



ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE
DELLA RICERCA

Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

CD49f+ mammary epithelial cells decrease in milk from dairy cows stressed by overstocking during the dry period / Baratta, Mario; Miretti, Silvia; Accornero, Paolo; Galeati, Giovanna; Formigoni, Andrea; Gabai, Gianfranco; Nucera, Daniele; Martignani, Eugenio. - In: THE JOURNAL OF DAIRY RESEARCH. - ISSN 0022-0299. - STAMPA. - 84:4(2017), pp. 414-417. [10.1017/S0022029917000589]

Availability:

This version is available at: <https://hdl.handle.net/11585/619156> since: 2020-10-27

Published:

DOI: <http://doi.org/10.1017/S0022029917000589>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

Mario Baratta, Silvia Miretti, Paolo Accornero, Giovanna Galeati, Andrea Formigoni, Gianfranco Gabai, Daniele Nucera, Eugenio Martignani *CD49f+ mammary epithelial cells decrease in milk in overstocked-stressed dairy cows during the dry period*, Journal of Dairy Research, 84 (4), 2017, pp.414-417.

The final published version is available online at:

DOI: <https://doi.org/10.1017/S0022029917000589>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

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

2 Period

3

4 Mario Baratta^{1*}, Silvia Miretti¹, Paolo Accornero¹, Giovanna Galeati², Andrea Formigoni²,

5 Gianfranco Gabai³, Daniele Nucera⁴, Eugenio Martignani¹

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

7 Italy

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

9 dell'Emilia (BO), Italy

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

11 Italy

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

13 Short title: Epithelial cells populations in milk in dairy cows

14 *Correspondence: Mario Baratta

15 Dept. Veterinary Science

16 University of Turin

17 Largo Braccini, 2

18 10095 Grugliasco (TO)

19 Italy

20 phone +39-11-670 9146

21 FAX +39-11-236 9146

22 *E-mail: 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⁺ and cytokeratin18⁺ 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 m² each, whereas the overstocked condition (OS) had 3 interference
35 animals in the same pen with 4.8 m² 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⁺ (p<0.01) and
39 cytokeratin 18⁺ (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)⁺ luminal cells and an outer layer of cytokeratin
62 14 (K14)⁺ myoepithelial cells while CD49⁺ 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 m² in total with 13,5 m² of resting area and 9 m² 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 m² each, while the overstocked condition (OS) had three interference
86 animals in the same pen with 5 m² 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- α -
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

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

106 *Statistical Analysis*

107 The two groups of cows (13 animals each) were compared on the following variables: living cells,
108 CD49f⁺, K14⁺, K18⁺, and K14⁺K18⁺; values were collected at the beginning and in the last month of
109 lactation. Considering that all variables were frequencies, non-parametric test were performed for
110 all the analyses. In particular, Mann-Whitney U test was chosen and, firstly, variables were
111 compared between the groups of cows at the first month of lactation. Secondly, the same analyses
112 were repeated for measures collected in the last month of lactation, in order to explore for
113 differences in significant results. Results were considered significant when associated at least to
114 $p < 0.05$ for all the comparisons.

115 **Results**

116 *Hormones concentrations*

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

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

121 Figure 1a shows total living cells (ranging from 66% to 78%) detected in the somatic cell
122 population, identified as CD45⁻ cells, in Holstein Friesian cows in response to stress induced by
123 overstocking (OS) or not (CTR) during the dry period. A significant difference between the two
124 groups was observed in CD49f⁺ cells ($p < 0.01$) with a decrease in OS group of frequency from 20 to
125 %. Interestingly, we observed a significant difference ($p < 0.05$) in the level of luminal cells (K18⁺)
126 with a decrease in OS group. Finally, no differences were detected in myoepithelial (K14⁺) and
127 CK14⁺/CK18⁺ cells.

128 *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⁻ 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⁺) were present at low frequency in both
133 group (2-3 %) while myoepithelial cells (K14⁺) still showed a greater concentration ranging from
134 18 to 21%. Finally, no differences were detected in myoepithelial, luminal and CK14⁺/CK18⁺ 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 ± 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

158 received or not the stressful experience of overstocking monitored by the change of DHEA during
159 the last days of dry period. We observed a different pattern of expression between the two groups of
160 animal in the first month of lactation but not at the end of the physiological period indicating that
161 the stressful-experienced cows showed a lower expression in CD49f⁺ and K18⁺ cell populations.
162 We were interested on CD49f population evaluation since they belong to more primitive MEC
163 (mammary precursors). They appear to decrease during the decline of lactation and in this way may
164 exert a role in the reduction of the mammary secretory function, which adjusts the number of active
165 secretory cells. We hypothesize that this subpopulation may be considered the signal of a reduction
166 in mammary efficiency. The presence of CD49f positive cells, even if in a low number, may be
167 related also to the reduction in the myoepithelial compartment that indicated the modification of the
168 myoepithelial genetic program (Garbe *et al.*, 2012). This integrin has been shown to be a
169 component of a feedback circuit that regulates the myoepithelial phenotype in mammary epithelial
170 cells from humans and mice (Deugnier *et al.*, 1999; LaBarge *et al.*, 2009) suggesting that the basal
171 regulatory machinery may be disrupted in myoepithelial cells and inappropriately engaged in
172 luminal epithelial cells, maybe during the aging process. We did not observe a significant difference
173 in K14/K18 double positive cells, in term of activation of regenerative functional tissue of
174 mammary gland, in particular during the final phase of lactation. On the contrary, we have observed
175 a difference in K18⁺ cells with a decrease in OS group during the first month of lactation. This
176 subpopulation are specifically linked to the secreting cells since they are referred as luminal cells.
177 We would expect this difference to be associated with a reduction in milk yield during the period of
178 milking, although we did not observed any decrease in milk production. The exposure to stressful
179 conditions might influence the numerical relationship between luminal cells that produce milk in
180 the mammary gland and epithelial cells that are shed in milk. One aspect that deserves to be
181 thoroughly investigated is the number of functional cells found in milk needed to detect an effect on
182 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.

