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CD49f+ mammary epithelial cells decrease in milk from dairy cows stressed by overstocking during the dry period

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1 **CD49f+ mammary epithelial cells decrease in milk in overstocked-stressed dairy cows during the**  
2 **dry**

3 **2 Period**

4

5 Mario Baratta<sup>1\*</sup>, Silvia Miretti<sup>1</sup>, Paolo Accornero<sup>1</sup>, Giovanna Galeati<sup>2</sup>, Andrea Formigoni<sup>2</sup>,

6 Gianfranco Gabai<sup>3</sup>, Daniele Nucera<sup>4</sup>, Eugenio Martignani<sup>1</sup>

7 Dept. Veterinary Science, University of Turin, Italy, Largo Braccini 2, 10095 Grugliasco (TO)

8 Italy

9 Dept. Veterinary Medical Sciences (DIMEVET), University of Bologna, 40062 Ozzano

10 dell'Emilia (BO), Italy

11 Dept. Comparative Biomedicine and Food Science, University of Padua, 35020 Legnaro (PD),

12 Italy

13 Dept. Agricultural, Forest and Food Sciences, University of Torino, 10095 Grugliasco (TO), Italy

14 Short title: **Epithelial cells populations in milk in dairy cows**

15 \*Correspondence: Mario Baratta

16 Dept. Veterinary Science

17 University of Turin

18 Largo Braccini, 2

19 10095 Grugliasco (TO)

20 Italy

21 phone +39-11-670 9146

22 FAX +39-11-236 9146

*E-mail: [mario.baratta@unito.it](mailto:mario.baratta@unito.it)*

## 23 Summary

24 This study aims to describe the modification in epithelial cells populations during the first and the  
25 last month of milking in Holstein Friesian cows that have undergone different management during  
26 the dry period. We report the differential expression of CD49f<sup>+</sup> and cytokeratin18<sup>+</sup> cell  
27 subpopulations managed in overstocking during the dry period (from 21 ± 3 d to the expected  
28 calving until calving). Twenty six cows were randomly divided into 2 groups (13 animals each),  
29 balanced for the number of lactations, body condition score, and expected date of calving. Cows in  
30 the far-off phase of the dry period (from 60 to 21 d before the expected calving date) were housed  
31 together in a bedded pack. Then, animals (from 21 ± 3 d before the expected calving) until calving  
32 were housed in pens with the same size but under different crowding conditions due to the  
33 introduction of heifers (interference animals) into the pen. The control condition (CTR) had 2  
34 animals per pen with 12.0 m<sup>2</sup> each, whereas the overstocked condition (OS) had 3 interference  
35 animals in the same pen with 4.8 m<sup>2</sup> for each animal. Cells collected from milk samples were  
36 directly analyzed for: CD45, CD49f, cytokeratin 14, cytokeratin 18 and cell viability. Milk samples  
37 were collected in two different periods of lactation: early lactation (EL = d 0–30) and late lactation  
38 (LL = 270–300). We observed a differential expression with a reduction in CD49f<sup>+</sup> (p<0.01) and  
39 cytokeratin 18<sup>+</sup> (p<0.05) cells in EL. These observations suggest that mammary epithelial cell  
40 immunophenotypes could be associated to different animal management in dry period and we  
41 hypothesize they may have a role as biomarkers for mammary gland function in dairy cows.

## 42 Introduction

43 Increased stocking density is a common practice among dairy producers. One of the main tools to  
44 evaluate the effect of such management is to determine the threshold of stressful situation that  
45 triggers a number of changes such as activation of the sympathetic nervous system and  
46 hypothalamic-pituitary-adrenal axis that have been considered a well-known source of biomarkers  
47 for animal welfare (Prunier *et al.*, 2013). Very recently it has been demonstrated that overstocking  
48 during the dry period in Holstein Friesian dairy cow is associated with changes also in DHEA  
49 (Fustini *et al.*, 2017).

50 However, the determination of hormonal pattern to evaluate stressful situation presents some  
51 difficulties for the sampling and comparison of hormone levels in a given time interval. Also it  
52 would be interesting to include the ability to insert other physiological parameters that are to some  
53 extent related to the animal's well being. We have reported the expression of epithelial precursors  
54 and fully differentiated cells in bovine milk, highlighting possible variations in the number and  
55 features of mammary epithelial cells (MEC) subsets in dairy cows (Baratta *et al.*, 2015). MEC are  
56 found in milk, caused by shedding during the lactation phase, but the range of cell frequency differ  
57 from total somatic cell count (SCC) if only the live cell fraction is analyzed. The total amount of  
58 somatic cells in milk is affected by different factors, such as species, breeds, lactation phase, milk  
59 yield, individual animal differences, and management practices (Rupp *et al.*, 2000). A specific  
60 pattern of epithelial cell types has been found in the milk according to the stage of lactation. Cell  
61 types include an inner layer of cytokeratin 18 (K18)<sup>+</sup> luminal cells and an outer layer of cytokeratin  
62 14 (K14)<sup>+</sup> myoepithelial cells while CD49<sup>+</sup> cells are probably derived from a more primitive stage  
63 of cell differentiation (Martignani *et al.*, 2015). In this study we show different mammary epithelial  
64 cell types present in milk of dairy cows that have been undergone overstocking during the dry  
65 period and hypothesize that specific cell types variations may be related to stressful management.

