

## Research Article

**Cite this article:** Christensen B, Zachariae ED, Poulsen NA, Buitenhuis AJ, Larsen LB and Sørensen ES (2021). Factors influencing milk osteopontin concentration based on measurements from Danish Holstein cows. *Journal of Dairy Research* **88**, 89–94. <https://doi.org/10.1017/S0022029921000054>

Received: 21 April 2020

Revised: 16 November 2020

Accepted: 23 November 2020

First published online: 24 February 2021

**Keywords:**

Bioactive milk protein; ELISA; heritability; Osteopontin

**Author for correspondence:**

Esben S. Sørensen,  
Email: [ess@mbg.au.dk](mailto:ess@mbg.au.dk)

# Factors influencing milk osteopontin concentration based on measurements from Danish Holstein cows

Brian Christensen<sup>1,2</sup>, Elias D. Zachariae<sup>1</sup>, Nina A. Poulsen<sup>2,3</sup>,  
Albert J. Buitenhuis<sup>2,4</sup>, Lotte B. Larsen<sup>2,3</sup> and Esben S. Sørensen<sup>1,2</sup>

<sup>1</sup>Department of Molecular Biology and Genetics, Science Park, Aarhus University, Aarhus, Denmark; <sup>2</sup>CiFOOD, Aarhus University Centre for Innovative Food Research, Aarhus, Denmark; <sup>3</sup>Department of Food Science, Aarhus University, Aarhus, Denmark and <sup>4</sup>Center for Quantitative Genetics and Genomics, Aarhus University, Tjele, Denmark

**Abstract**

Our objective was to determine the content of the bioactive protein osteopontin (OPN) in bovine milk and identify factors influencing its concentration. OPN is expressed in many tissues and body fluids, with by far the highest concentrations in milk. OPN plays a role in immunological and developmental processes and it has been associated with several milk production traits and lactation persistency in cows. In the present study, we report the development of an enzyme linked immunosorbent assay (ELISA) for measurement of OPN in bovine milk. The method was used to determine the concentration of OPN in milk from 661 individual Danish Holstein cows. The median OPN level was determined to 21.9 mg/l with a pronounced level of individual variation ranging from 0.4 mg/l to 67.8 mg/l. Breeding for increased OPN in cow's milk is of significant interest, however, the heritability of OPN in milk was found to be relatively low, with an estimated value of 0.19 in the current dataset. The variation explained by the herd was also found to be low suggesting that OPN levels are not affected by farm management or feeding. Interestingly, the concentration of OPN was found to increase with days in milk and to decrease with parity.

Osteopontin (OPN) is a multifunctional integrin binding phospho-glycoprotein involved in a wide range of physiological processes such as bone mineralization and remodeling, tissue transformations and immune modulation (Christensen and Sørensen, 2016; Lok and Lyle, 2019). OPN is synthesized by a variety of cell types and is present in most tissues and body fluids, with by far the highest levels reported in human milk (Schack *et al.*, 2009a; Bruun *et al.*, 2018). The amino acid sequences of bovine and human OPN are very similar and all important structural elements such as the integrin binding sequences, sites for post-translational modifications and regulatory proteolytic cleavage sites are conserved between the two species (Christensen and Sørensen, 2016). In human and bovine milk, OPN is extremely heterogeneously phosphorylated and O-glycosylated and consequently exists in many different isoforms (Sørensen *et al.*, 1995; Christensen *et al.*, 2005). Furthermore, it has been shown to form complexes with other milk proteins (Azuma *et al.*, 2006; Yamniuk *et al.*, 2009). In addition, in bovine milk, OPN is present both as the intact full-length protein (constituting approximately 25% of the total amount) and as a group of N-terminal-derived fragments (75%), primarily consisting of residues 1–151 resulting from proteolysis in the milk (Christensen and Sørensen, 2014). The C-terminal fragment consisting of residues 152–262 is presumably degraded by milk proteases (Christensen and Sørensen, 2014).

