Intrauterine exposure to diethylhexyl phthalate disrupts gap junctions in the fetal rat testis

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Fetal exposure to certain phthalate esters can disrupt testis development in rodents and lead to male reproductive disorders, but with a causal link less certain in humans. Di(2-ethylhexyl) phthalate (DEHP) is one of the most common phthalates found in the environment and in rodents it is known to induce serious testis toxicity, as well as male reproductive disorders including cryptorchidism, hypospadias, impaired spermatogenesis and reduced fertility. In this study, we show that perinatal DEHP exposure disrupts gap junction localization in fetal and postnatal rat testis and correlate these findings to morphological changes. The protein Connexin 43 (CX43), normally expressed strongly in testicular gap junctions, was markedly downregulated in Leydig cells of DEHP-exposed fetal testes. In the postnatal testes, CX43 expression was recovered in the DEHP-exposed animals, even though Leydig cell clusters and malformed cords with intratubular Leydig cells were still present.

1. Introduction

In recent decades, there has been a rise in the incidences of male reproductive disorders such as cryptorchidism, hypospadias, poor semen quality and impaired fertility in many parts of the world (Skakkebaek et al., 2016). Environmental factors are proposed to play a major role, not least exposure and impaired fertility in many parts of the world (Skakkebaek et al., 2016; Beverly et al., 2014). The abundantly used plasticizer di(2-ethylhexyl) phthalate (DEHP) is classified as a reproductive toxicant and also considered a substance of very high concern due to its endocrine disrupting properties (ECHA, 2019). Adverse effects, including hypospadias, short anogenital distance (AGD), prostate disorders or reduced fertility, have all been observed in rodents after exposure to DEHP or other phthalates, for instance dibutyl phthalate (DBP) (Christiansen et al., 2010; Di Lorenzo et al., 2018; Furr et al., 2014; Gao et al., 2017; Gray Jr. et al., 2000; Kay et al., 2014; Mylchreest et al., 1998). In humans, on the other hand, associations between phthalate exposure and male reproductive disorders are not as strong. Weak associations between short male AGD in newborn boys and higher exposure to certain phthalates in early pregnancy have been reported (Bornehag et al., 2015; Suzuki et al., 2012), but whether or not the “phthalate syndrome” is rodent-specific or also relevant for humans remains unsettled.

Phthalate exposure during fetal life can also induce focal dysgenesis of seminiferous cords in rodents; abnormalities associated with human testicular dysgenesis (Hutchison et al., 2008; Larsen et al., 2017; Mahood et al., 2005). In the fetal testis, these focal dysgenetic cords typically present as ectopic Sertoli and germ cells (Fisher et al., 2003; Borch et al., 2005), which can develop from seminiferous cords that break down during late gestation (Lara et al., 2017). After birth, malformed seminiferous tubules are histologically visible and persist into adulthood (Fisher et al., 2003; Jarfelt et al., 2005).

There is still a knowledge gap with regard to how phthalates cause structural alterations in the testes (Arzuaga et al., 2019). Interference with gap junctional intracellular communication or signalling through peroxisome proliferation activated receptors (PPARs) have been proposed as potential mechanisms (David, 2006). In the testis, gap junctions are located between adjacent Leydig cells, between Sertoli cells, and between Sertoli...
and germ cells. Gap junctions are assembled by connexins forming hemichannels crucial for the exchange of ions and small molecules and for the control of cell growth, metabolic support, intercellular homeostasis and hormone responsiveness (Pointis et al., 2010). Connexin 43 (Cx43) is one of the most abundant gap junction connexin proteins of the testis and is critical for maintaining spermatogenesis and the blood-testis barrier, as well as for steroidogenesis (Batias et al., 2000; Goldenberg et al., 2003; Kidder and Cyr, 2016). Further, Cx43 is the major connexin expressed in Leydig cells (Li et al., 2013) and may coordinate the androgenic secretory activities of these cells (Kibschull et al., 2015).

Disrupted gap junction formation has been described in testes of young adult rats after exposure to phthalates (Sobarzo et al., 2009), but any effects of phthalates on gap junctions in the fetal testes is poorly described. Down-regulation of Cx43 (Gja1) in fetal rat testes has been observed at the transcript level after exposure to a range of phthalates, including DBP and DEHP (Liu et al., 2005), but how this effect translates into changes in protein expression and testis morphology is unknown. Hence, we investigated the histopathological effects of perinatal exposure to different doses of DEHP on gap junction formation in fetal and postnatal rat testis.