193 Acknowledgments

194 This work was supported by PRIN 2010-2011 (project of national interest from the Italian Ministry
195 of Education, Universities and Research, Rome, Italy). None of the authors of this manuscript
196 declare a conflict of interest.

197 **References**

198

199 Baratta M, Volpe MG, Nucera D, Gabai G, Guzzo N, Fustini M & Martignani E 2015 Differential
200 expression of living mammary epithelial cell subpopulations in milk during lactation in
201 dairy cows. *Journal of Dairy Science* **98** 6897-6904

202 Bayer J, Grunwald D, Lambert C, Mayol JF & Maynadie M 2007 Thematic workshop on
203 fluorescence compensation settings in multicolor flow cytometry. *Cytometry B Clinical*
204 *Cytometry* **72** 8-13

205 Deugnier MA, Faraldo MM, Rousselle P, Thiery JP & Glukhova MA 1999 Cell-extracellular
206 matrix interactions and EGF are important regulators of the basal mammary epithelial cell
207 phenotype. *Journal of Cell Science* **112** 1035-1044

208 Fustini M, Galeati G, Gabai G, Mammi LE, Bucci D, Baratta M, Accorsi PA & Formigoni A 2017
209 Overstocking dairy cows during the dry period affects dehydroepiandrosterone and cortisol
210 secretion. *Journal of Dairy Science* **100** 620-628

211 Garbe JC, Pepin F, Pelissier FA, Sputova K, Fridriksdottir AJ, Guo DE, Villadsen R, Park M,
212 Petersen OW, Borowsky AD, Stampfer MR & Labarge MA 2012 Accumulation of
213 multipotent progenitors with a basal differentiation bias during aging of human mammary
214 epithelia. *Cancer Research* **72** 3687-3701

215 LaBarge MA, Nelson CM, Villadsen R, Fridriksdottir A, Ruth JR, Stampfer MR, Petersen OW &
216 Bissell MJ 2009 Human mammary progenitor cell fate decisions are products of interactions
217 with combinatorial microenvironments. *Integrative Biology* **1** 70-79

218 Martignani E, Cravero D, Miretti S, Accornero P & Baratta M 2015 Clonogenic assay allows for
219 selection of a primitive mammary epithelial cell population in bovine. *Experimental Cell*
220 *Research* **338** 245-250

221 Nguyen AD & Conley AJ 2008 Adrenal androgens in humans and nonhuman primates: production,
222 zonation and regulation. *Endocrine Development* **13** 33-54

223 Prunier A, Mounier L, Le NP, Leterrier C, Mormede P, Paulmier V, Prunet P, Terlouw C &
224 Guatteo R 2013 Identifying and monitoring pain in farm animals: a review. *Animal* **7** 998-
225 1010

226 Rupp R, Beaudou F & Boichard D 2000 Relationship between milk somatic-cell counts in the first
227 lactation and clinical mastitis occurrence in the second lactation of French Holstein cows.
228 *Preventive Veterinary Medicine* **46** 99-111

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

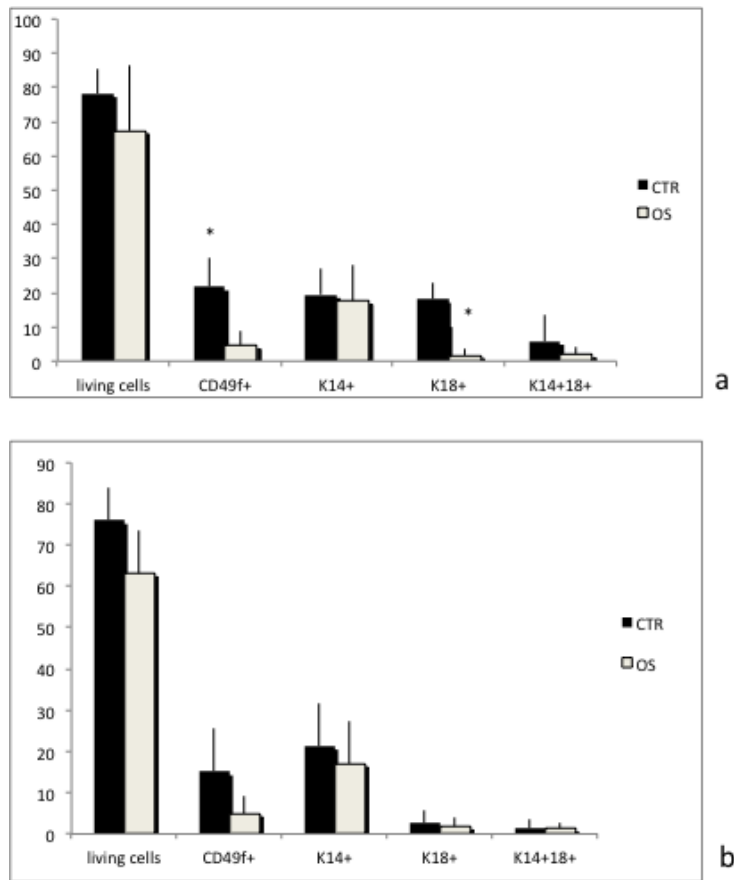


Table 1

| 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 |