The role of OPN in milk is not well defined, but studies suggest that it plays a role in immunological processes and development in infants. OPN has been shown to possess cytokine-like properties and regulate the Th1/Th2-type cytokine balanced immune response (Ashkar *et al.*, 2000), induce the expression of interleukin-12 from intestinal mononuclear cells (Schack *et al.*, 2009a) and mark bacteria for opsonization by immune cells (Schack *et al.*, 2009b). OPN is relatively resistant to proteolysis by gastric juices (Chatterton *et al.*, 2004) and a bioactive fragment consisting of residues Trp<sup>27</sup>-Phe<sup>151</sup> has been shown to resist simulated gastrointestinal digestion in a form that enables interaction with integrins (Christensen *et al.*, 2020), suggesting a mechanism for the beneficial effects of orally administered OPN reported in intervention studies.

Supplementation of formulas with bovine milk OPN shifts the intestinal gene expression in infant rhesus monkeys significantly towards that found in breast-fed infants (Donovan *et al.*, 2014). Likewise, a clinical trial with human infants administered formula supplemented with bovine milk OPN showed significantly reduced levels of pro-inflammatory cytokines and fewer days of fever compared with infants fed regular formula (Lønnerdal *et al.*, 2015).

Thus, it is hypothesized that milk OPN provides an important immunological signal to the infant during development and bovine milk OPN is now marketed as an ingredient for use in infant formulas.

In cattle, OPN has been suggested to be an important regulatory protein during lactation that specifically plays a role for milk protein gene expression (Sheehy *et al.*, 2009; Strucken *et al.*, 2015). Furthermore, specific gene variants of OPN have been associated with milk production traits (Leonard *et al.*, 2005; Khatib *et al.*, 2007) and lactation persistency (Bissonnette, 2018). Current knowledge on the OPN level in bovine milk is based on two smaller studies, comprising very few cows (Dudemaine *et al.*, 2014; Schack *et al.*, 2009a). In the present study we have developed a milk sample preparation process and an enzyme linked immunosorbent assay (ELISA) method for measurement of OPN in bovine milk. By this method, the OPN concentration in milk from 661 individual Danish Holstein cows was determined and the data was used for identification of factors underlying the variation of OPN concentration in this large population, as well as to estimate heritability.

## Material and methods

All procedures to collect the samples followed the protocols approved by the National Guidelines for Animal Experimentation and the Danish Animal Experimental Ethics Committee. All sampling was restricted to routine on-farm procedures that did not cause any inconvenience or stress to the animals.

### Milk samples

Milk samples were collected once from 661 Danish Holstein cows during morning milking and skimmed as described (Poulsen *et al.*, 2012; Gebreyesus *et al.*, 2017). Briefly, milk from 322 cows were collected in 2009 (Poulsen *et al.*, 2012) and milk from 339 cows in 2013 (Gebreyesus *et al.*, 2017). All herds used an indoor total mixed ration feeding strategy without grazing. Cows were sampled from 21 herds at different stages of lactation (days 4 to 877 in milk) and parity (1 to 6). P1 = parity 1 ( $n = 261$ ), P2 = parity 2 ( $n = 243$ ), P3 = parity 3 ( $n = 141$ ), P4 = parity 4, 5, and 6 ( $n = 16$ ). Representative milk samples of at least 0.5 l were placed on ice during transport to the laboratory, where skim milk samples were prepared by centrifugation ( $2643 \times g$  for 30 min at 4 °C) and stored at  $-80$  °C until analysis.

### Preparation of osteopontin standards and antibodies

OPN was purified from bovine milk as described (Sørensen and Petersen, 1993), and the full-length protein and the N-terminal fragments were separated by gel filtration on a Superdex 200 HR 10/30 column (GE Healthcare, Uppsala, Sweden) connected to a GE Healthcare LKB system as described (Christensen and Sørensen, 2014). The purity of the OPN was determined by SDS-PAGE and Edman sequencing, and the concentration was determined by amino acid analysis. A polyclonal antibody using the purified OPN as antigen was raised in rabbits at Dako A/S (Glostrup, Denmark). In the ELISA, the purified N-terminal milk OPN fragment was used as standard. The monoclonal antibody MAB193P, which recognizes an epitope located in the N-terminal part of both human and bovine OPN was purchased from Maine Biotechnology Services (Portland, ME)