2. Materials and methods

2.1. Chemicals

The test compound was DEHP (di(2-ethylhexyl) phthalate), CAS No. 117-81-7, purity 99% (Sigma-Aldrich; cat.no. 80030). Corn oil (Sigma Aldrich; cat.no. C8267) was used as both vehicle and control compound.

2.2. Animals and treatment

Animal experiments were performed in accordance with relevant guidelines and regulations. Ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553) was obtained before commencement of the study. The animal study has been described previously (Borch et al., 2006). Time-mated, young adult Wistar rats with body weights approx. 200 g (Han-Tac: WH, Taconic M&B, Denmark) were supplied at day 3 of pregnancy. The day following overnight mating was designated gestational day (GD) 1 and the day of birth was designated postnatal day (PND) 1. The dams were randomly distributed in pairs upon arrival and housed in semitransparent plastic cages (15 cm × 27 cm × 43 cm) with Aspen bedding (Tapvei). The animal room had controlled environmental conditions (12 h light-dark cycles with light starting at 9 p.m., light intensity 500 lx, temperature 21 ± 2 °C, humidity 50 ± 5%, ventilation 8 air changes/h). The opposite light-dark cycle was applied to cause minimal stress to animals by handling them in the (dark) hours when they are normally awake. Dams were fed a complete rodent diet (Altromin ad libitum access to acidified tap water.

Animals were acclimatized for 4 days prior to treatment and the animals were weighed daily to calculate a dosing volume of 2 ml/kg bw. Dams were dosed once daily by oral gavage between GD 7 and PND 16 to either vehicle (corn oil) or 30, 300, 900 mg DEHP/kg bw/day (n = 16 control litters and 8 litters per exposure group). Animals were monitored for general toxicity twice daily. A subset of dams were administered vehicle or 300 mg DEHP/kg bw/day from GD 7 to GD 21 (n = 8 litters), euthanized at GD 21 in CO2/O2 anaesthesia, and testes of fetuses were removed and fixed overnight with either Bouin’s fixative or 10% formalin. On PND 6 and 16, one or two pups per litter were euthanized and testes were removed and fixed overnight with either Bouin’s fixative or 10% formalin. Fixed tissues were processed and embedded in paraffin for histopathological analyses.

2.3. Histology and immunohistochemistry

Paraffin-embedded Bouin’s fixed testes were sectioned at 3 μm and stained with haematoxylin and eosin (H&E; alternately right and left testis) following standard protocols. At PND 6, H&E stained tissue sections from all dose groups (n = 4–12 testes from separate litters of all dose groups) were evaluated for the presence or absence of multinucleated gonocytes, small or large Leydig cell clusters and testis cord dysmorphology. Testis cord diameters were analysed by measuring the diameter of tubular cross-sections perpendicular to the tubular length, as previously described (Dalgaard et al., 2002).

Immunofluorescence (IF) was carried out essentially as described (Svingen et al., 2012) in n = 3 testes (from separate litters of the 300 mg/kg bw/day dose group). Briefly, 4 μm tissue sections were dewaxed, heat-treated (microwave) in Tris-EDTA (pH 9.0) antigen-retrieval buffer and washed in phosphate-buffered saline (PBS). Sections were pre-blocked with 1% bovine serum albumin (BSA), then incubated with primary antibody (in blocking solution) overnight at 4 °C. The next day, sections were washed in PBS and incubated with secondary antibodies in 1% BSA in PBS for 1 h at room temperature. Primary antibodies were: goat-anti-HSD3β (1:200; Santa Cruz, sc-30820), rabbit-anti-CX43 (1:8000; Abcam, ab11370), mouse-anti-SMA (1:1600; Sigma, A2547), goat-anti-CYP11A1 (1:100; Santa Cruz, sc-18,043), and mouse-anti-DDX4 (1:2000; Abcam, ab27591). Secondary antibodies were: donkey-anti-goat AlexaFluor-488, donkey-anti-mouse AlexaFluor-488, and donkey-anti-rabbit AlexaFluor-588 (Molecular Probes) all used at 1:500 dilution. Sections were counter-stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) diluted 1:1000 in PBS for 3 min. Slides were then washed in PBS and mounted with ProLong Gold AntifadeMountant (ThermoFisher Scientific; cat.no. P10144).

