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Oxidative stress in donor mares for ovum pick-up delays embryonic development

Mohamed Hedia [a,b,c,*], Daniel Angel-Velez [a,d], Marion Papas [a,e], Sofie Peere [a], Ilse Gerits [a], Tine De Coster [a], Emma Van den Branden [a], Jan Govaere [a], Ann Van Soom [a], Jo L.M.R. Leroy [c], Katrien Smits [a,**]

[a] Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[b] Theriogenology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[c] Gamete Research Centre, Department of Veterinary Sciences, University of Antwerp, Wilrijk, Belgium
[d] Research Group in Animal Sciences-INCA-CES, Universidad CES, 050021, Medellin, Colombia
[e] Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

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ABSTRACT

The *in vitro* production of equine embryos via ovum pick-up (OPU) and intracytoplasmic sperm injection (ICSI) has increased rapidly. There is a marked effect of the individual mare on the outcome of OPU-ICSI, but little is known about the influence of the mare's health condition. This study aimed to investigate the potential associations between the concentrations of interleukin-6 (IL-6), reactive oxygen metabolites (d-ROMs), and biological antioxidant potential (BAP) in serum of oocytes' donor mares and the subsequent embryonic development. Just before OPU, a blood sample was collected from 28 Warmblood donor mares, that were subjected to a routine OPU-ICSI program. The serum concentrations of IL-6, d-ROMs, and BAP were assayed photometrically. The maturation, cleavage and blastocyst rate as well as the kinetics of blastocyst development were recorded. The average blastocyst rate was 24.68 ± 5.16% and the average concentrations of IL-6, d-ROMs, and BAP were 519.59 ± 157.08 pg/mL, 171.30 ± 4.55 carratelli units (UCARR), and 2711.30 ± 4.55 μmol/L, respectively. Serum concentrations of IL-6, d-ROMs, and BAP were not significantly different between mares yielding at least one blastocyst (552.68 ± 235.18 pg/mL, 168.36 ± 5.56 UCARR, and 2524.80 ± 159.55 μmol/L) and mares yielding no blastocysts (468.47 ± 179.99 pg/mL, 175.85 ± 7.89 UCARR, and 2999.50 ± 300.13 μmol/L, respectively). Serum concentrations of d-ROMs were significantly lower in mares with fast growing (at day 7–8 post ICSI; 148.10 ± 8.13 UCARR) compared to those with slow growing blastocysts (≥ day 9 post ICSI; 179.41 ± 4.89 UCARR; $P = 0.003$). Taken together, the serum concentration of IL-6, d-ROMs, and BAP do not determine the mare's ability to produce blastocysts *in vitro*. Although it may be questioned whether a single sample is representative of the mare's health status, changes in serum metabolites related to oxidative stress at the time of oocyte retrieval were linked to a delayed blastocyst development in a clinical OPU-ICSI outcome.

1. Introduction

Ovum pick-up (OPU) and intracytoplasmic sperm injection (ICSI) are substantially used to produce equine embryos *in vitro* [1–3]. The OPU-ICSI program is multi-advantageous and is as effective as embryo flushing when measured by the number of day 45 pregnant recipients per mare [4]. Regardless the stage of the ovarian cycle, follicular health and season, OPU-ICSI allows the production of a high number of foals, even from old, subfertile [5], and euthanized mares [6]. The success rate of OPU-ICSI is mainly evaluated by the mare's ability to produce a blastocyst and by the rate of (transferable) blastocysts [2], which are repeatable for an individual mare between two consecutive sessions [7].

There are several known mare related factors that can affect the success rate of OPU-ICSI program. Aged mares (>20 y) have a relatively

* Corresponding author. Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium.
** Corresponding author.
E-mail addresses: mohammedhedia@cu.edu.eg, mohamed.hedia@ugent.be (M. Hedia), Katrien.smits@ugent.be (K. Smits).