### Osteopontin ELISA

Skimmed milk samples were incubated at 95 °C for 30 min and subsequently the caseins and denatured whey proteins which can form complexes with OPN, were precipitated by addition of hydrochloric acid to a concentration of 40 mM, followed by centrifugation at 10 000 g at 4 °C for 10 min. It was verified by Western blotting that no OPN was precipitated in the milk samples by this treatment (data not shown). The supernatant was collected and diluted 400–1600 times in phosphate-buffered saline (PBS) (pH 7.4) containing 0.1% Tween 20 and the biotinylated MAB193P antibody (2.5 µg/ml). The ELISA was performed using MaxiSorp immunoassay plates (ThermoFisher Scientific, Roskilde, Denmark) coated overnight at 4 °C with polyclonal antibodies against bovine milk OPN (5 µg/ml in 0.1 M of sodium carbonate, pH 9.8). The plates were washed extensively with PBS and blocked with 2% ovalbumin (Merck, Rødovre, Denmark) in PBS for 1 h at 37 °C and washed three times with the washing buffer (PBS containing 0.1% Tween 20). The milk supernatant and standard samples pre-incubated with the MAB193p antibody were added and incubated for 1 h at 37 °C followed by extensive washing. Captured OPN (both full-length and N-terminal OPN fragments) was detected by incubation with 100 µl of horseradish peroxidase-conjugated streptavidin (diluted 1:10 000) for 1 h at 37 °C. After the final wash, TMB-one Peroxidase Substrate (Kem-En-Tec Diagnostics, Copenhagen, Denmark) was added and the plates were incubated for 15 min, after which the reaction was quenched by addition of 0.2 M H<sub>2</sub>SO<sub>4</sub>. The plates were read at 450 nm using an ELISA reader (Bio-tek Instruments Inc., Winooski, VT). For determination of intra-assay variation, a milk supernatant sample was diluted and measured in eight wells on the same ELISA plate. For determination of inter-assay variation, a milk supernatant sample was measured on six different ELISA plates on six different days. Sample concentrations were calculated relative to the OPN standard using four-parameter logistic curve fitting.

### Genotypes

Of the 661 cows included in the study, DNA samples were available from 650 animals. Genomic DNA was extracted from ear tissue and genotyping was performed using the BovineHD Illumina Beadchip for 372 cows or the BovineSNP50 Beadchip for the remaining 278 cows. SNPs that overlapped between these two genotyping arrays were combined and subjected to quality control. Quality parameters used to select SNPs were: (1) minimum call rates of 90% for individuals and 95% for loci, (2) exclusion of SNPs with a minor allele frequency (MAF) lower than 5% and (3) detection from HW proportion at  $P < 0.05$ . Finally, 36 000 SNPs across the 29 bovine autosomes were available for the analyses (Gebreyesus *et al.*, 2017).

These 36 000 SNPs were used to generate the genomic relationship matrix **G** according to the first method as described (VanRaden, 2008). After matching the OPN phenotypes with the genotypes 637 animals remained for genetic analysis. The genetic variance and parameters were estimated using restricted maximum likelihood estimation (REML) as implemented in DMU (Madsen and Jensen, 2013). The general model used was:

$$Y_{ijkl} = \mu + \text{parity}_i + \beta_1 \text{DIM}_j + \beta_2 e^{-0.05 \text{DIM}_j} + \text{herd}_k + \text{Animal}_l + e_{ijkl}, \quad (1)$$

where  $Y_{ijkl}$  was the observation of the individual, in parity  $i$  and herd  $k$ ;  $\mu$  was the fixed mean effect;  $\text{parity}_i$  was a fixed effect

