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Placental disposition of the immunosuppressive drug tacrolimus in renal transplant recipients and in *ex vivo* perfused placental tissue

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ABSTRACT

Currently, tacrolimus is the most potent immunosuppressive agent for renal transplant recipients and is commonly prescribed during pregnancy. As data on placental exposure and transfer are limited, we studied tacrolimus placental handling in samples obtained from renal transplant recipients. We found transfer to venous umbilical cord blood, but particularly noted a strong placental accumulation. In patient samples, tissue concentrations in a range of 55–82 ng/g were found. More detailed *ex vivo* dual-side perfusions of term placentas from healthy women revealed a tissue-to-maternal perfusate concentration ratio of 113 ± 49 (mean \pm SEM), underlining the placental accumulation found *in vivo*. During the 3 h *ex vivo* perfusion interval no placental transfer to the fetal circulation was observed. In addition, we found a non-homogeneous distribution of tacrolimus across the perfused cotyledons. In conclusion, we observed extensive accumulation of tacrolimus in placental tissue. This warrants further studies into potential effects on placental function and immune cells of the placenta.

1. Introduction

The incidence of pregnancy in the renal transplant patient population is relatively high and increasing (OPTN/SRTR, 2014; McKay & Josephson, 2006). Treatment with immunosuppressive drugs needs to be continued during pregnancy to prevent graft rejection. Maintenance treatment usually consists of a calcineurin inhibitor combined with a glucocorticoid and/or an antimetabolite drug (Armenti et al., 2002). The optimal use of the calcineurin inhibitors tacrolimus and cyclosporine A in pregnancy has been discussed in literature (Kim et al., 2015; Liu et al., 2016).

Although > 90% of all kidney transplant recipients in the US receive tacrolimus as part of their immunosuppressive regimen, resulting in a considerable use during pregnancy, data on placental handling of tacrolimus are scarce (OPTN/SRTR, 2014). Since pregnant women are generally excluded from randomized controlled clinical trials because of ethical, legal, and practical considerations, there is a paucity of studies with respect to safety of drug use during pregnancy. Data on fetal drug exposure are therefore limited to either case reports or pharmacoepidemiological studies describing pregnancy outcome after

kidney transplantation under tacrolimus or cyclosporine A, as for instance performed by the National Transplantation Pregnancy Registry (NTPR) (Coscia et al., 2010). This large voluntary registry included almost 2000 pregnancies in female kidney recipients in the period 1990–2010 in North America and reported that of all neonates born, 53% was premature and 46% had a low birthweight (< 2500 g), which is significantly higher than the general prevalence in the US in 2010 of approximately 12% and 8%, respectively. In addition, 50–60% of all neonates were delivered by caesarean section, compared to approximately 33% for general pregnancies (National Vital Statistics Report, 2012). In a large systematic review and meta-analysis of 4706 pregnancies in kidney transplant recipients, an increased rate of pregnancy complications was also observed compared to the general population (Deshpande et al., 2011). Nevertheless, the limited available literature does not report any clinically relevant differences in pregnancy outcome when comparing immunosuppressive drugs (Perales-Puchalt et al., 2012).

Particularly in the absence of conclusive evidence on drug safety from clinical trials, mechanistic pharmacological knowledge regarding placental drug handling can help to decide which calcineurin inhibitor

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may be preferred during pregnancy. Tacrolimus (MW 804 g/mol, logP 3.3) and cyclosporine A (MW 1202 g/mol, logP 2.9) have been reported to cross the placental barrier to a similar extent. For both drugs venous umbilical cord concentrations have been reported to be lower than maternal blood concentrations (Venkataramanan et al., 1988; Zheng et al., 2013). However, when considering the safety of tacrolimus during pregnancy, one should not only be concerned about placental drug transfer, but also take a possible interference with placental function into account. It is known that placental insufficiency may lead to the pregnancy outcomes associated with the use of calcineurin inhibitors. Therefore, it is possible that tacrolimus may indeed affect placental function (Morgan, 2016). As placental drug exposure will drive such potential adverse effects, particularly more information on tacrolimus tissue concentrations is needed.

