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Polymorphisms of the melatonin receptor 1A (*MTNR1A*) gene do not affect sexual activity, plasma testosterone concentrations or testicular characteristics of 8-month-old ram-lambs born in autumn

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Abstract Thirty-nine autumn-born ram-lambs were used to study whether polymorphisms of the melatonin receptor 1A (*MTNR1A*) gene influence some reproductive parameters of Oct-born ram-lambs at 8 months of age. Animals were genotyped for their RsaI (CC, n=24; CT, n=6; TT, n=9) and Mn1I (GG, n=27; GA, n=6; AA, n=6) allelic variants of the *MTNR1A* gene. Liveweight (LW) was recorded bi-weekly until Jun, and scrotal perimeter (SP) was recorded once per month. From mid-Jan, blood samples were collected weekly to measure plasma testosterone concentrations. In mid-Jun, individual serving-capacity tests were performed. Testicular ultrasonography was performed at the end of the experiment. The effects of the week and gene polymorphism on LW, scrotal perimeter, and plasma testosterone concentrations were evaluated statistically by the GLM for repeated measures procedure. LW, SP, and plasma testosterone concentrations were significantly (P<0.001) affected by week, but neither of the polymorphisms of the melatonin receptor 1A (*MTNR1A*) gene affected these variables. In the serving tests, genotypes did not differ significantly in individual behaviors or the number of events (CC: 24.1±3.2, CT: 26.3±13.0, TT: 16.8±4.8, GG: 22.0±3.0, GA: 21.0±6.7, AA: 27.8±12.7). Testicular features assessed by ultrasonography did not differ significantly among genotypes. In conclusion, the polymorphisms of the melatonin receptor 1A (*MTNR1A*) gene did not have an effect on the reproductive characteristics of autumn-born ram lambs, based on serving capacity tests, testosterone secretion, and testicular measurements at 8 months of age.

Keywords: gene, melatonin, polymorphism, sheep

1. Introduction

In sheep, puberty is the process in which a lamb develops the ability to reproduce and, in ewe lambs, it is indicated by the first ovulation or estrus (Foster et al 2015). In the ram-lamb, puberty is less clear-cut. Some have based the timing of puberty on the week of age at which an ejaculate first contains a minimum number of sperm cells (Wheaton and Godfrey 2003) or on the first ejaculate that contains motile sperm (Khalifa et al 2013). Accelerating puberty in ram lambs might lower production costs, hasten the effects of genetic selection, enable earlier progeny and libido testing, and enhance overall lamb production of the flock (Wheaton and Godfrey 2003). Another benefit of early reproductive development is the advancement of puberty in ram lambs intended for artificial insemination and kept in stud books.

The month of birth influences age at puberty often, ewe-lambs born in winter or spring enter puberty at a younger age than those born in fall, which reach it 10-12 mo later (Valasi et al 2009). Prolonged susceptibility to negative estradiol feedback delays puberty in fall-born ewe lambs (Foster 1994). Although the lack of photoperiodic stimulation caused by changes in day length between 155 d and 200 d of age causes testis weight to decrease earlier in autumn-born young rams, Courot et al (1975) demonstrated that ram-lambs born in autumn could mature more quickly than can those born in spring.

Melatonin affects the pars tuberalis, which affects the seasonal reproductive activity in females in several mammals (see review by Dardente 2012). The central nervous system's numerous nuclei, including those that control reproduction, are affected by the action of melatonin on certain receptors

(Sliwowska et al 2004). Although animals contain several types of melatonin receptors, MT1, only appears to be involved in the reproductive activity (Weaver et al 1996; Dubocovich et al 1998). The MT1 receptor is a member of the G protein-coupled receptor family, and the genes for this receptor have been cloned and mapped for numerous animal species (Reppert et al 1994; Messer et al 1997). Numerous polymorphisms in the melatonin receptor 1A (*MTNR1A*) gene are associated with seasonal reproductive activity in sheep and other animals (Pelletier et al 2000; Carcangiu et al 2009). Previous research has shown that the polymorphisms in the *MTNR1A* gene sequence affect the ability of Rasa Aragonesa rams to reproduce as young and adults (Abecia et al 2020). Specifically, the T/T or G/G genotype was correlated with earlier ram-lamb mating activity, and adult T/T or G/G rams exhibited the most reproductive behavior in spring.

