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Abstract

The application of trans-vaginal ovum pick up (OPU) and intracytoplasmic sperm injection (ICSI) is well established for commercial *in vitro* embryo production in horses. These assisted reproductive techniques are especially applied during the non-breeding season of the mare. However, little is known about how the health of the oocyte donor may affect the biochemical composition of the follicular fluid (FF) in small and medium-sized follicles routinely aspirated during OPU. This study aimed to investigate associations between systemic and FF concentrations of interleukin-6 (IL-6), total cholesterol, triglycerides, non-esterified fatty acids (NEFA), reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), and oxidative stress index (OSI) during the non-breeding season in mares. At the slaughterhouse, serum and FF of small (5-10 mm in diameter), medium (>10-20 mm in diameter), and large (>20-30 mm in diameter) follicles were sampled from 12 healthy mares. There was a strong positive association ($P<0.01$) between the concentration of IL-6 in serum and those measured in small ($r= 0.846$), medium ($r= 0.999$), and large ($r= 0.996$) follicles. Serum concentrations of NEFA were positively correlated ($P<0.05$) with those measured in small ($r= 0.726$), medium ($r= 0.720$), and large ($r= 0.974$) follicles. Values of total cholesterol and OSI in serum and medium follicles were significantly associated ($r= 0.736$ and $r= 0.696$, respectively). The serum concentrations of all lipid metabolites were markedly higher than those measured in FF of small- and medium-sized follicles. Values of IL-6 and OSI did not change significantly between serum and all follicle classes ($P\geq0.05$). To conclude, changes in the blood composition associated with inflammation, oxidative stress, and disturbed lipid metabolism of mares may lead to an inadequate oocyte microenvironment, which could affect oocyte quality and the success rate of OPU/ICSI programs. Further research should indicate whether these changes may ultimately affect *in vitro* oocyte developmental capacity and subsequent embryo quality.

Keywords: Follicular fluid; Lipid metabolites; IL-6; Oxidative stress index; OPU/ICSI; Mare

Introduction

The *in vitro* embryo production (IVP) via ovum pick-up (OPU) and intracytoplasmic sperm injection (ICSI) has been commonly used in warmblood mares (Galli et al. 2014, Claes et al. 2018). The OPU/ICSI program allows breeders to produce relatively high numbers of embryos from genetically valuable mares of old age, diminished fertility, and even after death (Hinrichs, 2010). This program can be efficiently conducted, irrespective of follicular health, size, and composition, all year round, but OPU/ICSI is most extensively performed during the non-breeding season (Lazzari et al. 2020). Under these conditions, the percentages of freezable embryos typically range between 16-20% in warmblood mares (Lazzari et al. 2020). Several mare related factors such as the total number of recovered oocytes (Cuervo-Arango et al. 2019), maternal age and breed (Lazzari et al. 2020; Claes and Stout, 2022), and the serum concentrations of anti-Müllerian hormone of the donor mare during OPU (Papas et al. 2021) may affect the success rate of embryo production by IVP.

The crosstalk between the mare's health, the follicular microenvironment, and the oocyte quality has been scarcely investigated. In obese mares, there was a marked increase in serum concentrations of inflammatory cytokines (Sessions-Bresnahan and Carnevale, 2014) and lipid metabolites (Sessions-Bresnahan et al. 2016), which was directly associated with their concentrations in the preovulatory follicle. Ageing in mares affects follicular fluid (FF) exosomal microRNAs and granulosa cell transforming growth factor β during follicle development (de Silveira et al. 2015). The follicular microenvironment is further affected by follicular size. All year round, the maturation rate of equine oocytes was directly correlated with follicle size (Hinrichs and Schmidt, 2000). The follicular development in mares influences the follicular metabolites, electrolytes (Satué et al. 2019) and steroid hormones (Satué et al. 2020). Interestingly, FF components, related to the oocytes developmental competence, have been clearly described in cows (Annes et al. 2019), camels (El-Shahat et al. 2018), goats (Junior et al. 2018), and sows (Bertoldo et al. 2013). However, so far, this concept remains underexplored in mares.