To obtain more insight into the placental handling of tacrolimus during pregnancy, we studied tacrolimus concentrations in placental tissue samples from 6 renal transplant patients as well as in available corresponding umbilical cord blood samples. In addition, we determined placental accumulation and transfer of tacrolimus in an *ex vivo* model using isolated dual-side perfusions of cotyledons from healthy females.

2. Materials and methods

2.1. Collection of whole blood and placental tissue from renal transplant patients

Pregnant renal transplant recipients (n = 6) signed informed consent, according to the Dutch Medical Research Involving Human Subjects Act (WMO file number: 2014-232). Maternal peripheral blood was collected in EDTA tubes during routine visits to the clinic to monitor tacrolimus trough levels during pregnancy. Venous umbilical cord blood samples were available from 2 patients. Placentas were collected immediately after delivery and pieces of approximately 1 cm³ of the central part of the placental bed were stored at –80 °C until processing.

2.2. Conduction of placental perfusion experiments

The conduction of *ex vivo* placental perfusion studies with human tissues was approved by the local ethics committee (file number: 2014-1397) and an informed consent was signed which allowed use of placental tissue for *ex vivo* studies, but no reporting of clinical data or pregnancy outcomes. Placentas were collected from healthy pregnant women, who were not infected with HIV, hepatitis B or hepatitis C. Women who had a retained placenta or with a multiple pregnancy were also excluded. The perfusion procedure was based on the method described by Schneider et al. (Schneider et al., 1972; Schneider & Huch, 1985), with minor modifications as reported previously (Schalkwijk et al., 2016). In brief, placentas were collected immediately after delivery and time until perfusion was kept below 45 min. The perfusion medium consisted of Krebs-Henseleit buffer, supplemented with 11.1 mM glucose, 30 g/L human serum albumin (Albuman®, Sanquin, Nijmegen, the Netherlands) and 2500 IU/L heparin (LEO Pharma, Amsterdam, The Netherlands). The medium was oxygenated with 95% O₂/5% CO₂, while being kept on 37 °C at a pH between 7.2 and 7.5. An intact cotyledon was selected and a closed fetal circulation was re-established by cannulation of the matching vein and artery (6 mL/min). Subsequently, four cannulas were inserted through the maternal decidual plate into the intervillous space, resulting in an inflow of 12 mL/min. Medium flowing from the intervillous space into the perfusion chamber was recirculated *via* the maternal reservoir ensuring a closed system. In a pre-perfusion period, both circulations were initially left open in order to wash out residual blood. Subsequently, at the start of the experiment, maternal and fetal circulations were closed and 10 ng/mL tacrolimus (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the closed maternal circulation, which had a volume of 200 mL. After 10, 30, 45, 60, 90, 120, 150 and 180 min of perfusion, samples were taken from both the maternal and fetal

reservoir and stored at –20 °C until analysis. At the end of the experiment, the perfused cotyledon was excised from the remainder of the placenta, snap frozen in liquid nitrogen and stored at –80 °C until further use. To test for adherence of tacrolimus to our perfusion system a control perfusion was conducted in the absence of placental tissue.

For every placental perfusion, control substances were included to determine whether the placental capillary bed remained intact throughout the period of perfusion and to confirm overlap of the fetal and maternal circulation. Because of its size, FITC-dextran (molecular weight: 40 kDa) is expected not to cross the placental barrier after addition to the fetal circulation (36 mg/L), and therefore served as a marker for integrity of the fetal capillary bed. Antipyrine (molecular weight: 188 Da, logP: 0.38) was added to the maternal circulation (100 mg/L). It undergoes rapid passive diffusion across the placental barrier and was used to confirm overlap of the maternal and fetal circulation. These markers have been used previously in placental perfusion studies by Myllynen et al., Mathiesen et al. and Schalkwijk et al. (Schalkwijk et al., 2016; Myllynen et al., 2010; Mathiesen et al., 2010).