Immunohistochemistry (IHC) with peroxidase was carried out essentially as described (Borch et al., 2005) on n = 6 testes (from separate litters) at GD 21 and n = 4–7 testes (from separate litters) at PND 6. Briefly, 4 μm tissue sections were dewaxed, heat-treated (microwave) in citrate (pH 6.0) antigen-retrieval buffer and washed in PBS. Sections were treated with 3% H2O2 in PBS for 10 min to diminish endogenous peroxidase activity, then blocked for 30 min in 1% BSA in PBS. Sections were incubated with primary antibody overnight at 4 °C and the following day, sections were washed in PBS and incubated with HRP-conjugated secondary antibody for 30 min. Finally, sections were stained in diaminobenzidine (DAB + Substrate Chromogen System, DAKO) and counterstained with Mayer’s haematoxylin. Primary antibodies were: CX 43 (1:8000; Abcam, ab11370), SMA (1:100; clone 1A4, DAKO), HSD3β (1:200; gift from Dr. I. Mason, Edinburgh, UK). Secondary antibody used for CX43 was anti-rabbit Envision+ (DAKO). Double staining for SMA and HSD3β was performed by simultaneous incubation with the two primary antibodies followed by two consecutive staining procedures: first, incubation with anti-mouse EnVision+ and staining for SMA with AEC, then blocking in H2O2, incubation with anti-rabbit EnVision+ and staining with Vector SG peroxidase staining kit (Vector Laboratories, CA).

2.4. Imaging

Nonfluorescent histology slides were imaged with a LEICA DMR microscope fitted with Leica DFC295 Digital Camera and captured using Image Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD, USA). Fluorescent histology slides were imaged with an Olympus BX-53 microscope fitted with a QImaging Retiga-6000 monochrome camera and captured using the Cell Sense Dimensions V1.16 software (Olympus Ltd., UK). Adobe Photoshop 7.0 (Adobe System Inc., Mountain View, CA, USA) was used for subsequent image processing.

3. Results and discussion

Apart from disrupting steroidogenesis, some phthalates, including DEHP, can disrupt testis morphology. This can manifest as abnormal Leydig cell foci and disrupted seminiferous tubules (Spade et al., 2018; Zhang et al., 2013). Disruption of gap junctions has been proposed as a possible contributing factor to these effects (David, 2006). Herein, we focused on one of the main gap junction connexins found in the testis, CX43.
3.1. Phthalate-induced changes in fetal connexin expression

Expression of Gja1, the gene transcript encoding for CX43, is downregulated in testes following exposure to a range of phthalates (Liu et al., 2005). To explore if this effect translated to the protein level, we compared the expression pattern of CX43 in rat testes after perinatal exposure to DEHP with that of controls. As shown previously (Borch et al., 2006), a sub-set of Leydig cells clustered abnormally in the DEHP-exposed testes. Interestingly, CX43 expression was visibly weaker in the Leydig cells from DEHP-exposed animals compared to controls (Fig. 1A-D), an effect not limited to the Leydig cell clusters, but also occurring in areas with normal histology.

We next performed co-staining experiments to confirm dysregulated CX43 expression, but also to verify cellular integrity by marker expression. Smooth muscle actin (SMA) in peritubular myoid cells was unchanged between groups and helped demarcate testis cord boundaries (Fig. 1E-H). Leydig cells were stained for CYP11A1 (Fig. 1I-L) and HSD3β (Fig. 1M-P) which were expressed in both control and DEHP-exposed testes. CYP11A1 showed markedly weaker staining in exposed testes, whereas HSD3β was only marginally weaker in intensity. CX43, on the other hand, was almost absent in Leydig cells from DEHP-exposed animals (Fig. 1K-L and O-P), corroborating both the downregulation of the transcript (Liu et al., 2005) and the DAB-stained sections (Fig. 1C-D). That is, Leydig-to-Leydig cell gap junctions appear to be lost following phthalate exposure, as assessed by CX43 expression.