This study aimed to determine whether the lower seasonality of Rasa Aragonesa rams of several genotypes of the *MTNR1A* gene sequence in spring in northern Spain is associated with advanced reproductive performance in autumn-born ram lambs.

2. Material and methods

The experiment was conducted at the experimental farm of the University of Zaragoza, Spain (41°40'N). The care and use of animals were following the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. The Ethics Committee for Animal Experiments at the University of Zaragoza approved the procedures performed in the study.

Thirty-nine Rasa Aragonesa ram-lambs were chosen in mid-Jan based on their genotypes of the RsaI and Mn1I polymorphisms: CC (n = 24), C/T (n = 6), and T/T (n = 9), and GG (n=27), G/A (n=6), or A/A (n=6), respectively. The rams were born in the second half of Oct (mean birth date=25 Oct ± 9 d). From 12 Jan, blood samples were collected weekly to measure plasma testosterone concentrations, and LW was recorded every two weeks until 20 Jun. Scrotal perimeters (SP) were measured once per month.

DNA testing was performed on whole blood taken from each ram at 3 mo to determine the specific allelic variations. Jugular vein blood samples (10 ml) were drawn and placed in vacuum tubes that contained ethylenediaminetetraacetic acid (EDTA) as an anticoagulant.

A genomic DNA extraction kit (NucleoSpin® Blood, Macherey-Nagel, Germany) was used to extract the DNA. Following Messer et al (1997), the polymerase chain reaction (PCR) was performed on 150 ng of genomic DNA from each ram and specific primers (Sigma Genosys Ltd., Pampisford, Cambs, UK). The primers were in locations 285 to 304 for the sense primer (5' e TGT GTT TGT GGT GAG CCT GG e 30), and in positions 1108 to 1089 for the antisense primer (5'e ATGGAGAGGGTTTGCGTT Tae 3') (Reppert et al 1994) (GenBank accession number U14109). After that, we referenced the newest ovine *MTNR1A* gene sequence that was included in the latest ovine genome version (Oar_rambouillet_v1.0 - GenBank assembly accession number: GCA_002742125.1). The PCR reaction was performed following Mura et al (2019). The PCR products were subjected to a double restriction enzyme analysis involving the Mn1I and RsaI endonucleases (New England Biolabs, Beverly, MA, USA). The Mn1I restriction enzyme recognizes an A to a G substitution at position g.17355452, and RsaI recognizes a C to a T substitution at position g.17355458 of the GCA_002742125.1 genome sequence (corresponding, respectively, to position 612 and 606 of the older *MTNR1A* exon II U14109 nucleotide sequence). The digestion reactions were performed following Carcangiu et al (2009).

To induce synchronized oestrus, 40 adult Rasa Aragonesa ewes received intravaginal pessaries (Syncro-Part, CEVA Salud Animal, Spain) for 12 d in mid-Jun, when ram-lambs were 8-months old. Each ewe received i.m. 300 IU eCG at the time of pessary withdrawal (Syncro-Part PMSG, CEVA Salud Animal, Spain). Ewes were used in an individual ram serving-capacity test 48 h later (20 Jun) (Kilgour and Whale 1980; Damián et al 2015). For 20 min, individual rams were exposed to five oestrous ewes in a 15-m^2 pen. One of four observers recorded the number of acts of flehmen (elevating the head and upper lip in response to taste and odour of urine ewe or ambient odour), ano-genital sniffing (sniff in the genital region of ewe), approaches (rub, licks, or superficially nibbling the flank of the ewe with intensity), pawing (the ram stands behind the ewe at a small angle to her and kicks her flank with one of his forelegs), attempted mounting (stands behind the ewe and moves with the intention to copulate, with front legs in the air, but not placed safely on the ewe), and mounting (intrusion of the penis into vagina of ewe with one or more thrusts and, thereby, ejaculation can occur, which is indicated by the backward elevation of the ram's head) (Calderón-Leyva et al 2018).

Plasma testosterone concentrations were measured by radioimmunoassay following Hochereau-de Reviers et al (1990). Samples were run in a single assay (intra-assay CV = 6%). Sensitivity was 0.3 ng/ml.