## 66 Materials and Methods

### 67 *Animals, housing and diet*

68 Twenty six Holstein dairy cows were enrolled in this experiment. All animals were housed at the  
69 farm of the University of Bologna (Ozzano Emilia, Italy) and used according to EEC animal care  
70 guidelines. The experimental procedures had been approved by the Ethical Committee of Bologna  
71 University. Animals were randomly divided into two groups (13 animals each), balanced for

72 number of lactations, BCS (body condition score) and expected date of calving. Cows in the far-off  
73 phase of the dry period (from 60 to 21 days before the expected calving date) were housed together  
74 in a bedded-pack and received water and grass hay ad libitum. From 21±3 days until calving  
75 animals were housed in two bedded-pack groups where they had ad libitum access to water and  
76 were fed daily using total mixed ration. After calving cows were housed together in a bedded pack  
77 area for the first 2 weeks of lactation and then moved to a free-stall pen for the rest of lactation. The  
78 total mixed rations (TMR) were fed approximately at 7 am for lactating cows and 9 am for dry  
79 cows.

#### 80 *Experimental design, blood sampling and hormone assays*

81 Animals, dried off 8 weeks before the expected calving, were housed in pens with the same size  
82 (22,5 m<sup>2</sup> in total with 13,5 m<sup>2</sup> of resting area and 9 m<sup>2</sup> of feeding area) but in different crowding  
83 conditions due to the introduction in the pen of heifers (interference animals) having a body weight  
84 of 450-550 Kg. Control condition (CTR) had 2 animals per pen (one animal of the study with an  
85 interference animal) with 11 m<sup>2</sup> each, while the overstocked condition (OS) had three interference  
86 animals in the same pen with 5 m<sup>2</sup> for each animal. Cow were allocated to CTR or OS group based  
87 on parity, at 21 days before expected calving dates. The resting area is a deep-bedded pack with  
88 straw added twice a day. On days -30, -21, -7 before and 4, 10, 30, 60 relative to calving blood  
89 samples were collected from each cow for the determination of plasma DHEA and cortisol (C)  
90 concentrations by RIA..

#### 91 *Flow Cytometry Analysis: Sample Processing*

92 Quarter foremilk samples were obtained in accordance with the Veterinary Services Standards of  
93 the Italian National Health Service, branch of the Ministry of Health. Before morning milking, teats  
94 were scrubbed with 70% ethanol and the first 2 strips of milk were discarded. Aliquots of 200 mL  
95 of milk per udder were collected aseptically.. Cells were collected and analyzed according to  
96 previously reported (Baratta *et al.*, 2015). Briefly, the determination of epithelial subpopulations in  
97 milk was carried out utilizing a 6-color flow cytometry assay. Anti-CD45 antibody (VMRD Inc.,  
98 Pullman, WA) was used to gate immune cells, anti-human- CD49f-FITC antibody (anti-h- $\alpha$ -  
99 integrin-6-FITC, Novus Biological, Littleton, CO), monoclonal anti-CK peptide 18 antibody (clone  
100 KS-B17.2, Sigma, St. Louis, MO), and anti-CK14 antibody (Covance, Life Technology, Thermo  
101 Fisher).. Stained samples were analyzed using an Attune Acoustic Focusing Cytometer (Life

Technologies). Cells without antibody labeling served as a negative control and were regarded to be a measure for background fluorescence. Fluorescence Minus One (FMO) controls were used to identify data spread due to the multiple fluorescent signals (2000; Bayer *et al.*, 2007). Epithelial cells were identified and counted in the total living CD45<sup>+</sup> cell population.

#### *Statistical Analysis*

The two groups of cows (13 animals each) were compared on the following variables: living cells, CD49f<sup>+</sup>, K14<sup>+</sup>, K18<sup>+</sup>, and K14<sup>+</sup>K18<sup>+</sup>; values were collected at the beginning and in the last month of lactation. Considering that all variables were frequencies, non-parametric test were performed for all the analyses. In particular, Mann-Whitney U test was chosen and, firstly, variables were compared between the groups of cows at the first month of lactation. Secondly, the same analyses were repeated for measures collected in the last month of lactation, in order to explore for differences in significant results. Results were considered significant when associated at least to  $p < 0.05$  for all the comparisons.