Optimal conditions to support the developmental competence of the oocyte rely on a delicate balance between the supportive effect of lipids, cytokines and energy metabolites, and the detrimental influence of excessive concentrations of these components in the follicular environment. Lipid metabolites are crucial during oocyte maturation (Liu et al. 2022), as fatty acids are supplying oocytes with energy and as cholesterol is the precursor of steroid hormones (Dunning et al. 2014). On the other hand, maternal obesity (Sessions-Bresnahan et al. 2016) and dietary fat composition (Catandi et al. 2022) alters the lipid content of equine oocytes, which deteriorates their developmental competence. High concentrations of free fatty acids in bovine (Leroy et al. 2005), murine (Wu et al. 2010), and human follicles (Jungheim et al. 2011), as well as high cholesterol concentrations (Yesilaltay et al. 2014), also affected the oocytes' developmental competence.

Interleukin (IL)-6 is an intraovarian regulatory cytokine that promotes steroidogenesis and follicular rupture (Field et al. 2014, Adamczak et al. 2021). On the other hand, high FF concentrations of IL-6 have been associated with a dysregulated expression of genes related to oocyte maturation and cumulus expansion in mares (Sessions-Bresnahan and Carnevale, 2014). *In vitro*, excess IL-6 has been associated with decreased estradiol synthesis and aromatase activity in granulosa cells of women (Deura et al. 2005) and with inhibited expression of luteinizing hormone receptor mRNA during the maturation and differentiation of cultured rat granulosa cells (Tamura et al. 2001).

Cumulus-oocytes complexes with optimal number of mitochondria, sufficient levels of ATP, and proper reactive oxygen species (ROS) neutralization produce higher quality blastocysts in women (Assou et al. 2006) and cows (Marei et al. 2019). Accordingly, an oxidative imbalance in FF is associated with mitochondrial malfunctions, and DNA fragmentation in oocytes (Chaube et al. 2005, Zhang et al. 2006). The evaluation of serum and intrafollicular oxidative stress index (OSI), measured by reactive oxygen metabolites (d-ROMs; marker for oxidative stress) and biological antioxidant potential (BAP; marker for antioxidant defense status) has been well established in women (Luti et al. 2021), but not in mares. Interestingly, the balance between d-ROMs and BAP in FF (Terao et al. 2019)

and serum (Di Rosa et al. 2016) at the time of oocyte retrieval is important in the processes of fertilization and embryo growth in women (Terao et al. 2019).

There is a lack of knowledge concerning the concentrations of lipid metabolites (total cholesterol, triglycerides and total non-esterified fatty acids; NEFA), inflammatory status (IL-6), and oxidative stress markers (d-ROMs, BAP, and OSI) within the FF of mares during the non-breeding season. In this study, the hypothesis that the concentrations of FF components may be associated with their serum concentrations was tested. These insights are of crucial importance as they may impact the success rate of OPU/ICSI programs in horses. Therefore, the aims of the current study were (1) to measure the intrafollicular concentrations of total cholesterol, triglycerides, total NEFA, IL-6, d-ROMs, BAP, and oxidative stress index in different-sized follicles during the non-breeding season (the intense period of OPU/ICSI program) in mares and (2) to correlate these values with their serum concentrations.

Materials and methods

Animals and sampling

Samples were collected from 12 nonpregnant mares (warmblood), aged between 12 and 26 y during the non-breeding season (January and February 2022) in the abattoir of Anderlecht, Belgium (50°50'37.7"N 4°19'40.3"E). Only healthy mares were selected, with a body condition score between 5 and 6 (Henneke et al. 1983) and normal reproductive tracts upon macroscopical examination after slaughter. During exsanguination, blood samples were placed into serum clot activator tubes (10 ml) without separating gel to allow coagulation. Immediately after evisceration, ovaries were collected, and cooled to 4° C. Finally, both ovaries and coagulated blood samples were allocated per mare and transported on ice (4 °C) to the laboratory within 2 h after slaughtering.

Ovaries were washed two times with normal saline (NaCl 0.9%) and blotted dry. A conventional caliper was used to measure the follicles and FF was collected from three different follicle categories; small follicles (5-10 mm, n=10), medium follicles (>10-20 mm, n=11) and large

follicles (>20-30 mm, *n*=4). FF was aspirated by an 18 G needle and a 10 ml syringe and pooled per follicle class per mare. A different needle and syringe were used for each follicle class per mare. To avoid blood contamination, the needle was inserted within the follicular antrum, and the aspiration was stopped before the complete collapse of the follicle. A cooled centrifuge (4 °C) was used to remove the cellular pellets from FF (1500 × *g* for 10 min) and coagulated blood samples (2460 × *g* for 20 min). Supernatants of serum and FF samples were aliquoted into sterile 1.5 ml Eppendorf tubes and stored at –80 °C until further laboratory analysis. As a preliminary validation of our sampling protocol, the FF concentrations of all the studied variables were not different between the samples which were collected before transportation (in the slaughterhouse) and those aspirated after transportation (in the laboratory).