( $i = 1, \dots, 4$ ),  $\beta_1$  was the regression coefficient for days in milking (DIM)<sub>*i*</sub>; and DIM<sub>*j*</sub> was a covariate describing the effect of days *j* in milk. Wilmink adjustment ( $e^{-0.05DIM}$ ) was used for DIM,  $\beta_2$  was the regression coefficient for the Wilmink adjustment (Wilmink, 1987); herd<sub>*k*</sub> was the random herd effect of the herd with distribution  $N(0, I\sigma_h^2)$ ; animal<sub>*l*</sub> was the random additive genetic effect based on *G* of animal *l* with distribution  $N(0, G\sigma_a^2)$  and  $e_{ijkl}$  was the random residual effect, which was assumed to be normally distributed with  $e \sim N(0, I\sigma_e^2)$ .

The heritability ( $h^2$ ) was estimated using:

$$h^2 = \sigma_a^2 / (\sigma_a^2 + \sigma_h^2 + \sigma_e^2), \tag{2}$$

where  $\sigma_a^2$  was the genetic variation,  $\sigma_h^2$  was the herd variation and  $\sigma_e^2$  was the residual variation obtained from model 1.

The proportion of phenotypic variance explained by herd was defined as:

$$h_{herd} = \sigma_h^2 / (\sigma_a^2 + \sigma_h^2 + \sigma_e^2), \tag{3}$$

where  $\sigma_a^2$  was the genetic variation,  $\sigma_h^2$  was the herd variation and  $\sigma_e^2$  was the residual variation obtained from model 1.400

For the association study, model 1 was extended with an allele substitution effect ( $\beta_3$ ) and a covariate SNP<sub>*m*</sub> indicating if a SNP is heterozygote (1), or homozygote (0.2). The effect of the SNP was tested using a Wald test with a null hypothesis of  $H_0: \beta_3 = 0$ . To correct for multiple testing the false discovery rate (FDR) was

**Table 1.** Intra- and inter-assay variation for the bovine milk OPN ELISA

	<i>N</i>	Mean (mg/l)	SD	% CV
Intra-assay variation <sup>a</sup>	8	14.47	0.78	5.3
Inter-assay variation <sup>b</sup>	6	32.86	4.33	13.1

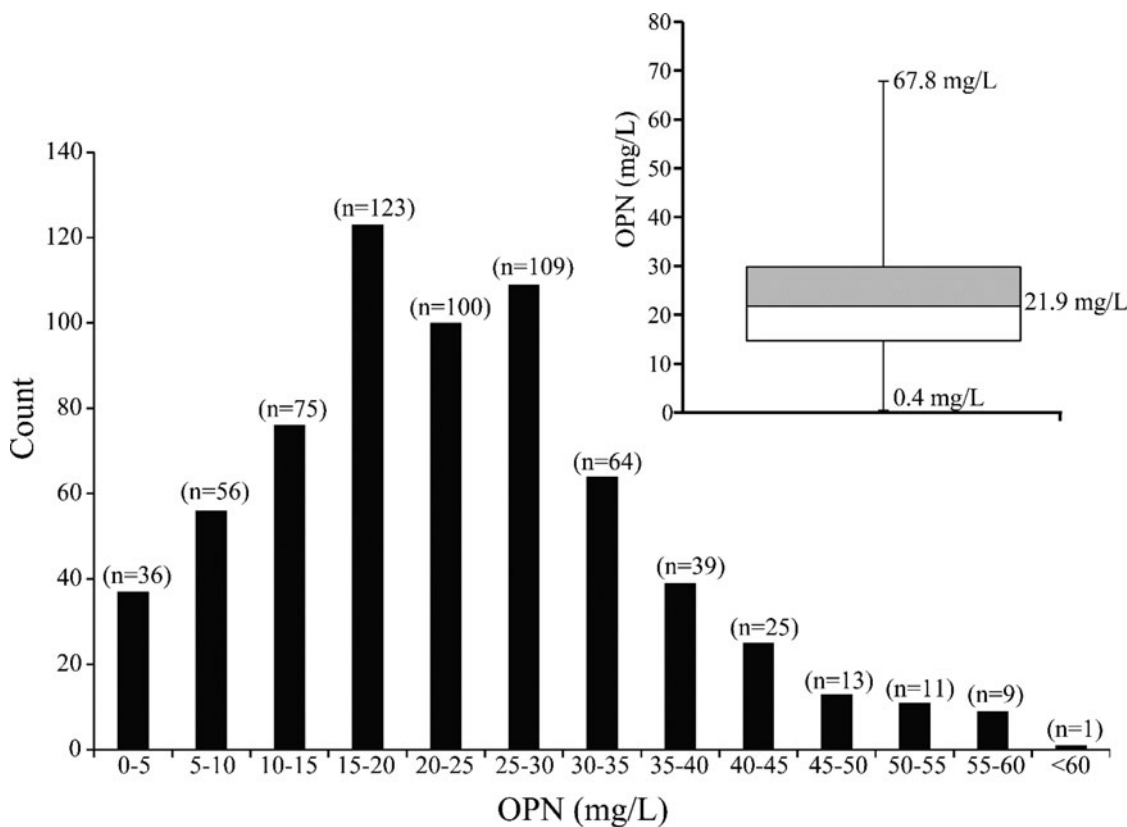
<sup>a</sup>The intra-assay variation of the OPN ELISA was determined by analysing eight replicates of the same milk sample.