On the day before the serving-capacity test, rams were subjected to ultrasonography by a portable ultrasound scanner (EXAGO, France) that was coupled to a 7.5 MHz transducer. Three movies (124 frames each) were recorded while the probe was positioned transverse to the main axes of each testicle. To characterize testicular structure, the following image features were measured by Ecotext software (Humeco, Spain): Number of black (ECOTEXT 1, Ec1), white (ECOTEXT 2, Ec2), or gray (ECOTEXT 3, Ec3) pixels, tubules /cm^2, proportion (%) of the total area occupied by the tubule lumen in the parenchyma, and mean diameter (µm) of the seminiferous tubule lumen.

The statistical significance of the effects of timing and gene polymorphism on LW, scrotal perimeter, and plasma testosterone concentrations was assessed by the GLM procedure for repeated measurements (SPSS v. 26). To identify significant differences in the characteristics measured in the scanning, an ANOVA was used. The

statistical significance of differences among groups was evaluated by post-hoc Least Significant Difference (LSD) tests. To compare the distribution of behavioural events among groups in the serving-capacity tests, an Independent-Samples Kruskal-Wallis Test was used. Results are presented as mean ± S.E.M., and $P < 0.05$ was the level for statistical significance.

3. Results

LW (Figure 1), SP (Figure 2), and plasma testosterone concentrations (Figure 3) were significantly ($P < 0.001$) influenced by week, but neither of the polymorphisms of the melatonin receptor 1A (*MTNR1A*) gene affected these variables.

In the individual serving-capacity tests, genotypes did not differ significantly in individual behaviors (Figure 4) or the total number of events (CC: 24.1±3.2; CT: 26.3±13.0; TT: 16.8±4.8; GG: 22.0±3.0; GA: 21.0±6.7; AA: 27.8±12.7). Ano-genital sniffing (56%) and approaches (22%) were the most, and attempt mounting (7%) and pawing (6%) were the least common behavior.

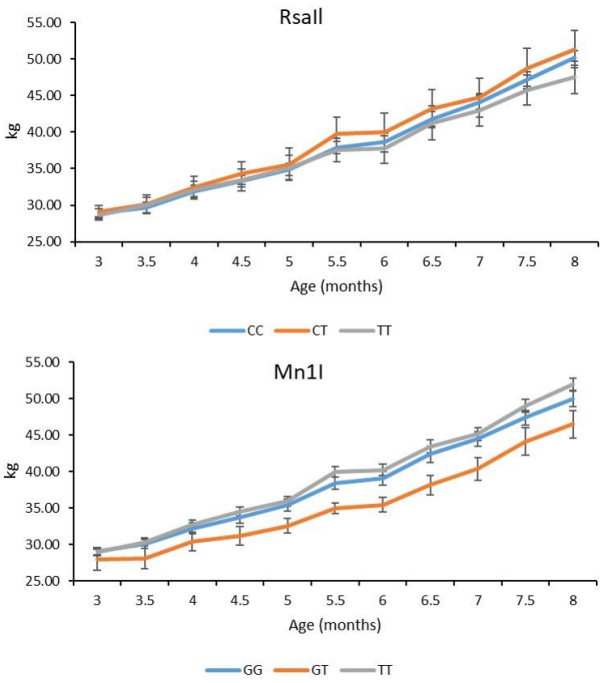

Figure 1 Mean (±S.E.M.) live weight (kg) of Rasa Aragonesa ram-lambs born in Oct that had been genotyped for their RsaI (CC, n=24 CT, n=6 TT, n=9) and Mn1I (GG, n=27 GA, n=6 AA, n=6) allelic variants of the *MTNR1A* gene.