Biochemical analyses

Total cholesterol, triglycerides, and NEFA

Concentrations of cholesterol, triglycerides, and NEFA in serum and FF were measured once by Roche Cobas chemistry analyzers (c501 module; Modular, Roche Diagnostics, Mannheim, Germany). According to the manufacturer guidelines, enzymatic-colorimetric assays were used (CHOL2 and TRIGL kits; Roche Diagnostics, Germany) to assess the concentrations of cholesterol and triglycerides, respectively, both at 700/505 nm bichromatic absorbance. For NEFA concentrations an enzymatic-end point method was performed, using the NEFA FS kit (DiSys Diagnostic Systems GmbH, Holzheim, Germany) at 546/600 nm bichromatic absorbance. The intra-assay coefficients of variation at the lowest, medium, and highest concentrations were 0.7, 1, and 1.1% for cholesterol, 0.7, 0.85, and 1.1% for triglycerides and 0.95, 1.05, and 1.1% for NEFA, respectively. The lowest limits of detection were 3.86 mg/dL, 8.85 mg/dL, and 0.18 mg/dL for total cholesterol, triglycerides, and NEFA, respectively.

IL-6 assay

The concentration of IL-6 in serum and FF was measured in duplicate using a commercial ELISA kit (Nori® equine IL-6 kit, Genorise Scientific, USA) according to the manufacturer's procedures without any modifications. The optical density was determined twice at 450 and 540 nm by Multiskan GO spectrophotometer (Thermo Fisher Scientific, Finland; room temperature), and the values at 540 nm were subtracted from the values at 450 nm for wavelength correction. A standard curve was created using excel software equipped with MyCurveFit® tool to generate a four-parameter logistic curve-fit. The intra-assay coefficient of variation at the lowest, medium, and highest concentration was 7.28, 8.35, and 4.90%, respectively. The lowest detection limit was 16 pg/mL, with a < 0.5% cross-reactivity.

Determination of d-ROMs, BAP, and OSI

Serum and FF concentratons of d-ROMs and BAP were measured in duplicate using the photometric Diacron® kits (Diacron International, Italy) according to the manufacturer's instructions. For both kits, the photometric readings were determined at 505 nm using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Finland; at 37° C). The coefficients of variation at the lowest, medium, and highest concentrations were 0.55, 2.78, and 5.94% for d-ROMs and 8.76, 6.55, and 0.03% for BAP, respectively. Analytical sensitivity for d-ROMs and BAP was 11 UCARR and 150 µmol/L, respectively. As described by Shono et al. (2020), the OSI was calculated using the formula (d-ROMs / BAP ×100).

Statistical analysis

A Kolmogorov Smirnov test was applied to check the distribution of data. Pearson's correlation coefficients between serum and intrafollicular concentrations for each follicle class were tested. Within the same mares, a paired samples t-test was conducted to compare the concentration of each variable in serum and those measured in small (n=10), medium (n= 11), and large (n= 4) follicles. The data were analyzed using the Statistical Package for Social Science SPSS® (SPSS Inc., version 16.0,

156 Chicago, IL. USA), and a *P*- value <0.05 was considered significant. Results are shown as mean ±SEM
157 for all studied variables in serum and FF from the different categories.

158 **Results**

159 For each variable, the relationships between systemic and intrafollicular values of small, medium,
160 and large follicles are presented in Table 1. There was a significant positive ($P < 0.05$) association
161 between NEFA concentrations in serum and FF of all follicle categories. Concentrations of IL-6 in
162 serum were positively associated with those found in all follicle classes ($P < 0.01$). Values of
163 cholesterol and OSI in serum were positively correlated ($P < 0.05$) with those measured in medium
164 follicles.

165 As shown in Table 2, concentrations of lipid metabolites were significantly higher in serum
166 compared to those found in FF of small and medium-sized follicles ($P < 0.05$). Concentrations of IL-
167 6 did not show any significant differences between systemic and intrafollicular levels. The average
168 serum concentrations of IL-6 in two mares (mare 4 and mare 8, respectively; Fig. 1. d) were
169 approximately 8- and 39-folds higher than the other 10 mares, which was associated with an increase
170 in the average of their FF values in small (67-folds), medium (97-folds), and large (878-folds) follicles.
171 Average and range serum and FF concentrations of all studied variables for individual mares are
172 depicted in the Supplementary Material.