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low number of ovarian follicles [8]. As such, the number of embryos per OPU session declines in old mares, but the mare's age does not have a significant effect on the developmental competence of the oocytes [7,9]. A second factor which markedly affects the success rate of OPU-ICSI is the mare's breed [2]. The oocytes of Arabian donor mares show significantly lower cleavage and blastocyst rates compared to those of Warmblood mares [10]. Still, maternal age and breed are constant factors, and they cannot explain short-term fluctuations in the success rates of OPU-ICSI for an individual mare. The relationship between maternal health, oocyte quality, and OPU-ICSI outcome has been scarcely investigated in mares. On the one hand, it has been shown that the physiological status (transitional vs. cycling [7,11]) and the presence of reproductive disorders [11] are not significantly affecting the blastocyst rate. On the other hand, mares engaged in intense sporting activities [11] and obese mares [12,13] display a decreased oocyte developmental competence *in vitro*. Nevertheless, more research is needed to study the impact of the mare's health condition on the success rate of OPU-ICSI.

Female gametes are vulnerable to oxidative stress [14]. Estimation of the systemic oxidative stress index (OSI), measured by derivatives of reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) has been well established in women undergoing *in vitro* fertilization [15,16]. There is a direct association between serum and follicular fluid values of d-ROMs and OSI in women [17], where higher values in serum were accompanied with abnormal fertilization, while increased values in follicular fluid were associated with diminished embryo quality [18]. In horses, we recently showed that the values of OSI in serum and follicular fluid are correlated too [19].

Pro-inflammatory cytokines play a vital role in maintaining the ovarian physiology during folliculogenesis, oocyte maturation and ovulation [20]. There is a strong association between serum and follicular fluid concentrations of IL-6 both in women [21] and mares [19]. Excess IL-6 has been associated with decreased estradiol synthesis and aromatase activity in granulosa cells of women *in vitro* [22]. Higher IL-6 concentrations inhibited the expression of luteinizing hormone receptor mRNA during the maturation and differentiation of cultured rat granulosa cells [23]. Higher follicular fluid IL-6 values in women were associated with decreased clinical pregnancy rate [24]. In mares, higher concentrations of IL-6 within the preovulatory follicle have been correlated with diminished oocyte quality [25].

The relationship between maternal inflammation or oxidative stress, and the OPU-ICSI outcome has not been previously investigated in mares. We hypothesize that there is an association between the serum concentrations of oxidative stress markers (d-ROMs, BAP, and OSI) and the pro-inflammatory cytokine IL-6 at the time of oocytes retrieval (OPU) and the oocyte developmental competence in mares. Therefore, the objective of the present study was to investigate the associations between the serum concentrations of d-ROMs, BAP, OSI, and IL-6 and the OPU-ICSI outcome in mares.

2. Materials and methods

For this study, no specific samples were acquired from or extra procedures were performed with the mares included in this study as analyses were performed during routine clinical OPU-ICSI services. For this reason, no extra ethical clearance was necessary for the present study.

2.1. Animals

Twenty-eight Warmblood mares, with a body condition score ranged between 3 and 6 [26] and aged 2–23 years old were used between mid-January and mid-March 2022. These mares regularly participated in the OPU-ICSI program at the equine reproduction clinic, Faculty of Veterinary Medicine, Ghent University.

2.2. OPU procedures

Just before conducting the OPU and after blood sampling, a preoperative regime [9] of benzylpenicillin (20000 IU/kg intramuscular; Penikel®, Kela, Sint Niklaas, Belgium) and flunixin meglumin (1.1 mg/kg intravenous; Wellicox®, Ceva Santé Animale, Naaldwijk, The Netherlands) was used. During the OPU, detomidine hydrochloride (0.01 mg/kg intravenous; Domidine®, Eurovet Animal Health BV, Bladel, The Netherlands) and butorphanol tartrate (0.01 mg/kg intravenous; Dolorex®, MSD Animal Health, Sint-Lambrechts-Woluwe, Belgium) were used for sedation. To subside intestinal contractions, N-butylscopolammonium bromide (0.3 mg/kg intravenous; Buscopan®, Boehringer Ingelheim, Brussel, Belgium) was injected. Urinary bladder catheterization and epidural anesthesia were not applied. After proper aseptic preparation for the perineal region, the transvaginal transducer (7.5 MhZ linear probe, MyLabOne, Esaote, Genoa, Italy [9]) equipped by a 12-G double-lumen needle attached via a double way tube system to a prewarmed collection bottle of flushing medium (Equiplus®, Minitube, Tiefenbach, Germany). All visible antral follicles were punctured, aspirated, scraped, and flushed 8 times.