2.3. Analysis of antipyrine and FITC-dextran concentrations in perfusate samples

Samples were processed according to the method of Brodie et al. after which antipyrine levels could be measured at 350 nm using a plate reading spectrophotometer (Brodie et al., 1949). A Fluorescence Multiwell Plate Reader (PerkinElmer, Ex 485 nm/Em 530 nm) was used to determine the FITC-dextran level in the maternal and fetal reservoir over time. The experiment was considered successful when the antipyrine maternal-to-fetal concentration ratio at the end of the perfusion was < 1.25 (*i.e.* good overlap of maternal and fetal circulation), the maternal-to-fetal concentration ratio of FITC-dextran was < 0.03, and volume loss from the fetal circulation did not exceed 3 mL/h during 180 min of perfusion (confirmed integrity of placental capillary bed).

2.4. Analysis of tacrolimus concentration in whole blood, tissue and perfusate samples

The tacrolimus assay in whole blood is based on validated UPLC–tandem mass spectrometry (Hoogtanders et al., 2007) and is used for therapeutic drug monitoring purposes in our medical center. To determine the tacrolimus concentration in placental tissue of renal transplant patients and in placental tissue after 180 min of perfusion, homogenates were made to allow subsequent LC-MS/MS analysis, by homogenizing 1 part of tissue in 4 parts of Krebs-Henseleit buffer. Rapamycin (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used as internal standard. To precipitate proteins, methanol was added to perfusate and homogenate samples. Following ultrasonification (5 min) and subsequent centrifugation (12,000 g for 3 min), supernatant was used to quantify tacrolimus. Tacrolimus levels were determined by LC-MS/MS analysis, using an Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Xevo TQ-S (Waters) triple quadrupole mass spectrometer. The elution gradient was as follows: 0 min, 55% B; 0.6–0.7 min, 70% B; 0.7–0.8 min, 90% B; 0.8–2.3 min 100% B and 2.3–2.5 min 55% B. The mobile phase consisted of solvent A (1 mM NH₄F + 0.1% formic acid in H₂O) and solvent B (1 mM NH₄F + 0.1% formic acid in MeOH). The following MRM transitions were used: for tacrolimus *m/z* 821.8 (parent ion) to *m/z* 768.7 and 786.8 (both product ions), for rapamycin *m/z* 931.9 (parent ion) to *m/z* 864.9 and 882.8 (both product ions). The detection limit of the assay was 0.24 nM. Data were analyzed using Graphpad Prism 5.03 (Prism, Graphpad software Inc., San Diego, USA) and results are presented as mean ± SEM.

3. Results

3.1. *In vivo* placental handling of tacrolimus in renal transplant patients

Patient characteristics of the 6 women participating in this part of our study can be found in Table 1. Based on analysis of venous

Table 1
Patient characteristics of renal transplant recipients at the time of delivery.

Patient	Age at delivery (yr)	Immunosuppressive regimen ^a	eGFR (mL/min/1.73 m ²)	Months between transplantation and delivery	Gestational age at delivery (wks + days)	Mode of delivery (vaginal or CS)	Hospital of delivery	Birthweight (g)
#1	30	AZA (125 mg) + TAC (8 mg)	41	34	36 + 2	CS	Radboudumc, Nijmegen	2342
#2	31	TAC (10 mg) + PRED (5 mg)	88	37	36 + 3	CS	University Medical Center Utrecht	2405
#3	38	TAC (7 mg) + PRED (15 mg) + AZA (150 mg)	25	72	33 + 1	Vaginal	Radboudumc, Nijmegen	1735
#4	30	TAC (6 mg bid) + PRED (10 mg)	46	27	37 + 3	CS	Radboudumc, Nijmegen	3240
#5	33	TAC (3 mg bid) + AZA (100 mg) + PRED (alternately 5 mg and 7.5 mg)	72	28	38 + 2	Vaginal	Radboudumc, Nijmegen	2400
#6	30	TAC (5.5 mg bid) + PRED (alternately 5 mg and 7.5 mg)	79	12	38 + 0	Vaginal	Radboudumc, Nijmegen	2615

^a If not specified: once daily. Abbreviations: AZA = azathioprine; TAC = tacrolimus; PRED = prednisone; bid = twice a day; eGFR = estimated glomerular filtration rate; CS = caesarean section.

Table 2

Tacrolimus concentrations in maternal whole blood, placental tissue and venous umbilical cord blood of renal transplant recipients. The reported maternal tacrolimus concentration is the last C_{trough} level measured before delivery and derived from patient records. Hence sampling of maternal blood was not at the time of delivery but at varying times prior to delivery.