173 The serum concentration of d-ROMs (Table 2) was significantly higher than those measured in all
174 follicle classes ($P < 0.05$). Biological antioxidant potential values were significantly higher in serum
175 compared to the FF of medium follicles. For all metabolites, no significant differences were detected
176 between the differently sized follicle classes.

177 **Discussion**

178 In this study, the hypothesis that there is an association between the serum and FF concentrations
179 of lipid metabolites (cholesterol, triglycerides and NEFA), a pro-inflammatory cytokine (IL-6), and

oxidative stress markers (d-ROMs, BAP and OSI) during the intense period of OPU/ICSI program (non-breeding season) was tested. The results confirm that the concentrations of NEFA and IL-6 in all follicle classes and the values of cholesterol and OSI in medium-sized follicles were correlated with those in serum. These findings may indicate that a disturbance in the maternal health related to inflammatory conditions, oxidative stress, or lipid metabolism is reflected in the micro-environment of the oocyte.

An optimal follicular environment during oocyte maturation should guarantee a proper nuclear and cytoplasmic maturation to secure the developmental capacity (Hatirnaz et al. 2018). Knowledge of the *in vivo* composition of FF can be used to predict the competence of oocytes derived from particular follicles or to improve conditions for *in vitro* maturation of the equine oocyte. This study is the first report showing the biochemical composition for differently-sized follicles during the non-breeding season in mares, follicles which are routinely aspirated during OPU to collect the oocytes used for commercial IVP (Lazzari et al. 2020).

In agreement with our findings, systemic lipid metabolites were mirrored in FF and the concentrations of triglycerides and cholesterol (Sessions-Bresnahan et al. 2016) as well as fatty acids (Catandi et al. 2022) were significantly higher in plasma compared to FF of the preovulatory follicle. An increase in FF triglycerides and cholesterol is found in obese mares and alters the expression of granulosa cells' genes related to endoplasmic reticulum and oxidative stress (Sessions-Bresnahan et al. 2016) and embryonic marker genes related to inflammation and lipid metabolism (Sessions-Bresnahan et al. 2018). Moreover, the plasma, follicular, and oocyte lipid concentrations are influenced by the composition of polyunsaturated fatty acids in diet, which determine the oocyte's developmental competence in mares (Catandi et al. 2022). This effect of high NEFA levels on the oocyte's developmental capacity has also been noticed in women (Valckx et al. 2014a), mice (Valckx et al. 2014b), and cows (Leroy et al. 2005).

Concentrations of IL-6 showed a strong positive correlation between serum and FF of all follicle classes. Sessions-Bresnahan and Carnevale (2014) also found that concentrations of IL-6 were highly correlated in serum and FF of preovulatory follicle in mares with equine metabolic syndrome. In addition, the minimum and maximum concentrations of follicular IL-6 were extremely different between individual mares. Only two mares in our study caused this difference (Fig. 1d). Similarly, the range of FF IL-6 concentration in women was also highly different and seems to be influenced by health conditions (Chen et al. 2000), but not by age or follicle size (Piccinni et al. 2021). Also in the horse, the concentration of IL-6 in serum can be severely affected by the mares' health status (Burton et al. 2009; Ibrahim et al. 2021). Unfortunately, the lack of clinical history of the mares in the present study prevented us from expecting the definite cause of this extreme concentrations of IL-6 in serum and FF. Nevertheless, it will be highly interesting to check if these higher concentrations would have a carryover effect on oocyte developmental competence. The average concentration of IL-6 in serum and FF in our study was lower than those measured in equine preovulatory follicles (Sessions-Bresnahan and Carnevale, 2014) and higher than those measured in serum of Arabian mares (Ibrahim et al. 2022). These variations may be due to the differences in breeds, season, and the used protocol of analysis.

In the present study, oxidative stress and antioxidant capacity in the FF of mares was determined for the first time. A positive association between the values of OSI in serum and FF of medium follicles was found. It is known that excessive exposure to oxidative stress may lead to oocyte chromosomal segregation, and damage of cellular components (Tarin et al. 1996) which may hamper subsequent embryonic development (Lin et al. 2021). In women, higher concentrations of d-ROMs in serum significantly reduced the success rate of clinical pregnancy (Di Rosa et al. 2016). Lower values of d-ROMs and OSI in FF of women were associated with better fertilization rates and production of more good quality embryos when compared to higher concentrations of both oxidative stress markers (Terao et al. 2019). In agreement with our results, there were no associations between systemic and

intrafollicular concentrations of BAP in women undergoing ART cycles (Di Rosa et al. 2016). More studies are necessary to further explore the influence of oxidative stress index, measured in serum and FF, on the OPU/ICSI outcomes in horses.