<sup>b</sup>The inter-assay variation was determined by measuring the same milk sample on six different assays over six different days.

used as implemented in the R package ‘qvalue’ version 1.34.0. A SNP was considered significant at an FDR of  $P < 0.10$ .

**Results**

The OPN content in 661 Holstein milk samples was determined by ELISA. The intra- and inter-assay coefficients of variation (% CV) were 5.3% and 13.1%, respectively (Table 1). The sensitivity of the ELISA was 1.25 ng/ml, defined as the background optical density plus two standard deviations. The median value of OPN in the milk samples and the frequency distribution of the combined results are shown in Fig. 1. The determined OPN concentrations obtained for the individual bovine milk samples ranged from 0.4 mg/l to 67.8 mg/l, with a median value of 21.9 mg/l (SD 12.2 mg/l). The main part of the samples had OPN concentrations close to the median value, however, a substantial part of samples had OPN concentrations below 5 mg/l or above 45 mg/l (Fig. 1).



**Fig. 1.** The distribution of OPN concentrations (mg/l) in milk from 661 Holstein cows. The Box plot inset shows the median OPN concentration. The horizontal line in the box is the median concentration. The lower and higher horizontal lines are the 25th and 75th quartile values of the distribution, respectively. The ends of the vertical lines represent the lower and upper limit of the distribution.

**Table 2.** Least-square estimates ( $\pm$ SE) of the effect of parity and days in milking (DIM) on the OPN content in bovine milk.

Parameter	Effect	Significance <sup>a</sup>
Parity 1	17.90 $\pm$ 1.31	***
Parity 2	17.94 $\pm$ 1.30	***
Parity 3	15.04 $\pm$ 1.39	***
Parity >4	14.85 $\pm$ 0.04	***
DIM	0.04 $\pm$ 0.004	***
$e^{-0.05 \cdot \text{DIM}}$	-32.29 $\pm$ 5.57	***

<sup>a</sup>Significance level is shown for the effect of parity and DIM compared to the mean. \*\*\* $P < 0.01$

The OPN concentration in milk was significantly affected by both DIM and by parity (Table 2). The OPN concentrations increased with DIM, but decreased in concentration with

increasing parity. The analysis of OPN in bovine milk showed that apart from the phenotypic variation, there was genetic variation as well. The heritability of the OPN concentration in bovine milk was 0.19 (SE: 0.15) (Table 3). In addition, it was shown that the proportion of total phenotypic variation explained by the herd was low, 0.04 (SE 0.035).

In Fig. 2, a Manhattan plot is presented of the GWAS results of OPN in milk. No SNP markers reached the FDR  $P$  value  $< 0.1$  significance level.