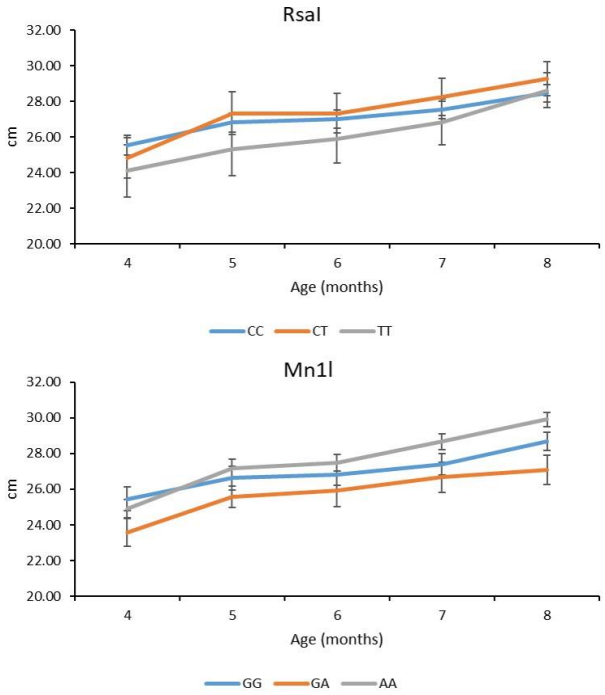

Figure 2 Mean (±S.E.M.) scrotal perimeter (cm) of Rasa Aragonesa ram-lambs born in Oct that had been genotyped for their RsaI (CC, n=24 CT, n=6 TT, n=9) and Mn1I (GG, n=27 GA, n=6 AA, n=6) allelic variants of the *MTNR1A* gene.

Rams did not differ significantly in the number of black, white, or grey pixels in the ultrasound images of their testicles, the number of tubules/cm^2, proportion (%) of the area occupied by the lumen of the tubules in the parenchyma, and mean diameter of the lumen of the seminiferous tubules (Table 1).

4. Discussion

The objective of this study was to ascertain whether having a specific *MTNR1A* polymorphism affected some reproductive variables of autumn-born ram-lambs at 8 months of age. The serving-capacity test, plasma testosterone levels, scrotal perimeter, and testicular echography measurements indicated that the *MTNR1A* gene did not have a significant effect on the age of sexual activity in those ram-lambs. In other studies in female sheep, the genotype at the *MTNR1A* gene did not have a significant effect on the age at which Sarda ewe-lambs first conceived (Mura et al. 2010; Luridiana et al 2016). Unlike adult buffaloes in which a polymorphism of the second exon of the MT1 receptor gene has an effect on the seasonality of reproduction, the genotype of the *MTNR1A* does not affect the timing of reproductive activity in pre-pubertal buffalo cows (Paludo et al 2011).

For rams, puberty is the week of age at which an ejaculate first includes a minimum number of sperm cells or the first ejaculate that contains motile sperm (Wheaton and Godfrey 2003; Khalifa et al 2013). In our study, electroejaculation was not used, and ram-lambs were not prevented from having previous interactions with females before the experiment; therefore, the precise moment when the ram-lambs began puberty could not be determined. In another study (Yarney and Sanford 1993), measurements of the two characteristics near the anticipated time of the onset of puberty provided the best indication of long-term adult reproductive function, and there were correlations between plasma testosterone concentration, testicular diameter, total

sperm per ejaculate, and ejaculation frequency. In our study, all of the ram-lamb groups had similar testosterone and scrotal features; therefore, probably, all of the groups matured at the same age. Furthermore, testicle size and spermatogenic function are correlated (Yarney et al 1990), and testicular ultrasound is a useful tool in the selection and morphophysiological evaluation of ram-lambs in the peripubertal and pubertal phases (Andrade et al. 2014) therefore, we suggest that the absence of differences in LW, scrotal perimeter, and testicular characteristics, reflects the lack of differences in sperm quality among groups.