Taken together, this study investigated the biochemical characterization of FF from differently sized follicles outside the breeding season, similar to the clinical OPU-conditions. For several parameters of lipid metabolism, inflammation and oxidative stress, follicular concentrations were correlated with those in serum. As such, our study generated new insights into the physiological concentrations of these metabolites in the horse and provides a solid basis for further research on the determination of their effect on the developmental competence of equine oocytes. Moreover, the correlations between serum and FF highlight the potential impact of the mare's health and metabolism on the composition of the FF and thus on oocyte quality as has been shown previously in other species.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CEediT authorship contribution statement

Mohamed Hedia: sampling, lab work, statistical analysis, and original draft writing. **Jo Leroy**: providing funding, methodology, data curation, review, and supervision. **Jan Govaere**: conceptualization and review. **Katrien Smits**: conceptualization, editing, review, and supervision. **Ann Van Soom**: editing, review, and supervision.

Declarations

Ethical approval

Prior ethical agreement was not necessary. Ovaries from horses were collected post-mortem in a commercial horse slaughterhouse.

Competing interests

The authors declare that they do not have any conflict of interest.

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Table 1. Correlation coefficients between serum and intrafollicular values of lipid metabolites (cholesterol, triglycerides, and NEFA), inflammatory cytokine (IL-6), and markers of oxidative stress (d-ROMs, BAP, and OSI) in small (**SF**; *n*= 10), medium (**MF**; *n*=11), and large (**LF**; *n*=4) follicles of warmblood mares.

	Cholesterol	**Triglycerides**	**NEFA**	**IL-6**	**d-ROMs**	**BAP**	**OSI**
Serum × small follicles	0.376	-0.125	0.726*	0.846**	0.234	-0.174	0.089
Serum × medium follicles	0.736**	-0.141	0.720*	0.999**	0.600	0.561	0.696*
Serum × large follicles	0.456	0.521	0.974*	0.996**	0.276	0.882	0.274

NEFA= non-esterified fatty acids; IL-6= interleukin 6; d-ROMs= reactive oxygen metabolites; BAP= biological antioxidant potential; OSI= oxidative stress index

* = Significant at 5% level. ** = Significant at 1% level.

Table 2. Mean ± S.E.M of lipid metabolites (total cholesterol, triglycerides, and NEFA), inflammatory biomarker (IL-6), and oxidative stress markers (d-ROMs, BAP, and OSI) in serum and follicular fluid of small (**SF**; *n*= 10), medium (**MF**; *n*=11), and large (**LF**; *n*=4) follicles in warmblood mares.

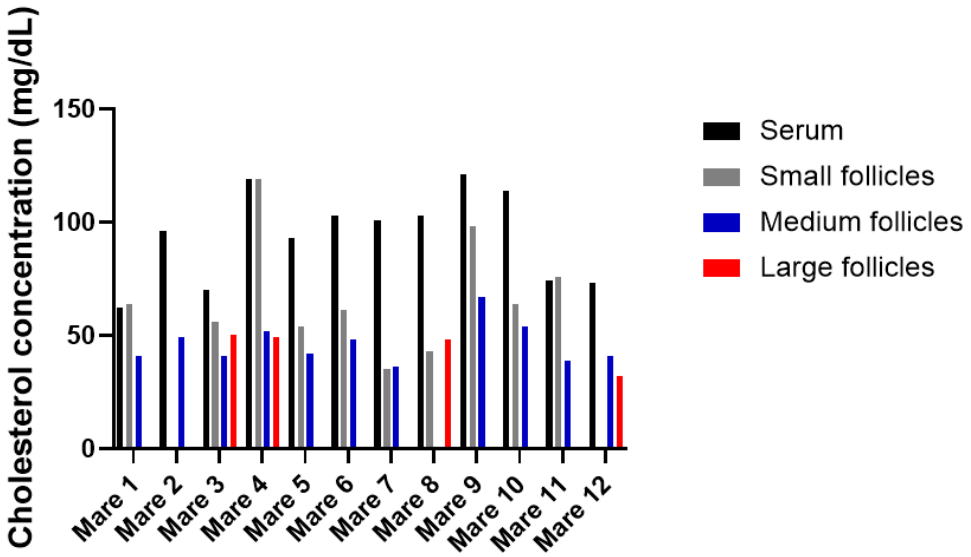
Category	Cholesterol (mg/dL)		Triglycerides (mg/dL)		NEFA (mg/dL)		IL-6 (pg/mL)		d-ROMs (UCARR)		BAP (µmol/mL)		OSI	
	S	**FF**	**S**	**FF**	**S**	**FF**	**S**	**FF**	**S**	**FF**	**S**	**FF**	**S**	**FF**
SF	96.00±6.60*	67.00±7.95	41.10±5.06*	19.30±1.93	10.97±1.93*	5.31±0.47	333.37±230.17	1029.10±640.46	140.42±10.55*	57.67±7.13	5726.30±647.16	4658..10±1116.10	2.88±0.48	2.11±0.77
MF	93.27±6.27*	46.10±2.95	46.27±6.03*	15.50±1.49	15.78±3.80*	6.22±0.55	99.06±38.34	693.49±628.50	141.24±9.25*	63.05±8.79	5295.90±608.22*	2521.90±464.44	3.15±0.47	3.47±0.73
LF	91.25±11.88*	44.75±4.27	29.25±5.34	16.75±1.65	18.85±9.71	6.85±1.20	742.38±551.71	26394.00±19635.00	132.88±7.66*	42.09±11.25	5319.00±1521.30	244.60±563.92	3.14±1.07	2.20±0.71

