.

The sensitivity or minimum detectable dose of human MMP14 was found to be 0.59 ng/ml. The maximum measurable dose of human MMP14 was found to be 100 ng/ml. No significant cross-reactivity or interference between human MMP14 and analogues was observed.

The sensitivity or minimum detectable dose of human TIMP 1 was found to be 0.059 ng/ml. The maximum measurable dose of human TIMP 1 was found to be 10 ng/ml.

No significant cross-reactivity or interference between human TIMP 1 analogues was observed.

The sensitivity or minimum detectable dose of human TIMP 2 was found to be 0.41 ng/ml. The maximum measurable dose of human TIMP 2 was found to be 60 ng/ml. No significant cross-reactivity or interference between human TIMP 2 analogues was observed.

The sensitivity or minimum detectable dose of human TIMP 3 was found to be 0.056 ng/ml. The maximum measurable dose of human TIMP 3 was found to be 10 ng/ml. No significant cross-reactivity or interference between human TIMP 3 analogues was observed.

IL-12p40, IL-17A, IP-10, sCD25

Concentration of IL-12p40, IL-17A, IP-10 and sCD25 was measured using commercially available ELISA kits (Diaclone, Medix Biochemica Group: Human IL-12p40 ELISA Kit 0p40-32; Human IL-17A ELISA Kit 117A-23; Human IP-10 ELISA Kit 1P10-16; Human sCD25 ELISA Kit 0025-80).

All test kits used are intended for *in-vitro* quantitative determination of the specific target antigen in serum, plasma, cell culture supernatants, cell lysates, tissue lysates and other biological fluids of human origin.

This assays employs the quantitative sandwich enzyme immunoassay (ELISA) technique using horseradish peroxidase (HRP) as the enzyme and tetramethylbenzidine (TMB) as the substrate. Absorbance value was determined of each well on a spectrophotometer (Infinite® F50; Tecan; Switzerland) using 450 nm as the primary wavelength and optionally 620 nm as the reference wavelength. The Magellan™ reader control and data analysis software is used to plot the calibration curve and read the concentrations of the test samples.

The sensitivity or minimum detectable dose of human IL-12p40 was found to be 20 pg/ml. The maximum measurable dose of human IL-12p40 was found to be 2000 pg/ml. No significant cross-reactivity or interference between human IL-12p40 and analogues was observed.

The sensitivity or minimum detectable dose of human IL-17A was found to be < 2.3 pg/ml. The maximum measurable dose of human IL-17A was found to be 100 pg/ml. No significant cross-reactivity or interference between human IL-17A and analogues was observed.

The sensitivity or minimum detectable dose of human IP-10 was found to be 5.7 pg/ml. The maximum measurable dose of human IP-10 was found to be 200 pg/ml. No significant cross-reactivity or interference between human IP-10 and analogues was observed.

The sensitivity or minimum detectable dose of human sCD25 was found to be 32.5 pg/ml. The maximum measurable dose of human sCD25was found to be 2200 pg/ml. No significant cross-reactivity or interference between human sCD25 and analogues was observed.

Bioethics Committee

The study was approved on 18 May 2021 by the Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Bioethical Committee, and was assigned a classification number: KB 334/2021. All patients gave informed written consent to participate in the study.

Statistical analysis

Mann-Whitney test was used to compare quantitative variables between two groups, while the Kruskal-Wallis test (followed by Dunn post hoc test) was used for more than two groups. The relationship between two quantitative variables was assessed with Spearman's coefficient of correlation. Odds ratios (OR), calculated with univariate logistic regressions, were used to analyse the impact of selected variables on the dichotomous outcomes (positive/negative pregnancy tests). The significance level for all statistical tests was set as 0.05. R 4.1.2, and MS Excel 365 were used for computations.

Results

The research group consisted of 40 women (age 27–44, mean ± SD: 34.4 ±4.5). All women underwent IVF; in 20 cases, the pregnancy test result was positive 10–15 days after transfer (positive group – PG), and in 20 cases, it was negative (negative group – NG).

Both groups were comparable for age distribution, ethnicity, height, weight, and causes of infertility, and went through a comparable course of ovarian stimulation and oocyte retrieval. The general characteristics of the research group have been already described [4].

The results of analysed cytokines in the population are presented in Table 1.

The mean age of the pregnancy positive group was 34.25 ±4.15 and in the pregnancy negative group it was 34.55 ±5.1. The mean body mass index (BMI) in the pregnancy positive group was lower (22.77 ±3.87) than in the pregnancy negative group (25.09 ±3.85, $p = 0.036$).