## Discussion

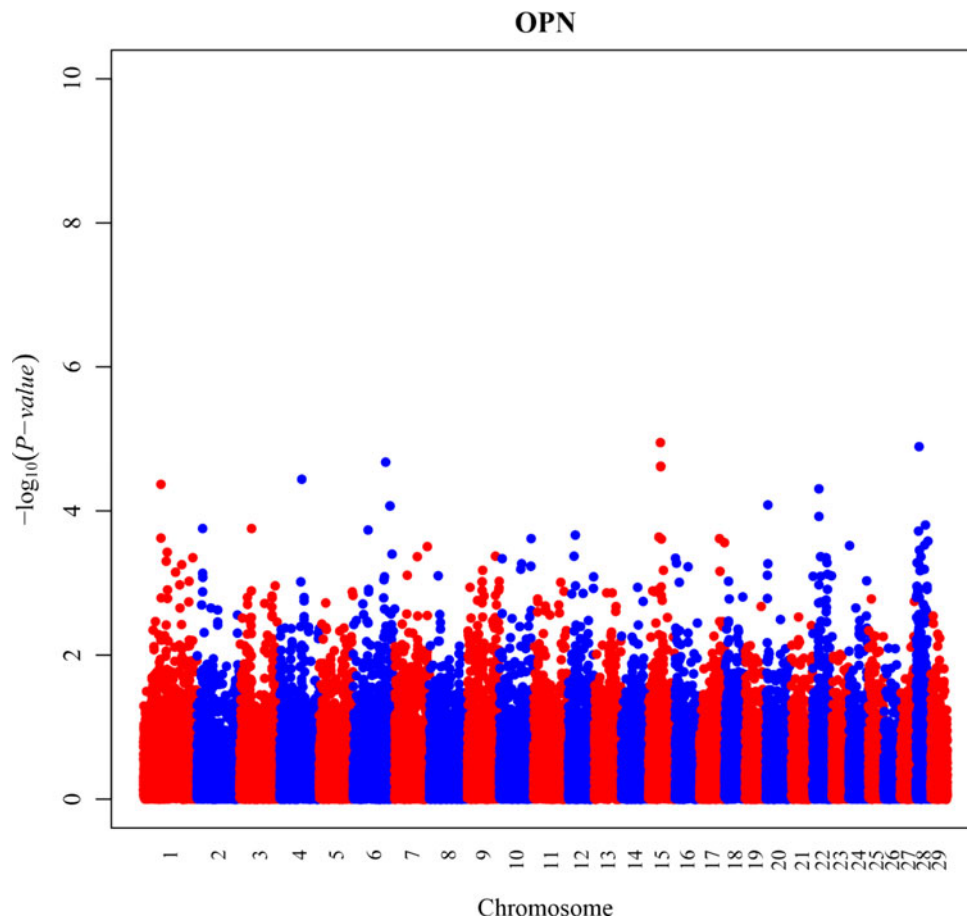
OPN is heterogeneously phosphorylated and subjected to proteolytic cleavage by endogenous milk proteases (Sørensen *et al.*, 1995; Christensen and Sørensen, 2014). Furthermore, OPN can form complexes with other milk proteins such as lactoferrin, lactoperoxidase and IgM through electrostatic or affinity interactions (Azuma *et al.*, 2006; Yamniuk *et al.*, 2009). These complications

**Table 3.** Variance components ( $\text{Var}_{\text{animal}}$ ,  $\text{Var}_{\text{herd}}$ ,  $\text{Var}_{\text{residual}}$ ), heritability<sup>a</sup> ( $h^2$ ) and part of phenotypic variance for milk OPN content explained by herd<sup>b</sup> ( $h_{\text{herd}}$ ) for Danish Holstein ( $n = 637$ ) cows

	OPN mean (mg/l) (sd)	$\text{Var}_{\text{animal}}$	$\text{Var}_{\text{herd}}$	$\text{Var}_{\text{residual}}$	$h^2$ (SE)	$h_{\text{herd}}$ (SE)
Danish Holstein	23.0 (12.22)	0.02	0.004	0.08	0.19 (0.15)	0.04 (0.035)

<sup>a</sup> $h^2 = \text{Var}_{\text{animal}} / \text{Var}_{\text{phenotypic}}$ ,  $\text{Var}_{\text{phenotypic}} = \text{Var}_{\text{animal}} + \text{Var}_{\text{herd}} + \text{Var}_{\text{residual}}$ .