S= serum; FF= follicular fluid; NEFA= non-esterified fatty acids; IL-6= interleukin 6; d-ROMs= reactive oxygen metabolites; BAP= biological antioxidant potential; OSI= oxidative stress index.

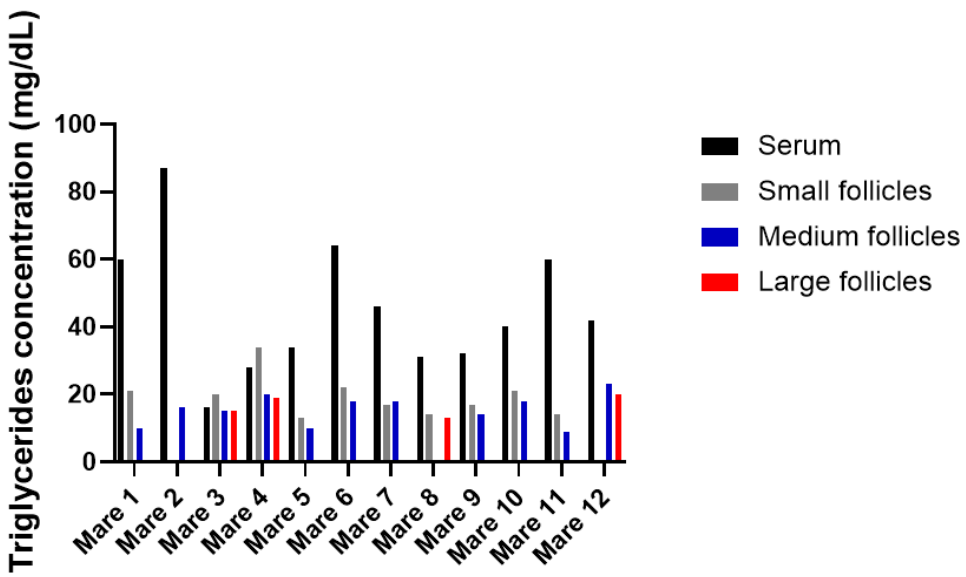
Superscript *: Serum concentration of the corresponding variable differ significantly ($P < 0.05$) from its concentration in the follicular fluid.

Fig. 1. Mean serum and follicular fluid concentrations of lipid metabolites (**a**: cholesterol, **b**: triglycerides, and **c**: NEFA), inflammatory cytokine (**d**: IL-6), and markers of oxidative stress (**e**: d-ROMs, **f**: BAP, and **g**: OSI) for individual mares.