The logistic regression model was used to assess if the analysed protein concentration in FF is a predictor of the positive pregnancy test. It was found that there was no statistically significant relationship between the positive pregnancy test and the results of immunoassay performed (Table 2).

Body weight, BMI, and age did not statistically significantly correlate with the concentration of analysed cytokines in FF.

What is interesting, in the research group the number of COC-1 correlates significantly and positively with RANTES ($r = 0.34$; $p = 0.038$) and IP-10 ($r = 0.329$; $p = 0.038$). MII correlates significantly and positively with RANTES ($r = 0.341$, $p = 0.031$).

Table 1. Results of immunoassay in FF of 40 patients that underwent IVF/ET

Parameter	Min.	Max.	Average	St. dev.	Median	Q1	Q3
IL-12p40 [pg/ml]	24.742	2000	213.4063	428.366	79.045	61.85575	121.785
IL-17A [pg/ml]	117.01	297.54	200.9473	47.18683	199.73	168.4	225.3
RANTES [ng/ml]	1.2644	7.4946	2.252758	1.175955	1.9235	1.4738	2.59815
IP-10 [pg/ml]	23.984	228.95	73.6142	41.41866	67.193	44.50925	81.92375
sCD25 [pg/ml]	852.55	5724	2541.666	1070.07	2337.25	1748.75	2917.7
TGFb [pg/ml]	344.46	1983.9	910.4788	468.7683	780.67	600.355	933.75
MIP4A [pg/ml]	29.809	569.19	245.7203	127.1584	267.95	155.79	317.6125
MMP9 [ng/ml]	9.9167	103.75	27.97034	25.26615	15.505	14.2135	34.79425
MMP7 [ng/ml]	0.1036	2.6899	0.495924	0.51852	0.365165	0.262653	0.434775
MMP14 [ng/ml]	0.481	1.3432	0.922845	0.188721	0.90558	0.81159	1.0421
TIMP 1 [ng/ml]	75.603	199.415	153.6967	24.8149	154.7795	141.5208	171.2113
TIMP 2 [ng/ml]	12.586	130.75	48.55637	32.91147	34.2925	23.1995	81.23575
TIMP 3 [ng/ml]	0.1166	1.6871	0.496006	0.293632	0.445965	0.30977	0.655025

Table 2. No relation between the concentration of any analysed cytokines and the pregnancy test result after IVF/ET

Cytokine	OR	95% CI		*P*-value
IL-12p40 [pg/ml]	1.00111	0.99894	1.00329	0.316
IL-17A [pg/ml]	1.00487	0.9915	1.01841	0.477
RANTES [ng/ml]	1.02305	0.60275	1.73643	0.933
IP-10 [pg/ml]	0.99984	0.98498	1.01493	0.984
sCD25 [pg/ml]	0.99987	0.99929	1.00046	0.673
TGFb [pg/ml]	1.00132	0.99977	1.00287	0.095
MIP4A [pg/ml]	0.99797	0.99301	1.00296	0.424
MMP9 [ng/ml]	1.00261	0.97814	1.02768	0.836
MMP7 [ng/ml]	5.88692	0.32279	107.36242	0.231
MM14 [ng/ml]	0.53173	0.01956	14.45597	0.708
TIMP 1 [ng/ml]	1.00195	0.9772	1.02733	0.879
TIMP 2 [ng/ml]	1.01114	0.99144	1.03123	0.27
TIMP 3 [ng/ml]	1.31855	0.15613	11.13548	0.799

P – univariate logistic regressions.

The number of top-quality embryo correlates significantly and positively with IL-17A (r = 0.451, p = 0.004) and TIMP 1 (r = 0.44, p = 0.005).

Progesterone and prolactin concentration correlates with MM14 (r = –0.445, p = 0.004 and r = 0.407, p = 0.009, respectively). AMH (Anti-Mullerian hormone) correlates with IP-10 (r = 0.372, p = 0.018) and TIMP 1 (r = 0.353, p = 0.025).

Analysed cytokines concentration did not have any relation with the primary cause of infertility in all analysed cases.

Discussion

FF is rich in cytokines, which was observed in our previous research [4, 7, 8]. The interpretation of FF cytokine concentration and its influence on ovarian follicles, the natural course of ovulation and corpus luteum formation, and eventually embryo implantation is difficult due to many confounding factors. There are many reasons for shifts in the immunological balance of different tissues of the human body, including rheumatological diseases, infections, allergies, smoking, autoimmune disorders, and many more [9–12].