<sup>b</sup> $h_{\text{herd}} = \text{Var}_{\text{herd}} / \text{Var}_{\text{phenotypic}}$ .

**Fig. 2.** Manhattan plot of the GWAS results of OPN content in milk of Danish Holstein. The Y-axis shows the  $-\log_{10}(P\text{-value})$  of single-marker association tests. The X-axis shows marker positions in base pairs.

have to be taken into account in development of an ELISA for OPN in bovine milk. The antibodies used in the presented ELISA recognize both phosphorylated and non-phosphorylated OPN variants, as well as both the N-terminal fragments of OPN and the full-length protein. Prior to the ELISA, milk samples were heated to 95 °C and acidified to eliminate OPN interactions with other milk components and to precipitate major milk proteins that may interfere with OPN binding to the antibodies. OPN is an intrinsically disordered protein, which remains fully soluble after the heat and acid treatment of milk (Sørensen and Petersen, 1993), so this treatment is known not to cause any loss of OPN in the samples to be analyzed. The developed ELISA detects the natural mixture of full-length OPN (approx. 25%) and N-terminal OPN fragments (approx. 75%) that is present in pooled bovine milk (Christensen and Sørensen, 2014). In the study, we have used the purified N-terminal OPN fragment as standard in the ELISA, as this represents the major form of OPN in milk. In an ideal ELISA set-up, the standard protein is identical to the protein to be measured, but as the degree of OPN fragmentation in milk varies among individual cows, such a standard is not obtainable. Using the N-terminal fragment as standard may result in a slight underestimation of OPN content when converting the ELISA read-out to concentration in the milk, as the N-terminal fragment has a molecular mass of 19.2 kDa (Boskey *et al.*, 2012) and the weighted average molecular mass of the mixture of full-length OPN and the N-terminal OPN fragment in milk is 22.8 kDa. In summary, this ELISA method and the preceding milk sample treatment was developed to avoid the complications of OPN heterogeneity, proteolytic fragmentation and complex formation, which is associated with measuring OPN in bovine milk.

Analyses of milk from 661 Danish Holstein cows showed a median OPN value of 21.9 mg/l (SD 12.2) (Fig. 1) and a corresponding average concentration of 23.0 mg/l. This concentration is similar to the average concentration of 18 mg/l reported for pooled bovine milk (Schack *et al.*, 2009a). In a study investigating the OPN content in milk from five cows, levels were found to fluctuate around ~70 mg/l from parturition to lactation week 25, whereafter the concentration increased to ~150–250 mg/l between week 25 and 30 (Dudemaine *et al.*, 2014). The cows in that study were selected based on specific genetic variations that had an effect on the OPN promoter activity (Dudemaine *et al.*, 2014). Furthermore, a commercially available full-length bovine OPN standard was used for the quantification of the protein. In our ELISA, this standard reacted very poorly with the antibodies resulting in unrealistic high OPN levels (data not shown). These factors could contribute to the differences in OPN levels reported in the two studies.

In the present study, a considerable individual variation was observed between the cows, with levels varying from 0.4 mg/l to 67.8 mg/l. Such large individual variation in OPN levels has not previously been reported, as earlier studies have only comprised a very limited number of cows (Schack *et al.*, 2009a; Dudemaine *et al.*, 2014). We also showed that the OPN content in cow's milk increases significantly with DIM (Table 2). This is in line with the study by Dudemaine *et al.* (2014), where a significant increase in bovine OPN levels were observed after lactation week 25. The present study is the first to indicate an effect of parity, as OPN levels decrease with increasing parity (Table 2). The reason for this is not known. However, OPN plays a role in tissue remodeling and has been associated with lactation persistency (Bissonnette, 2018), and the lower OPN levels in later parities

and DIM could suggest that changes occur in the mammary gland, which may affect the mammary gland directly, but also lead to changes in milk composition as reported by others (Leonard *et al.*, 2005; Khatib *et al.*, 2007).