Patient	Maternal C _{trough} level before delivery (ng/mL)	Placental tissue concentration (ng/g) as mean ± SEM	Venous umbilical cord blood concentration (ng/mL)
#1	4.2	55 ± 3	–
#2	7.6	68 ± 19	–
#3	3.9	82 ± 6	–
#4	6.6	75 ± 2	–
#5	2.9	62 ± 2	6.2
#6	4.0	82 ± 1	4.8

umbilical cord concentrations as well as corresponding placental tissue concentrations, placental handling of tacrolimus could be assessed *in vivo*. The maternal C_{trough} level at the last sampling time point before delivery varied between 2.9 and 7.6 ng/mL. In 2 cases, venous umbilical cord blood concentration were recorded in patient records, being 4.8 and 6.2 ng/mL, which is indicative of placental transfer of tacrolimus. A placental tissue concentration ranging between 55 and 82 ng/g demonstrated tacrolimus accumulation within the organ (Table 2).

3.2. Ex vivo placental perfusion experiments

A total of four out of eight perfusion experiments with placentas of healthy women met the quality control criteria as explained above; three from vaginal deliveries and one from an elective caesarean section. As can be seen in Fig. 1A, the antipyrine concentrations equilibrated in both circulations. Upon addition to the maternal circulation, a mean maternal-to-fetal concentration ratio of 1.07 ± 0.04 for antipyrine was observed. This is in accordance with good passive diffusion across the placental barrier, indicating overlap of the maternal and fetal circulations. Integrity of villous structures was confirmed by the poor placental passage (maternal-to-fetal concentration ratio of < 0.03) of FITC-dextran (Fig. 1B). For both the maternal and fetal reservoir, pH and volume loss were within the predefined required ranges (pH 7.2–7.5, volume loss < 3 mL/h).

After addition of tacrolimus (10 ng/mL) to the maternal circulation, maternal perfusate levels decreased to 1.9 ± 0.4 ng/mL at t = 180 min, while tacrolimus remained undetectable in the fetal reservoir (Fig. 2). The poor placental transfer from maternal to fetal circulation in the investigated perfusion interval and absence of the drug in the fetal circulation suggested placental retention of tacrolimus. Comparison of the tacrolimus concentration in placental tissue with tacrolimus concentration in maternal perfusate at the end of the experiment, indeed revealed a tissue-to-maternal perfusate concentration ratio of 113 ± 49, as shown in Table 3. Interestingly, our *ex vivo* perfusion studies also demonstrated that tacrolimus was not distributed homogeneously across the perfused cotyledon (Supplementary Table S1). The measured tissue concentrations differed up to a factor 19 in a series of samples taken from different locations in the same cotyledon. Nevertheless, in all samples obtained in the *ex vivo* perfusions, placental tissue concentrations exceeded maternal and fetal perfusate concentrations.

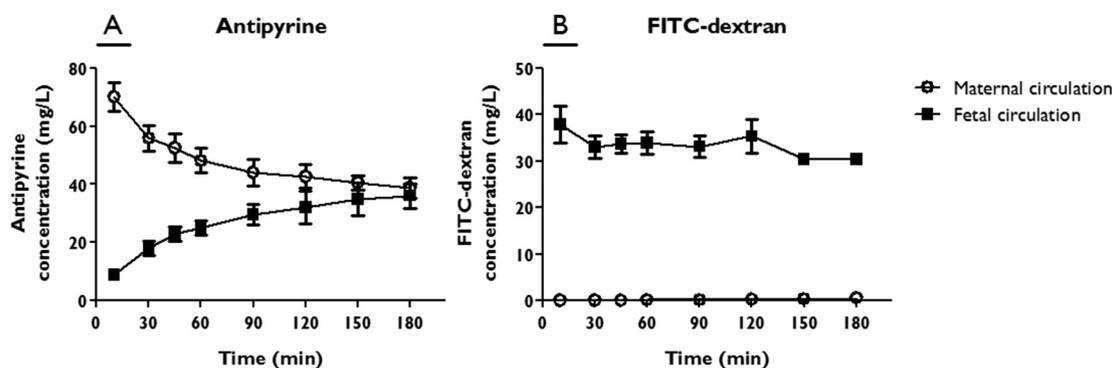


Fig. 1. Placental transfer of the control molecules antipyrine and FITC-dextran. Concentration–time profile of (A) antipyrine (100 mg/L) and (B) FITC-dextran (36 mg/L) in the maternal (open circles) and fetal circulation (squares), based on four successful experiments during 180 min of perfusion. Data are depicted as mean ± SEM.