The knowledge about cytokines in FF is limited for several reasons. First of all, it is problematic to obtain FF from healthy women due to the necessity of ovarian puncture. In practice, it is usually connected with some kind of gynaecological procedure, which makes it important to be aware of the patient's underlying medical condition, which led to an ovarian puncture. Secondly, the interactions between cytokines and the general condition of the female body are complex.

The BMI was lower in a group of women with successful IVF. There was no statistically important difference in the concentrations of MMPs and TIMPs in relation with BMI of patients. It is widely known that adipose tissue is immunologically active. Obesity leads to a shift from an anti-inflammatory to a pro-inflammatory profile in the adipose tissue, leading eventually to insulin-resistance and metabolic syndrome [13]. In other studies no association between adipose tissue and plasma levels of some MMPs, specifically MMP-2 and MMP-9, was observed [14].

The current study was based on FF obtained from women with different causes of infertility and was aimed to measure the metalloproteinase concentration in their

FF and to analyse the possible role in predicting the result of IVF/ET. There are several interesting aspects of the results we achieved.

MMP are a family of zinc endopeptidases. They are able to degrade all the components of the extracellular matrix. They are divided into subgroups depending on the specificity of the substrates. Inhibition of MMPs is associated with the activity of alfa-2 macroglobulin and tissue inhibitors of metalloproteinases (TIMPs).

Four types of TIMP proteins (TIPM-1 to 4) have been identified. MMP-2 and matrix metalloproteinase-9 (MMP-9) belong to the subgroup of gelatinases, and their activity is specifically inhibited by TIMPs. The tissue inhibitor of TIMP-1 exhibits a higher affinity for MMP-9, whereas the tissue inhibitor of TIMP-2 has a higher affinity for MMP-2. There is a consistent expression of MMP and TIMP in the ovary, especially in forming corpus luteum. MMP-TIMP system is involved in the proteolytic network of follicular development and rupture of the follicle wall with successful ovulation [3, 15–17].

The results of our study are in contrast to the previous findings. Yang *et al*. [3] found that human follicular fluid MMP-2 level, in 150 patients undergoing IVF/ET, was significantly associated with the rate of maturity of oocytes with a higher fertilization rate ($p < 0.01$). On the other hand, there was no significant correlation between follicular MMP-9, TIMP-1, and TIMP-2 and the maturation rate of oocytes [3].

Malvezzi *et al.* [18] found that the increase in MMP-2 and -9 activity, as well as a decrease in TIMP-1 expression in FF of women with endometriosis undergoing IVF, went together with poor oocyte and embryo development.

In general, endometriosis patients experience a wide range of changes in their immunological profile, leading to cytokine imbalance. Endometriosis is characterized by increased levels of cytokines and autoantibodies, what is suggesting the role of low-intensity inflammation in infertility associated with this disease [19]. The differential expression of anti-inflammatory cytokines (interleukin-4 and -10, and transforming growth factor-β1) occurs in women with endometriosis [20]. In one of the studies, the expression of MMP-9 in ectopic endometrial stromal cells was significantly higher than that in eutopic endometrium [21]. In our study only 3 women had endometriosis, a group being too small to achieve valid results. But the problem requires further investigation.

Singh *et al.* [22] published interesting research of 340 infertile patients and found, in women who qualified for IVF due to endometriosis, an extensive MMP-9/TIMP-1 imbalance. Poor oocyte and embryo development in those patients were associated with increased MMP-2 and -9 and decreased TIMP-1 expression. What is interesting, progesterone supplementation seemed effective in improving this imbalance. Horka *et al.* [23] investigated the cytokine concentration in FF and sera of 58 female patients undergoing IVF treatment (29 with positive and 29 with negative pregnancy tests). Patients with the positive pregnancy test have shown the highest MMP-9 concentrations in blood serum (833.5 (686.0; 958.7) ng/ml) and FF (9.6 (6.0; 17.0) ng/ml) compared to women with unsuccessful IVF. The authors concluded that MMP-9 concentration could be a good predictor of a successful IVF outcome.

The results of the studies cited above are not confirmed in our study. There was no statistically significant correlation between MMP and TIMP concentration of the successful IVF/ET. TIMP 1 concentration correlated significantly and positively with the number of the top-quality embryo. Our previously published research found that an important parameter in assessing the chances of successful IVF is the number of top-quality embryos achieved [8].