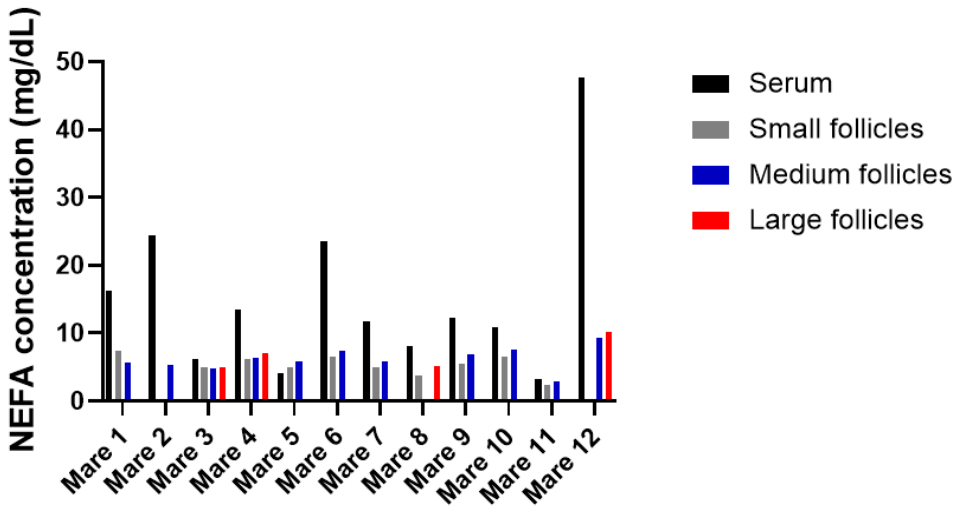
(a)



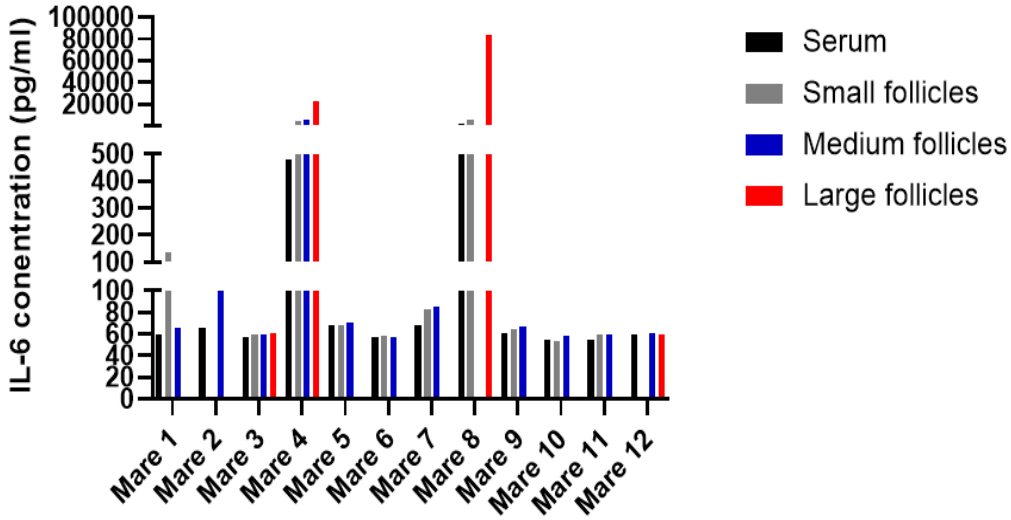
(b)



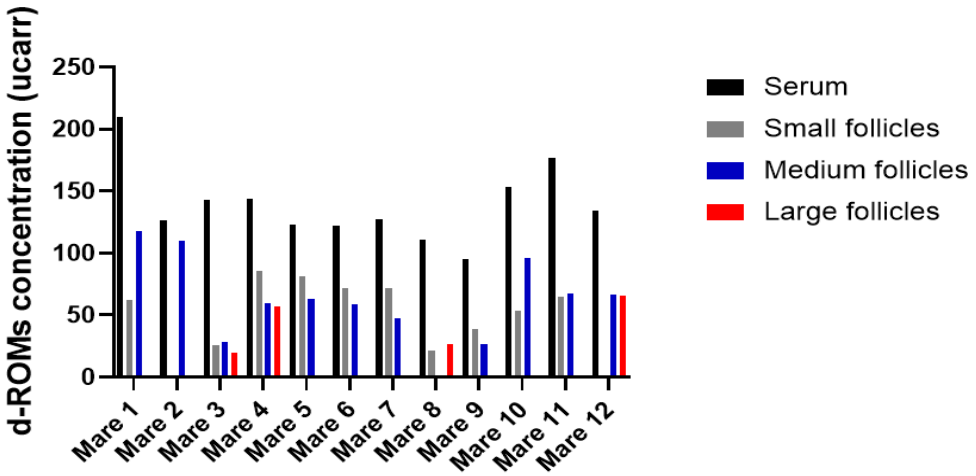
(c)


NEFA concentration (mg/dL)
50
40
30
20
10
0
Mare 1
Mare 2
Mare 3
Mare 4
Mare 5
Mare 6
Mare 7
Mare 8
Mare 9
Mare 10
Mare 11
Mare 12
Serum
Small follicles
Medium follicles
Large follicles