2.3. In vitro embryo production

The collection of oocytes [9] was carried out under sterile conditions using a laminar air flow equipped with a stereomicroscope (Olympus SZX7®, Olympus Corp., Japan). The whole contents of the collection bottle (follicular fluid, flushing medium, and scrapped follicular cells) were filtrated through a sterile 70 μm filter (Cell strainer®, BD Biosciences, Falcon, Erembodegem, Belgium) and the COCs were recovered from the filtrated contents in medium 199 with Hank's salts (Gibco, Life Technologies, Merelbeke, Belgium) supplemented with 10 % fetal bovine serum (FBS; Gibco). According to the schedule, the recovered COCs were either directly transferred to maturation medium (medium 199 with Earl's salts (Gibco) containing 10 % (v/v) FBS (Gibco), 9.4 μg/mL follicle stimulating hormone, and 1.88 g/ml luteinising hormone (Stimufol, Reprobiol, Ouffet, Belgium)) or were kept overnight in a commercial embryo holding medium (Emcare®, Agtech, Zulte, Belgium) at room temperature (~22 °C) prior to maturation. *In vitro* maturation was carried out in groups of 2–18 COCs in 100–500 μl maturation medium under oil (CooperSurgical, Venlo, The Netherlands) at 38.2 °C in 5 % CO_2 containing air for 28–32 h. A small piece of straw with frozen semen was thawed in 1 mL G-MOPS (38.2 °C; Vitrolife, Londerzeel, Belgium) and centrifuged twice (400×*g*/3 min at room] temperature; ~22 °C). After the first centrifugation, the supernatant was discarded and the pellet was re-suspended in 1 mL G-MOPs. After the second centrifugation, the supernatant was discarded and the pellet was resuspended in 200 μl G-MOPS. Immediately before ICSI, a small amount of the resuspended sperm was added to a 5 μl droplet of 7 % polyvinylpyrrolidone (CooperSurgical, Venlo, The Netherlands). Intracytoplasmic sperm injection, *in vitro* culture of presumptive zygotes, and the evaluation of embryonic development were performed until day 13 post ICSI [9].

2.4. Blood collection and laboratory analyses

A single blood sample per mare was collected from the jugular vein into vacutainer tubes with clot activator (BD Vacutainer®, BD-Plymouth, UK). To separate the serum, samples were centrifuged at 2460×*g* for 20 min at 4 °C. Serum was aliquoted into sterile 2 ml Eppendorf tubes and stored at −80 °C until further biochemical analysis.

Colorimetric kits (Diacron®; Diacron International, Italy) were used to measure the serum concentrations of d-ROMs and BAP, according to the manufacturer's guidelines [19]. A Multiskan GO spectrophotometer (Thermo Fisher Scientific, Finland; at 37 °C) was used to estimate the photometric measurements for both kits at 505 nm. The coefficient of variation was 1.72% for d-ROMs and 2.32% for BAP. The lowest limit of

detection for d-ROMs and BAP was 11 UCARR and 150 μmol/L, respectively. The OSI was determined from the concentrations of d-ROMs and BAP using the formula d-ROMs/BAP × 100 [27].

Serum concentrations of IL-6 were measured spectrophotometrically using an equine IL-6 ELISA kit (Nori®, Genorise Scientific, USA), according to the manufacturer's guidelines. A Multiskan GO spectrophotometer (Thermo Fisher Scientific, Finland; room temperature) was used to determine the optical density at 450 and 540 nm, which was followed by a wavelength correction. The average coefficient of variation was 6.49% and the lowest detection limit was 16 pg/mL.

2.5. Study design

Blood sampling was performed just before OPU. Immediately after OPU, the cumulus-oocyte complexes (COCs) were recovered, matured, and fertilized by ICSI.