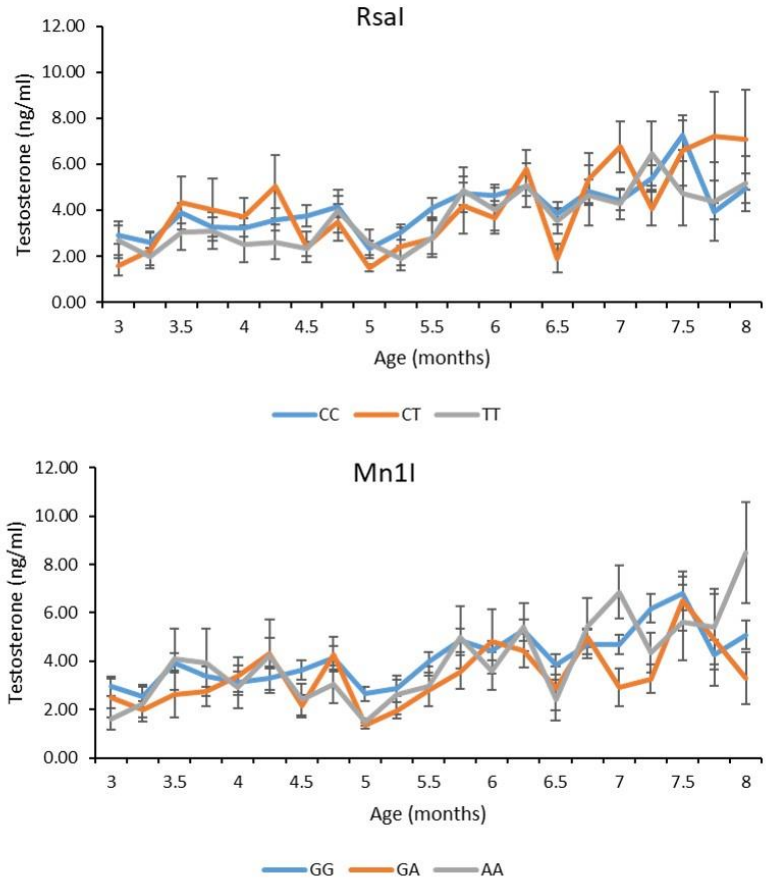

Figure 3 Mean (±SEM) plasma testosterone concentration (ng/ml) of Rasa Aragonesa ram-lambs born in Oct that had been genotyped for their RsaI (CC, n=24 CT, n=6 TT, n=9) and Mn1I (GG, n=27 GA, n=6 AA, n=6) allelic variants of the *MTNR1A* gene.

Nutrition, photoperiod, and genetic factors are the primary factors influencing the onset of puberty in sheep (Dyrmundsson 1973a,b). At Mediterranean latitudes, ewe-lambs born in autumn achieve a suitable age and body weight in late spring or early summer, but because of an unfavorable photoperiod, puberty does not begin until the following autumn (Forcada et al 1995). A few studies have attempted to influence the timing of puberty in sheep genetically. Xing et al. (2018) provided a basis for understanding the role of the Lin28A gene in the initiation of puberty in sheep. In the Davisdale line of productive sheep, mutations in the leptin receptor (LEPR) gene are significantly correlated with a delay in the onset of puberty and an elevated weight and body condition score at 18 months of age (Haldar et al 2014). However, the metabolic hormones IGF-I and leptin provide a physiological link between the developing tissues and the reproductive axis. Rosales Nieto et al (2013) found that selection for the genetic potential for growth can accelerate the onset of puberty and increase fertility and reproductive rate in Merino ewe lambs. In our study, any effect of growth rate or LW on the onset of puberty could not be assessed because genotypic groups did not differ in LW.

At the pre-pubertal stage, a ram's social rank can affect how well it reproduces in adulthood, although testosterone serum levels and semen output are not correlated with social rank (Ungerfeld and Lacuesta 2010). Our earlier research showed that the social hierarchy in the ram flock was not related to the increase in the rams' sexual performance in the spring that carried particular genotypes of the *MTNR1A* gene (Abecia et al 2022). Rams that carried the TT genotype at position g.17355458 C>T, and the GG genotype at position g.17355452G>A performed sexually better in the spring, but their social status was not significantly different from that of the rams that carried the other genotypes.