CX43 is essential for steroidogenesis (Li et al., 2013; Kibschull et al., 2015), yet testosterone levels can also affect CX43 expression in Leydig cell-like structures.}

![Fig. 1. Reduced CX43 expression in Leydig cells of DEHP-exposed rats at GD 21. A-D: Cross-sections of formalin-fixed testes were immunostained with Connexin 43 antibody (CX43) and counterstained with haematoxylin. Control sections showed intense staining of Leydig cell borders (A-B), whereas weak CX43 staining was seen in DEHP exposed animals (C-D). E-P: Cross-sections of formalin-fixed testes were stained by immunofluorescence with antibodies against Connexin 43 (CX43, red) and either smooth muscle actin (SMA, green, E and G), or the Leydig cell markers CYP11A1 (green, I and K) or HSD3β (green, M and O). In panels F, H, J, L, N and P where only CX43 staining is shown, a dashed line indicates cord borders. Samples were counterstained with DAPI (blue). Panels to the left show sections from control animals and panels to the right show sections from animals exposed from GD 7 to 21 to 300 mg DEHP/kg bw/day. TC: Testis cord; Int: Interstitial cells. Scale bars = 40 μm. Scale bar in panel C is representative of images A and C; scale bar in panel D is representative of images B and D; scale bar in panel P is representative of the scale of images E to P.](image-url)
cells (Zhang et al., 2016). Thus, it remains unclear whether reduced CX43 expression is a cause or an effect of the lower testosterone levels seen in fetal rat testes exposed to DEHP (Borch et al., 2006). The fact that gap junctions are dysregulated highlights a new mechanism by which phthalates can induce male reproductive disorders. A common notion with regard to the ’phthalate syndrome hypothesis’ is that phthalates disrupt steroidogenesis which causes a drop in testosterone synthesis, ultimately leading to undervirilization of the developing fetus. Interestingly, disruption of steroidogenesis is primarily seen at high doses and in adult Leydig cells or adult testis culture (Desdoits-Lethimonier et al., 2012; Svechnikov et al., 2016), whereas the causes of low expression of steroidogenesis related genes in fetal rat testes could have other causes such as maturation delay (Svechnikov et al., 2016). In human adrenal cells DEHP and its metabolite MEHP reduce testosterone production at high doses in some (Desdoits-Lethimonier et al., 2012; Kambia et al., 2019), but not all studies (Kjaerstad et al., 2010; Lee et al., 2019). This fact, together with our present data, points to a mechanism whereby phthalate esters can reduce testosterone synthesis by mechanisms other than directly affecting enzymes in steroidogenesis pathway. We propose a second mechanism by which it is Leydig cell integrity that is affected, which subsequently could lead to lower levels of testosterone production. This could also explain observed effects of Leydig cell-specific gene transcripts.

It is clear from various studies that Leydig cell genes such as Cyp11a1, Cyp17a1, Hsd3b1 and Insl3 are often downregulated in phthalate-exposed testes (Gray Jr. et al., 2016; Beverly et al., 2014; Hannas et al., 2012; Lehmann et al., 2004). However, it is sometimes difficult to discern changes in tissue cellularity from actual changes in gene regulation. The testis comprise more than ten different cell types (Svingen and Koopman, 2013), so simply looking at expression levels of cell-specific genes does not necessarily answer the question if the observed effect are caused by bona fide changes to gene transcription of if the effects is a readout of changes to cellularity. In other words, if there are fewer Leydig cells (or fewer Leydig cells with complete phenotype), it will translate as downregulation of Leydig cell-specific genes at the tissue (testis) level, whereas the transcript levels within individual cells may be unchanged. Of note, action through one mechanism doesn’t necessarily exclude action through another. Here, from in vitro data, and the fact

![Fig. 2. Ectopic germ cells without CX43 expression in DEHP-exposed rats at GD 21. A-C: Cross-sections of formalin-fixed testes were immunostained with antibodies against Connexin 43 (CX43, red) and germ cell marker DDX4 (green). Samples were counterstained with DAPI (blue). Dashed lines demarcate testis cord boundary. A: Control, B-C: DEHP 300 mg/kg bw/day. Scale bar = 50 μm (inset = 10 μm), representative for all panels.](image)