At each OPU-ICSI session, (a) the number of aspirated follicles, (b) the number of recovered oocytes, (c) the recovery rate (b/a × 100), (d) the number of mature oocytes, (e) the maturation rate (d/b × 100), (f) the number of cleaved presumptive zygotes, (g) the cleavage rate (f/d × 100), (h) the number of produced blastocysts, (i) the blastocyst rate (h/d × 100), (j) the proportion of the cleaved zygotes that developed to blastocysts (h/f × 100), (k) the time of blastocyst formation, and (l) the serum concentrations of d-ROMs, BAP, OSI, and IL-6 were recorded. The mares were distributed into different groups according to (1) their ability to produce blastocysts: blastocyst producing (≥1 blastocyst; n = 17) and non-producing (0 blastocyst; n = 11) mares, (2) the required time for embryonic development: mares with fast growing (first blastocyst developed at day 7–8 post ICSI; n = 6) and mares with slow growing (first blastocyst developed at day ≥9 post ICSI; n = 11) embryos, and (3) age: young (≤14 y), middle-aged (15–19 y), and old (≥20 y) mares [28].

2.6. Statistical analyses

The assessment of the normality of data was performed using a Shapiro-Wilk test. The mean values of d-ROMs, BAP, and OSI, but not IL-6, were normally distributed. For the blastocyst producing mares, Spearman's correlation coefficients between the blastocyst rate, the proportion of cleaved zygotes that developed to blastocysts, and the serum concentrations of d-ROMs, BAP, OSI, and IL-6 were calculated. Differences in serum parameters between groups based on the mare's ability to produce embryos (blastocyst producing vs. non-producing mares) and the onset of embryonic development (fast vs. slow growing blastocysts) were determined using the independent t-test or Mann-Whitney U test. Differences between groups based on maternal age were explored using Welch one-way ANOVA followed by Games-Howell or Kruskal-Wallis test. The data were analyzed using the statistical package for social science SPSS® (SPSS Inc., version 16.0, Chicago, IL. USA), and a P- value < 0.05 was considered significant. Data are presented as mean ± SEM.

3. Results

The mean ± SEM values of all the studied parameters are presented in Table 1. There were no significant correlations between the blastocyst rate, the proportion of cleaved zygotes that produced blastocysts, and the serum concentrations of d-ROMs, BAP, OSI, and IL-6.

As shown in Table 2, serum concentrations of BAP were significantly higher in old mares (3544.60 ± 218.07 μmol/L) compared to the young ones (2461.40 ± 133.56 μmol/L). Values of OSI were significantly increased in young mares (7.41 ± 0.52) compared to old ones (4.95 ± 0.24). Serum concentrations of d-ROMs and IL-6 were not significantly different between young, middle-aged, and old mares. The blastocyst rate was not significantly affected by age.

Serum concentrations of d-ROMs, BAP, OSI, and IL-6 were not significantly different between the blastocyst producing and non-producing mares (Table 3). Serum concentrations of d-ROMs (Table 4) were significantly (P = 0.003) higher in mares with slow growing blastocysts (179.41 ± 4.89 UCARR) compared to those with fast growing ones (148.10 ± 8.13 UCARR).

Table 1
Mean values of parameters in donor mares (n = 28).

Parameters	Mean ± SEM	Range
Age (years)	11.32 ± 1.21	2–23
BCS	4.64 ± 0.17	3–6
Number of aspirated follicles	16.43 ± 1.44	6–31
Number of recovered oocytes	8.21 ± 0.87	2–18
Recovery rate (%)	51.45 ± 3.73	13.30–100
Number of mature oocytes	5.32 ± 0.61	1–15
Maturation rate (%)	66.84 ± 3.97	33.30–100
Number of cleaved presumptive zygotes	4.14 ± 0.54	1–13
Cleavage rate (%)	80.55 ± 4.54	20–100
Number of blastocysts	1.45 ± 0.41	0–9
Blastocyst rate (%)	24.68 ± 5.16	0–100
d-ROMS (UCARR)	171.30 ± 4.55	127.39–213.53
BAP (μmol/L)	2711.30 ± 155.74	1161.54–4846.67
OSI	6.89 ± 0.43	3.51-13.57
IL-6 (pg/mL)	519.59 ± 157.08	29.88–3828.21

BCS = body condition score; d-ROMs = reactive oxygen metabolites; BAP = biological antioxidant potential; OSI = oxidative stress index; IL-6 = interleukin-6.