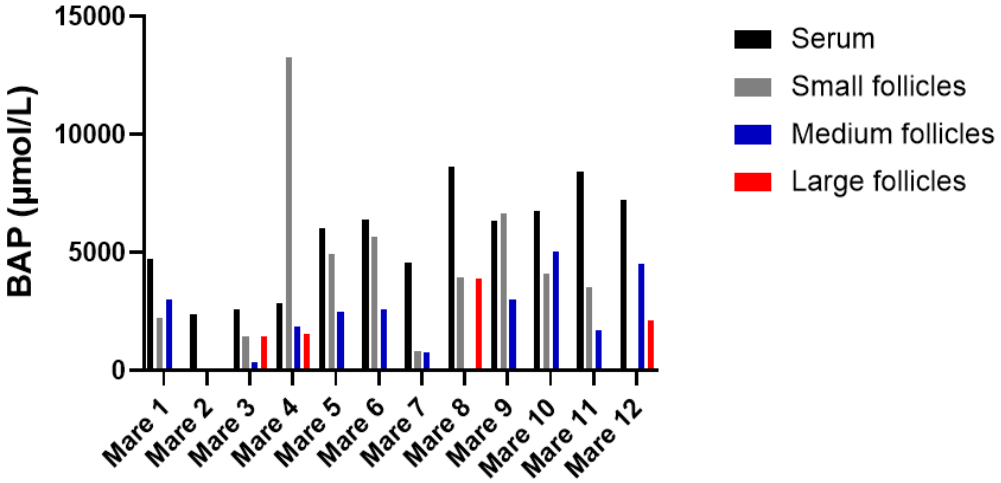
(d)


IL-6 conentration (pg/ml)
100000
80000
60000
40000
20000
500
400
300
200
100
100
80
60
40
20
0
Mare 1
Mare 2
Mare 3
Mare 4
Mare 5
Mare 6
Mare 7
Mare 8
Mare 9
Mare 10
Mare 11
Mare 12
Serum
Small follicles
Medium follicles
Large follicles

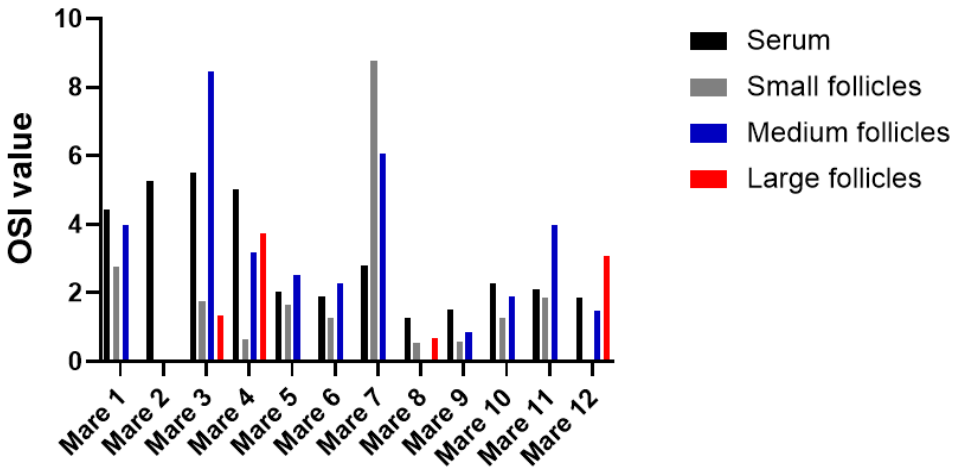
(e)


d-ROMs concentration (ucarr)
250
200
150
100
50
0
Mare 1
Mare 2
Mare 3
Mare 4
Mare 5
Mare 6
Mare 7
Mare 8
Mare 9
Mare 10
Mare 11
Mare 12
Serum
Small follicles
Medium follicles
Large follicles

(f)


BAP (µmol/L)
15000
10000
5000
0
Mare 1
Mare 2
Mare 3
Mare 4
Mare 5
Mare 6
Mare 7
Mare 8
Mare 9
Mare 10
Mare 11
Mare 12
Serum
Small follicles
Medium follicles
Large follicles

(g)


OSI value
10
8
6
4
2
0
Mare 1
Mare 2
Mare 3
Mare 4
Mare 5
Mare 6
Mare 7
Mare 8
Mare 9
Mare 10
Mare 11
Mare 12
Serum
Small follicles
Medium follicles
Large follicles