![Fig. 3. Leydig cell clustering (black arrows) and presence of dysgenic cords (#) with weaker SMA expression in DEHP-exposed rats at PND 6. A-C: H&E stained rat testis. D-F: Double immunostaining of rat testis for the marker of peritubular myoid cells smooth muscle actin (SMA) and the Leydig cell marker HSD3β. Panels to the left show sections from control animals and panels to the right show sections from animals exposed from GD 7 to 21 to 300 mg DEHP/kg bw/day. Black arrows indicate Leydig cell clustering and # indicates presence of dysgenic seminiferous cords. Scale bar = 40 μm, representative for all panels.](image)
that the net effect on Leydig cell genes typically are downregulation, we see strong evidence to suggest that phthalates have adverse effects on Leydig cell integrity and overall numbers.

Although the end result of reduced testosterone levels, male reproductive disorders, would be the same regardless of mechanisms of action, this knowledge would be of great value for how we test chemicals for potential endocrine disrupting properties. That is, a chemical does not have to be positive in classical in vitro tests such as H295 steroidogenesis assay or Androgen receptor reporter gene assays to cause anti-androgenic effects in vivo. Further mechanistic understanding would facilitate development of Adverse Outcome Pathways (AOPs) and related novel test methods. Thus, a next step towards improved human safety testing should be to characterize whether phthalates or other chemicals can also disrupt gap junctions in the human fetal testis and cause Leydig cell dysfunction.

3.2. Loss of CX43 expression in seminiferous cords and in ectopic germ cells

CX43 expression was also altered inside the seminiferous cords in DEHP-exposed testes. In control to contrast tests, where CX43 expression is most prominent in the basal compartment of the seminiferous cords because of Sertoli-Sertoli and Sertoli-germ cell gap junctions localization (Fig. 1A), CX43 expression was displaced in DEHP-exposed testes. CX43 was absent in the center of cords (Fig. 1C). Co-staining with the germ cell marker DDX4 in GD 21 testes (Fig. 2A-B) showed clear presence of CX43 between Sertoli cells, but little CX43 expression in the center of cords. At this stage, gonocytes of DEHP-exposed testes were centrally located with little connection with Sertoli cells (Fig. 1C), and thus lack of CX43 expression. This disorganization may have long-term consequences, and disrupted spermatogenesis in rats exposed in utero to phthalates could result from failure of gonocytes to migrate to the basal membrane postnatally (Barlow and Foster, 2003; Kleymenova et al., 2005).

Previous studies have shown disrupted seminiferous cords and ectopic gonocytes or Sertoli cells in phthalate-exposed fetal testes (Lara et al., 2017; Borch et al., 2005). Lara et al. demonstrated that the presence of ectopic gonocytes and Sertoli cells was not due to disrupted cord formation, but rather rupture of already established cords (Lara et al., 2017). We also observed ectopic germ cells within the testis interstitium, and in these regions there was no obvious CX43 expression in any cell type (Fig. 2C). This observation adds to our understanding of how fetal phthalate exposure leads to impaired adhesion between Sertoli and germ cells, as was previously observed in explanted rat and human testes (van den Driesche et al., 2015).

3.3. Structural changes in postnatal testes

Clustering of Leydig cells was still present in DEHP-exposed animals at PND 6 (Fig. 3B, E and Table 1). Some seminiferous cords were malformed and showed presence of intratubular Leydig cells positively stained for HSD3β (Fig. 3F). Inside the seminiferous cords, gonocytes appeared enlarged, the diameter of the cords were dose-dependently shorter, and multinucleated gonocytes were present at high dose DEHP testes (Table 1).