Table 2
Serum concentrations of oxidative stress markers (d-ROMs, BAP, and OSI) and the pro-inflammatory cytokine IL-6 in young (≤14 y), middle-aged (15–19 y), and old (≥20 y) mares.

Items	Young mares (n = 19)	Middle-aged mares (n = 5)	Old mares (n = 4)	P value
d-ROMS (UCARR)	171.77 ± 6.40	166.96 ± 2.99	174.51 ± 10.41	0.689
BAP (μmol/L)	2461.40 ± 133.56[a]	2994.00 ± 604.26[a,b]	3544.60 ± 218.07[b]	0.017
OSI	7.41 ± 0.52[a]	6.47 ± 1.13[a,b]	4.95 ± 0.24[b]	0.006
IL-6 (pg/ml)	515.25 ± 207.61	838.49 ± 367.06	141.58 ± 107.42	0.300
BR (%)	26.10 ± 6.74	28.33 ± 18.56	13.33 ± 8.16	0.806

Data with superscripts a and b within the same row are significantly different (P < 0.05).
d-ROMs = reactive oxygen metabolites; BAP = biological antioxidant potential; OSI = oxidative stress index; IL-6 = interleukin-6; BR= Blastocyst rate.

4. Discussion

In this study, we found an association between the serum concentrations of oxidative stress markers (d-ROMs, BAP, and OSI) and the pro-inflammatory cytokine IL-6 at the time of OPU and kinetics of embryo

Table 3
Serum concentrations of oxidative stress markers (d-ROMs, BAP, and OSI) and the pro-inflammatory cytokine IL-6 in blastocyst producing and non-producing mares.

Items	Blastocyst producing mares (n = 17)	Non-producing mares (n = 11)	P value
d-ROMS (UCARR)	168.36 ± 5.56	175.85 ± 7.89	0.432
BAP (μmol/L)	2524.80 ± 159.55	2999.50 ± 300.13	0.139
OSI	7.08 ± 0.46	6.59 ± 0.85	0.580
IL-6 (pg/mL)	552.68 ± 235.18	468.47 ± 179.99	0.335

d-ROMs = reactive oxygen metabolites; BAP = biological antioxidant potential; OSI = oxidative stress index; IL-6 = interleukin-6.

Table 4
Serum concentrations of oxidative stress markers (d-ROMs, BAP, and OSI) and pro-inflammatory cytokine (IL-6) in mares with fast (first embryo at day 7–8 post ICSI) and slow (first embryo at ≥ day 9 post ICSI) growing blastocysts.

Items	Mares with fast growing blastocysts ($n = 6$)	Mares with slow growing blastocysts ($n = 11$)	P value
d-ROMS (UCARR)	148.10 ± 8.13[a]	179.41 ± 4.89[b]	0.003
BAP (μmol/L)	2403.00 ± 274.54	2591.20 ± 202.83	0.590
OSI	6.65 ± 0.90	7.32 ± 0.54	0.508
IL-6 (pg/mL)	570.82 ± 262.25	542.78 ± 343.48	0.131

Data with superscripts a and b within the same row are significantly different ($P < 0.05$).
ICSI = intracytoplasmic sperm injection; d-ROMs = reactive oxygen metabolites; BAP = biological antioxidant potential; OSI = oxidative stress index; IL-6 = interleukin-6.

development. More specifically, high concentrations of d-ROMs at the time of OPU are linked to delayed embryonic development in mares. This may point out that a disturbance in maternal health related to oxidative stress can affect the OPU-ICSI outcome in mares.

Overall, the OPU-ICSI results in this study were consistent with those reported previously [2,9]. Serum concentrations of d-ROMs, BAP, and IL-6 were within the previously reported range in Warmblood mares during the non-breeding season [19]. In agreement with literature, ageing had neither significant effect on the serum concentrations of d-ROMs [29], nor on the blastocyst rate [7,9]. In our study, the serum concentrations of BAP were significantly higher and the values of OSI were significantly lower in old mares compared to young ones. In humans, the total antioxidant capacity in serum also increases with advancing age, which may be related to diet and daily routine [30]. The effect of age on the serum antioxidant status in horses is not clear. Some studies did not report any significant effect of ageing on the serum total antioxidant status [31] and BAP [29] in horses. On the other hand, Andriichuk et al. [32] found that physical exercise increases the plasma concentrations of thiobarbituric acid reactive substrates, catalase, and glutathione reductase in Warmblood horses. While our study indicates an effect of ageing on oxidative stress, further influence of diet and physical activity remains to be determined.