It is difficult to assess why the results are different. In some presented studies the population of patients was larger, but in others it was comparable. There are many factors that can contribute to differences in MMP expression, including underlying disease, medication, acute illnesses, and carcinoma [24, 25]. The expression of MMPs and TIMPs would require further investigation on a wide population of patients. The connection between MMP 9 concentration in FF and successful IVF/ET is not as clear and obvious as previously thought and might differ depending on the researched population.

Chemokine CCL5, also known as RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted), is chemotactic for T cells, eosinophils, and basophils. During the inflammation process, RANTES has a role in recruiting leukocytes into the site. Under the influence of IL-2 and IFN-γ that are released by T cells, CCL5 induces the proliferation and activation of certain NK cells [26].

RANTES is found in the follicular fluid. Xu *et al.* [27] examined thirty-two women who qualified for IVF/ET due to endometriosis-related infertility and 28 controls without endometriosis. The levels of RANTES in FF from patients with endometriosis were significantly higher (460.4 ±90.3 pg/ml) compared with concentrations in patients with tubal infertility (243.8 ±70.9 pg/ml; $p < 0.05$).

Liu *et al.* [28], in a study of 35 premature ovarian insufficiency patients and 37 controls, found that RANTES concentration in FF was decreased in the research group and positively correlated with the rate of day 3 good-quality embryos.

Ledee *et al.* [29] found that chemokine CCL5 was significantly higher in FF related to the best quality (Top) embryos.

In our study, the number of COC-1 correlated significantly and positively with RANTES and IP-10. What is more, MII correlated significantly and positively with RANTES. The number of the top-quality embryos was not related to RANTES concentration but correlated significantly and positively with IL-17A and TIMP 1. Although the result differs from the one obtained in previous research,

it still shows that RANTES concentration correlates with the oocyte maturity level. The pathophysiology of this phenomenon requires further investigation.

Sabbaghi *et al.* [30] found that the level of IL-17A is higher in follicular fluid of endometriosis, polycystic ovary syndrome (PCOS), and tubal factor patients than in the control group. Similar results were obtained in the blood serum. What is more, there was a correlation between the numbers of meiosis I (MI) oocytes and the level of blood serum and follicular fluid IL-17A in PCOS patients.

Mao *et al.* [31] found that in the follicular fluid of endometriosis patients undergoing *in vitro* fertilization, the levels of IL-4, IL-13, IL-3 and IL-1α were significantly increased, while levels of IFN-γ, IL-17A, MDC, and MIP-1α were decreased compared with the control subjects.

In another study it was found that the expression of IFN-γ, IL-4, IL-17A, and TGF-β1 in peripheral blood and follicular fluid of patients testing positive for anti-thyroid autoantibodies may have a role in predicting IVF/ET outcomes [32].

In our study, the number of top-quality embryos correlated significantly and positively with IL-17A and TIMP 1. The number of top-quality embryos plays an important role in assessing the chances of successful IVF/ET, defined as a positive pregnancy test, and IL-17A concentration might be a candidate for a marker in assessing the chances of a successful procedure. This requires further investigation.

The study limitations were described in detail in the previous paper [4]. In summary, the main limitation in the interpretation of the results of this case-control study is the relatively small and diverse research population. A sample size/power analysis was not performed, and the number of patients was based on previous research and the financial support received. Furthermore, the interpretation of our results is difficult because the measured cytokines play important roles in many other conditions. For example, in some cases of cancer development, MMPs and their inhibitors are known to play an important role [33]. RANTES are mediators of chronic inflammation of different origin, what can also play a role in the cytological profile of patients [34]. For example, inhibition of RANTES may improve the condition of patients with endometriosis [35].

The current study provides some insight into the cytokine profile of FF in patients undergoing IVF. The main discovery is that, contrary to previous studies, we did not observe a statistically significant association between TIMPs and MMPs and the result of the pregnancy test. In our study, the number of COC-1 and MII correlated significantly and positively with RANTES. The number of top-quality embryos correlated significantly and positively with IL-17A and TIMP 1, which may play a role in predicting IVF/ET success. Further studies are required on the influence of the follicular fluid immunological environment on the oocyte maturation and quality and, subsequently, embryo development.

Acknowledgments

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Collegium Medicum Ethics Committee – KB 331/2021 (18.05.2021).

Conflict of interest

The authors declare no conflict of interest.

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