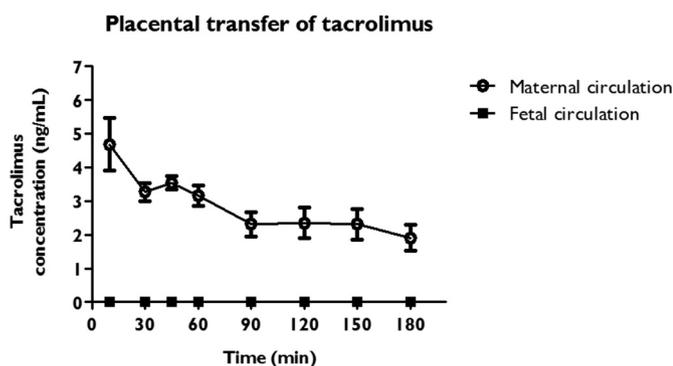


Fig. 2. Placental transfer of tacrolimus. Concentration–time profile of tacrolimus (10 ng/mL) in the maternal (open circles) and fetal circulation (squares), based on four successful experiments during 180 min of perfusion. Data are depicted as mean ± SEM.

Table 3

Tacrolimus concentrations in perfusate samples and perfused tissue after placental perfusion experiments. Placental tissue-to-maternal perfusate concentration ratio was calculated based on the tacrolimus concentration in maternal perfusate at the end of a perfusion and the perfused placental tissue concentration. Results are depicted as mean ± SEM.

Perfusion	Maternal perfusate concentration at t = 180 min (ng/mL)	Placental tissue concentration (ng/g) as mean ± SEM	Placental tissue-to-maternal perfusate concentration ratio
#1	1.8	54 ± 8	31
#2	2.1	138 ± 85	65
#3	2.8	287 ± 103	102
#4	0.9	236 ± 76	253
Mean	1.9	179	113
SEM	0.4	52	49

4. Discussion

We observed a substantial, > 10-fold accumulation of tacrolimus in placentas obtained from both renal transplant recipients as well as in *ex vivo* perfusion experiments using placental tissue from healthy females. To our knowledge, tacrolimus concentrations in placentas from renal transplant recipients have not been reported before. In a study performed by Jain et al., who focused on liver transplant patients, a 3-fold higher tacrolimus concentration in placental tissue compared to maternal plasma levels was reported (Jain et al., 1997). In our study we found a much higher degree of accumulation in placental tissue samples

from exposed patients. This could be due to differences in placental disposition between renal and liver transplant patients, but possibly also because of differences in methods of analysis, tissue preservation or sampling, which were only briefly described by the authors and did not allow us to compare these aspects between the studies. As tacrolimus is a drug with a high volume of distribution, it is likely that tissue accumulation of this drug is not limited to the placenta. Indeed, tissue distribution studies in rats showed accumulation within the heart, lung and spleen (Venkataramanan et al., 1990), while also accumulation was noted in liver and kidney biopsies obtained from liver and kidney transplant patients (Noll et al., 2013; Capron et al., 2007). Nevertheless, we now describe that in renal transplant patients, significant exposure of the placenta clearly takes place, indicating that this organ may be prone to tacrolimus-induced toxicity as well.

In contrast to our observations in short-term perfusion experiments that did not show detectable tacrolimus levels in the fetal circulation, analysis of venous umbilical cord blood samples indicated that *in vivo* fetal exposure to tacrolimus does occur. This discrepancy could mean that the extensive placental binding of tacrolimus that contributes to the placental retention in the *ex vivo* studies, may have become saturated upon chronic exposure in the clinical setting. Saturation of binding capacity would result in an increase in unbound tacrolimus concentrations within the placenta and therefore a stronger driving force for diffusion from the placenta to the fetal circulation. To further study whether saturation of tissue binding plays a role, *ex vivo* placental perfusion experiments could be performed with placentas from renal transplant recipients that have been chronically exposed to tacrolimus *in vivo*. In this case, an increased placental transfer to the fetal circulation would be expected to occur.