## **Results**

### *Hormones concentrations*

In overstocking group (OS) significantly ( $P < 0.01$ ) DHEA significantly increased compared to CTR group at day -7 ( $2.13 \pm 0.63$  vs  $1.47 \pm 0.46$  pmol/ml) while C did not differ between CTR and OS group (see supplementary data).

### *Frequency of epithelial subpopulations during the first month of lactation*

Figure 1a shows total living cells (ranging from 66% to 78%) detected in the somatic cell population, identified as CD45<sup>+</sup> cells, in Holstein Friesian cows in response to stress induced by overstocking (OS) or not (CTR) during the dry period. A significant difference between the two groups was observed in CD49f<sup>+</sup> cells ( $p < 0.01$ ) with a decrease in OS group of frequency from 20 to %. Interestingly, we observed a significant difference ( $p < 0.05$ ) in the level of luminal cells (K18<sup>+</sup>) with a decrease in OS group. Finally, no differences were detected in myoepithelial (K14<sup>+</sup>) and CK14<sup>+</sup>/CK18<sup>+</sup> cells.

### *Frequency of epithelial subpopulations during the last month of lactation*

129 Figure 1b shows total living cells ranging from 63% to 75% detected in the somatic cell population,  
130 identified as CD45<sup>+</sup> cells, in cows that were exposed to stress during the dry period induced by  
131 overstocking (OS). A tendency to a decrease in OS group was observed without reaching a  
132 statistical difference was ( $p= 0.066$ ). Luminal cell (K18<sup>+</sup>) were present at low frequency in both  
133 group (2-3 %) while myoepithelial cells (K14<sup>+</sup>) still showed a greater concentration ranging from  
134 18 to 21%. Finally, no differences were detected in myoepithelial, luminal and CK14<sup>+</sup>/CK18<sup>+</sup> cells  
135 between the two groups.

#### 136 *Milk yield in response to treatment over transition period*

137 Mean milk yield (kg/d) in response to treatment over the transition period was not different among  
138 treatments (Table 1). Among cows, treatment did not differ regarding previous lactation 305-d  
139 mature- equivalent milk yield (CTR =  $10.1 \pm 215.1$  kg, OS =  $9.5 \pm 187.7$  kg;  $P = 0.35$ ).

#### 140 **Discussion**

141 It has recently been reported that DHEA secretion is affected in response to overstocking during the  
142 dry period in Holstein Friesian cows (Fustini *et al.*, 2017). We reported that DHEA concentrations  
143 were affected only during the dry period, when the stressful stimulus was applied, while no  
144 differences in DHEA secretion were observed during the first two months of lactation. Since the  
145 placenta seems the most important DHEA source in the late pregnant cow (Gabai *et al.*, 2004), it is  
146 possible that overstocking stimulates the release of DHEA from the maternal-foetal units through a  
147 still unknown mechanism. In the present work we cannot investigate on the source of this  
148 metabolite; however, we can confirm that in dairy cow DHEA plasma levels are affected during the  
149 last part of pregnancy by stressful management like overstocking that usually occurring during the  
150 dry period.

151 We mainly focused our attention on the frequency and differential expression of epithelial cells  
152 subpopulations in milk. We have previously reported the expression of epithelial precursors and  
153 fully differentiated cells according to the phase of lactation (Baratta *et al.*, 2015). We report now a  
154 further information that lead us to consider the hypothesis that different distributions of MEC  
155 subpopulations may provide more detailed information on the physiology of the mammary gland  
156 during lactation in dairy cows. In particular, our data suggest that stressful situations can affect the  
157 somatic cell subpopulations. We considered the first and the last month of lactation period that