### Table 1
Testicular histopathology at PND 6 in male rat offspring exposed to 0, 30, 300 or 900 mg DEHP/kg bw/day from GD 7 to GD 21 (maternal gavage) and from PND 1 to PND 6 (via mother’s milk. Percentage of affected tests (in parenthesis number of affected of the total number).

| Condition                  | CTRL        | DEHP 30 mg/kg | DEHP 300 mg/kg | DEHP 900 mg/kg |
|----------------------------|-------------|---------------|---------------|---------------|
| Multinucleated gonocytes   | 25% (3/12)  | 83.3% (5/6)   | 75% (3/4)     | 100% (4/4)    |
| Small Leydig cell clusters | 25% (3/12)  | 83.3% (5/6)   | 25% (1/4)     | 25% (1/4)     |
| Large Leydig cell clusters | 0% (0/12)   | 16.6% (1/6)   | 75% (3/4)     | 75% (3/4)     |
| Malformed cords            | 0% (0/12)   | 0% (0/6)      | 0% (0/4)      | 50% (2/4)     |
| Cord diameter (μm)         | 161 ± 4     | 151 ± 4       | 149 ± 6       | 152 ± 6       |

![Fig. 4. No change in CX43 expression at PND 6 or 16 in normal or intratubular Leydig cells. A-E: Cross-sections of testes fixed in Bouin’s (PND 6, A-B) or formalin (PND 16, C-E) were immunostained with antibodies against Connexin 43 (CX43, red) and Leydig cell marker HSD3β (green). PND 16 samples were counterstained with DAPI (blue). Dashed lines indicate cord outline. A and C: Control; B, D and E: DEHP 300 mg/kg bw/day. Scale bar = 50 μm is representative for all images.](Image)
Clearly, the effects observed in fetal rat testis following exposure to DEHP persisted after birth. We thus also wanted to examine CX43 expression in postnatal testes from animals having been exposed to DEHP in utero.

At PND 6 and 16, CX43 of control testes was present at the Sertoli-germ cell junction, and the staining pattern did not differ between controls (Fig. 4A and C) and exposed animals (Fig. 4B and D-E). As opposed to the findings at GD 21, the expression pattern of CX43 in clusters of Leydig cells was comparable between control and exposed testes at PND 6 (Fig. 4B). At PND 16, no CX43 staining was seen in the single Leydig cells that had no visible contact points with neighboring cells, whereas occasional small Leydig cell clusters were CX43 positive. However, we still observed ectopic Leydig cells inside dysgenic tubules, again with CX43 present as in other clustering Leydig cells (Fig. 4E). Dysgenetic seminiferous tubules are described to follow fetal phalatate exposure (Sharpe and Skakkebaek, 2008; Lara et al., 2017; Fisher et al., 2003) and to persist into adulthood. Notably, however, the degree of tubular dysgenesis is markedly reduced as the normal seminiferous cords grow (Jarfelt et al., 2005; Barlow and Foster, 2003). Thus, it is likely that the formation of dysgenetic tubules is related to the morphological changes taking place in the interstitial ducts during gestation (Lara et al., 2017), but becomes less pronounced later during postnatal development. In fetal testes, downregulation of SMA expression has been reported at the time of seminiferous cord rupture (Lara et al., 2017), which could indicate loss of peritubular cells. Here, we observed weaker SMA expression in peritubular cells located in areas of cord dysgenesis at PND 6 (Fig. 3F), which suggests that loss of peritubular cell integrity is involved rather than simply loss of peritubular cells caused by tubular rupture.

4. Conclusions

Intrauterine exposure to certain phthalates can induce gross histological changes to testis architecture, manifesting as disrupted seminiferous tubules, ectopic intratesticular gonocytes and intratubular Leydig cells. Aside from confirming this, we have shown that disrupted expression of the gap junction protein CX43 is involved. The cause-effect relationship between loss of CX43 gap junction expression and the testicular histopathology remains unclear, but gap junction integrity is undoubtedly involved in phthalate-induced testis dysgenesis. It would be of interest to explore how phthalates cause gap junction dysregulation, i.e. if it is direct effects on connexin proteins or upstream regulators, or if the gap junction effect is downstream of cellular dysgenesis. The fact that tubular dysmorphology becomes less prominent during development of the postnatal testis, and that CX43 expression re-establishes, suggests that phalatates act directly on the regulation of gap junction formation or integrity. This, in turn, could disrupt cell-cell communications leading to tubular rupture and ectopic cell localizations. Finally, our findings point to an alternative mechanism by which phthalates can cause male reproductive disorders associated with perturbed androgen action during the fetal masculinization programming window. In fact, it remains unclear how exactly phthalates act as anti-androgenic compounds. Although reduced testosterone levels has been clearly established, it may not occur through directly blocking steroid biosynthesis, but rather by affecting Leydig cell integrity and function.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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