Although oxidative stress markers in serum did not determine the mare's ability to produce embryos, mares with slow growing blastocysts showed significantly higher serum concentrations of d-ROMs. In a previous study, we found that serum and follicular fluid values of OSI (d-ROMs/BAP × 100) are directly correlated in Warmblood mares [19]. Speed of embryo development affects both pregnancy and foaling rates in mares. Ducheyne et al. [33] found that fast growing embryos (formed before day 9 post ICSI) yield significantly higher pregnancy rates compared to the slow growing ones (formed after day 9 post ICSI). Foaling rate was significantly higher for day 7 and day 8 embryos (71.7 % and 53.3 %) compared to day 9 and day 10 embryos (38.5 % and 25 % [34]). In mammals, the oocyte developmental competence is linked to maternal health [35]. Maternal oxidative stress is increasing the concentrations of reactive oxygen species (ROS) in oocytes, which reduces their viability [36,37]. Oocytes with higher levels of ROS show delayed two-cell, four-cell, and blastocyst development in mice [36,38]. Oxidative stress in serum and follicular fluid significantly decreases the clinical pregnancy rate in women [15,39]. Higher values of d-ROMs in follicular fluid of women have been associated with abnormal fertilization and production of bad quality embryos [18]. Here, we hypothesize that the higher serum concentrations of d-ROMs may be reflected by increased d-ROMs in the follicular fluid, which may affect oocytes quality, resulting in delayed embryo development. More studies are necessary to further explore the effect of oxidative stress and antioxidants on the oocyte developmental competence in horses.

In this study, serum concentrations of IL-6 neither affected the mare's ability to produce embryos nor the blastocyst rate. There is a positive association between serum and follicular fluid concentrations of IL-6 in mares [19,25]. In the follicle, IL-6 is responsible for extracellular matrix formation and stabilization, which regulates cumulus cells expansion and increases the oocyte competence [40]. Several studies have been conducted, but there is no conclusive answer regarding the role of IL-6 in oocytes and subsequent embryos. Higher concentrations of IL-6 in FF can either increase [41] or decrease [24] the pregnancy outcome in women. Supplementation of culture media with IL-6 improved fetal development of IVF-embryos in cows [42] and supported embryonic compaction, blastulation and hatching in mice [43]. The expression of IL-6 and IL-6 signal transducer genes in granulosa cells was upregulated with advancing maternal age in mares [28]. In women with infertility, there was a downregulation in the expression of IL-6 signal transducer and IL-6 receptor genes in granulosa cells of older patients [44]. However, maternal age did not affect the concentrations of IL-6 in mares' serum (this study) and women's FF [45]. Therefore, it seems that the role of IL-6 in oocytes and embryos is species-specific and dose-dependent.

5. Conclusions

In conclusion, mares with higher serum concentrations of d-ROMs at the time of oocyte retrieval (OPU) show a delayed embryonic development. More studies should be conducted to investigate underlying mechanisms and potential therapy by antioxidants supplementation during the *in vitro* maturation of the oocytes collected from mares with oxidative stress. The measured ranges of d-ROMs, BAP, and IL-6 concentrations in serum at the time of OPU cannot be used to predict the mare's ability to produce embryos *in vitro*.

CRediT authorship contribution statement

Mohamed Hedia: Conceptualization, Lab work, Data curation, Original draft writing. **Daniel Angel-Velez:** Lab work, Data curation. **Marion Papas:** Animals work. **Sofie Peere:** Animals work. **Ilse Gerits:** Animals work. **Tine De Coster:** Lab work. **Emma Van den Branden:** Animals work. **Jan Govaere:** Conceptualization, Animals work, Providing fund. **Ann Van Soom:** Supervision. **Jo L.M.R. Leroy:** Supervision, Providing fund. **Katrien Smits:** Conceptualization, Lab work, Supervision, Providing fund.

Declaration of competing interest

The authors declare no competing interests.

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