received or not the stressful experience of overstocking monitored by the change of DHEA during the last days of dry period. We observed a different pattern of expression between the two groups of animal in the first month of lactation but not at the end of the physiological period indicating that the stressful-experienced cows showed a lower expression in CD49f<sup>+</sup> and K18<sup>+</sup> cell populations. We were interested on CD49f population evaluation since they belong to more primitive MEC (mammary precursors). They appear to decrease during the decline of lactation and in this way may exert a role in the reduction of the mammary secretory function, which adjusts the number of active secretory cells. We hypothesize that this subpopulation may be considered the signal of a reduction in mammary efficiency. The presence of CD49f positive cells, even if in a low number, may be related also to the reduction in the myoepithelial compartment that indicated the modification of the myoepithelial genetic program (Garbe *et al.*, 2012). This integrin has been shown to be a component of a feedback circuit that regulates the myoepithelial phenotype in mammary epithelial cells from humans and mice (Deugnier *et al.*, 1999; LaBarge *et al.*, 2009) suggesting that the basal regulatory machinery may be disrupted in myoepithelial cells and inappropriately engaged in luminal epithelial cells, maybe during the aging process. We did not observe a significant difference in K14/K18 double positive cells, in term of activation of regenerative functional tissue of mammary gland, in particular during the final phase of lactation. On the contrary, we have observed a difference in K18<sup>+</sup> cells with a decrease in OS group during the first month of lactation. This subpopulation are specifically linked to the secreting cells since they are referred as luminal cells. We would expect this difference to be associated with a reduction in milk yield during the period of milking, although we did not observed any decrease in milk production. The exposure to stressful conditions might influence the numerical relationship between luminal cells that produce milk in the mammary gland and epithelial cells that are shed in milk. One aspect that deserves to be thoroughly investigated is the number of functional cells found in milk needed to detect an effect on milk production.

### 183 Conclusions

184 In conclusion, we report the expression of epithelial precursors and fully differentiated cells during  
185 the first month of lactation in dairy cows that were overstocked during the previous dry period,  
186 highlighting variations in the number and features of MEC subsets in milk. Although we were not  
187 able to detect a correlation with milk production, it remains interesting to observe that overstocking  
188 associated with hormonal pattern during dry period shows different modulation of somatic cells

189 during the lactation. Further studies are necessary to determine if different distributions of MEC  
190 subpopulations may provide more detailed information on the physiology of the mammary gland  
191 during lactation in dairy cows and, potentially, have an application to evaluate mammary gland  
192 functionality as biomarkers.

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196 declare a conflict of interest.

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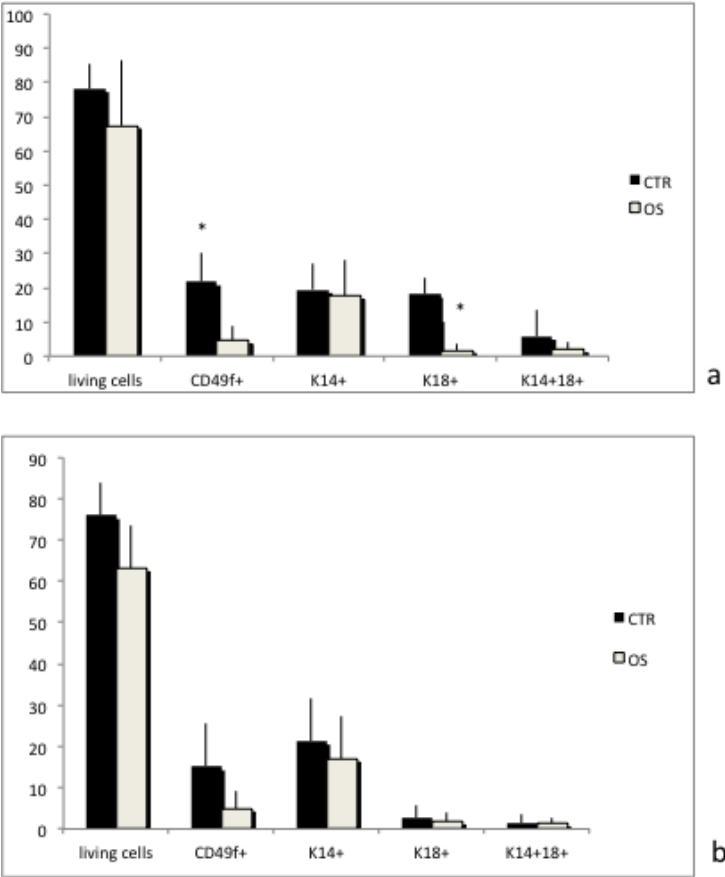
**229. Figure Legends**

230. **Fig.1** Frequency in percentage of cell viability and of epithelial cell subpopulations in bovine milk  
231. during the first month of lactation (**a**) and during the last month of lactation in control (**b**) in control  
232. (CTR) and overstocked condition (OS) groups. Cell subpopulations are identified according to the  
233. positive expression of CD49f, K14 and K18. \* mean at least  $P < 0.05$ . Error bars represent SD.

234.

235. **Tab.1** Mean ECM yield (kg/d) in response to treatment experienced in the transition period

Fig.1



**Table 1**

Week after calving	Control (CTR)	Overstock condition (OS)	SEM	P-value
1	23,5	22,3	1,4	0,65
2	34,9	31,9	1,5	0,13
3	35,8	34,4	1,6	0,23
4	37,1	35,9	1,3	0,18