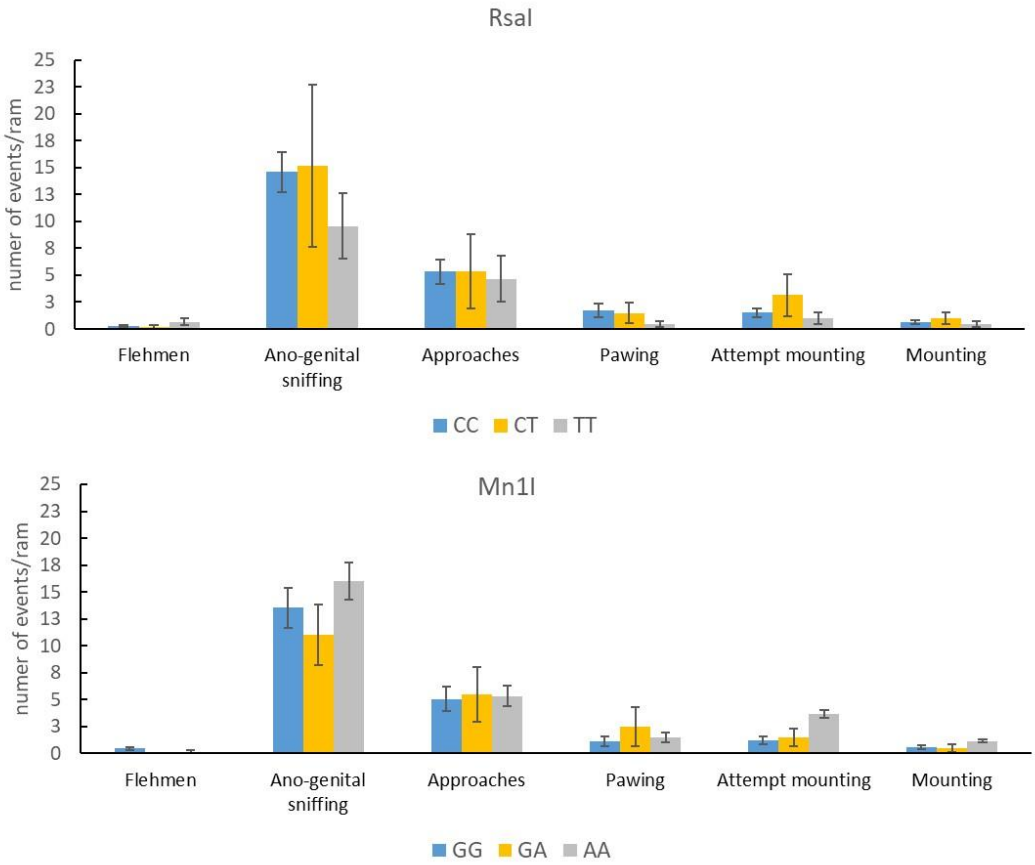

Figure 4 Mean (±SEM) number of flehmen, anogenital sniffing, approaches, pawing events, mounting attempts, and mountings in a 20-min (one ram-lamb with three estrous ewes) serving-capacity test by Rasa Aragonesa ram-lambs born in Oct that had been genotyped for their RsaI (CC, n=24 CT, n=6 TT, n=9) and Mn1I (GG, n=27 GA, n=6 AA, n=6) allelic variants of the *MTNR1A* gene.

Table 1 Mean (±SEM) image characteristics from ultrasonography of the testicles of Rasa Aragonesa ram-lambs born in Oct that had been genotyped for their RsaI (CC, n=24 CT, n=6 TT, n=9) and Mn1I (GG, n=27 GA, n=6 AA, n=6) allelic variants of the *MTNR1A* gene.

	RsaI			Mn1I		
	CC	CT	TT	GG	GA	AA
Ecotext 1 (black pixels)	17.7±3.3	14.5±3.0	13.3±4.7	17.1±2.8	14.8±8.5	13.8±3.2
Ecotext 2 (white pixels)	244.0± 47.3	304.8±58.3	235.4±38.2	214.7±30.1	366.8±140.5	301.3±58.7
Ecotext 3 (grey pixels)	101.6±2.1	105.2±1.7	103.3±1.8	100.9±1.6	107.2±4.4	105.3±1.8
Tubular area (%)	7.6±0.5	7.1±0.5	7.2±0.6	7.7±0.4	6.7±1.1	6.9±0.6
Tubular diameter (µm)	104.9±10.4	161.0±19.2	152.1±18.3	145.2±10.3	140.1±19.0	158.8±18.5
Tubular density (tubules/cm^2)	140.7±2.2	138.6±5.3	141.0±3.3	141.7±1.9	136.6±5.7	138.6±5.3

5. Conclusions

In conclusion, the melatonin receptor 1A (*MTNR1A*) polymorphisms did not affect autumn-born ram lambs' reproductive characteristics based on serving capacity tests, testosterone secretion, and testicular measurements at 8 months of age.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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