Our *in vivo* data demonstrated that the venous umbilical cord blood concentrations were somewhat higher than the maternal C_{trough} levels. However, because the sampling times of venous umbilical cord blood and maternal blood did not match in our observational study, it was not possible to give an accurate estimate of the maternal tacrolimus level around the time of delivery, and hence calculate an exact placental transfer ratio. Zheng et al. collected maternal blood immediately after delivery and observed venous umbilical cord blood concentrations in exposed pregnant patients that were 71 ± 18% (range 45–99%) of maternal concentrations (Zheng et al., 2013).

As tacrolimus displays extensive erythrocyte binding *in vivo*, but no erythrocytes were added to the *ex vivo* perfusion buffer, the total tacrolimus concentration of 10 ng/mL used in this study probably results in higher free concentrations than present in whole blood of renal transplant recipients (Staatz & Tett, 2004). Still, physiological albumin concentrations were present in the *ex vivo* perfusion buffer, as well as some circulating erythrocytes since they cannot be washed out completely from the placenta. We did not measure the ultimate unbound

concentrations in the *ex vivo* experiments, but this could be considered relatively high. On the other hand, it should be noted that the tacrolimus fraction unbound *in vivo* also increases during pregnancy (Zheng et al., 2012). After addition of tacrolimus to the maternal perfusate, we found that rapid extraction occurred to the placenta, while also some adherence to components of the perfusion system was observed. Despite the high unbound tacrolimus exposure in the maternal perfusate and therefore also a large driving force for passive placental transfer, the amount of drug transported to the fetal circulation still remained negligible during 180 min of perfusion, further pointing to a high tacrolimus binding capacity of placental tissue.

Information about differences in placental accumulation and transfer between different calcineurin inhibitors may aid in the decision-making process of the drug of first choice during pregnancy. Nandakumaran et al. studied *ex vivo* placental transfer of cyclosporine A in a perfusion set-up and reported < 5% passage along with placental accumulation of cyclosporine A. This is in line with our findings with tacrolimus (Nandakumaran & Eldeen, 1990). The question now arises to which extent placental accumulation of either cyclosporine A and tacrolimus is causally linked to placental toxicity and how this relates to the adverse pregnancy outcomes reported in literature. Considering the vasoconstrictive effects of calcineurin inhibitors as well as their toxic effects on the kidney and pancreatic beta cells, potential cytotoxic effects on the placental level might be expected as well (Hoskova et al., 2017; Ozbay et al., 2011). Placental cytotrophoblasts, syncytiotrophoblasts and endothelial cells may be differentially affected in the face of the high concentrations we described here. Moreover, since the immune system is crucial for proper placentation, placental tissue accumulation of calcineurin inhibitors could also interfere with placental lymphocyte function and maturation, leading to pregnancy complications (Saito et al., 2010). Finally, rat embryo culture experiments showed that tacrolimus was more likely to induce apoptosis and cause more morphological abnormalities than cyclosporine A (Fazliogullari et al., 2016). Translation of the data from such studies to a toxicological risk for adverse placental drug effects in human is still difficult, because of species differences and because effects were not linked to the placental drug concentrations that were reached. We propose to combine our data on human placental drug concentrations with dose-response data for effects of tacrolimus on key placental physiological processes measured *in vitro* or *ex vivo* in human placental cells and tissues. This will help to further delineate risks of adverse placental effects of calcineurin inhibitors during pregnancy. In this respect, physiologically-based pharmacokinetic modeling approaches can also provide an opportunity to further advance our understanding of the link between placental pharmacokinetics and pharmacodynamics of immunosuppressive drugs (Schalkwijk et al., 2017).

We conclude that tacrolimus accumulates in placental tissue of renal transplant recipients treated with this drug, which is in line with observations in the *ex vivo* perfused human cotyledon. The build-up of a placental reservoir of tacrolimus in renal transplant patients warrants further studies into potential adverse pharmacological and immunological effects on placental function.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejps.2018.04.017>.

Disclosure

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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