The genetic analysis of OPN variance showed that the OPN concentration in bovine milk is heritable, indicating that there is both phenotypic as well as genetic variation for OPN in Danish Holstein milk. In addition, the proportion of the total phenotypic variance explained by the herd was low, indicating that management factors such as feed regime have little or no impact on the OPN concentration in bovine milk. The OPN gene (*SPP1*) is associated with milk production traits and lactation persistency (Leonard *et al.*, 2005; Khatib *et al.*, 2007; Bissonnette, 2018). This association of *SPP1* to production traits is interesting. However, in our data-set it was not possible to associate the *SPP1* gene to OPN performing a genome-wide association approach (Fig. 2).

In conclusion, the presented ELISA method provides a tool for investigation of bovine OPN in complex mixtures, as it measures OPN regardless of fragmentation and phosphorylation state, and in complexes with other proteins. To our knowledge this is the first study to document large variability in OPN content between cows. This variation was related to DIM and parity. In case one would like to pursue selective breeding this study showed that the heritability was 0.19, however with a relatively large standard error. In contrast, the variation explained by the herd was very low, which suggests that the milk OPN content cannot be influenced by farm management or feeding, but rather relates to some unexplained individual physiological effects that is not accounted for in the current study.

**Acknowledgements.** The project received funding from the Danish Milk Levy Fund and Arla Foods Ingredients Group P/S, Denmark.

## References

- Ashkar S W, Panoutsakopoulou V GF, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ and Cantor H (2000) Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science (New York, N.Y.)* **287**, 860–864.
- Azuma N, Maeta A, Fukuchi K and Kanno C (2006) A rapid method for purifying osteopontin from bovine milk and interaction between osteopontin and other milk proteins. *International Dairy Journal* **16**, 370–378.
- Bissonnette N (2018) Short communication: genetic association of variations in the osteopontin gene (*SPP1*) with lactation persistency in dairy cattle. *Journal of Dairy Science* **101**, 456–461.
- Boskey A L, Christensen B, Taleb H and Sørensen ES (2012) Post-translational modification of osteopontin: effects on in vitro hydroxyapatite formation and growth. *Biochemical and Biophysical Research Communications* **419**, 333–338.
- Bruun S, Jacobsen LN, Ze X, Husby S, Ueno HM, Nojiri K, Kobayashi S, Kwon J, Liu X, Yan S, Yang J, Zachariassen G, Chen L, Zhou W, Christensen B and Sørensen ES (2018) Osteopontin levels in human milk vary across countries and within lactation period: data from a multi-center study. *Journal of Pediatric Gastroenterology and Nutrition* **67**, 250–256.
- Chatterton DEW, Rasmussen JT, Heegaard CW, Sørensen ES and Petersen TE (2004) In vitro digestion of novel milk protein ingredients for use in infant formulas: research on biological functions. *Trends in Food Science & Technology* **15**, 373–383.
- Christensen B and Sørensen ES (2014) Osteopontin is highly susceptible to cleavage in bovine milk and the proteolytic fragments bind the  $\alpha V\beta_3$ -integrin receptor. *Journal of Dairy Science* **97**, 136–146.
- Christensen B and Sørensen ES (2016) Structure, function and nutritional potential of milk osteopontin. *International Dairy Journal* **57**, 1–6.

- Christensen B, Nielsen MS, Haselmann KF, Petersen TE and Sørensen ES** (2005) Post-translationally modified residues of native human osteopontin are located in clusters: identification of 36 phosphorylation and five O-glycosylation sites and their biological implications. *The Biochemical Journal* **390**(Pt 1), 285–292.
- Christensen B, Karlsen NJ, Jørgensen SDS, Jacobsen LN, Ostenfeld MS, Petersen SV, Müllertz A and Sørensen ES** (2020) Milk osteopontin retains integrin-binding activity after in vitro gastrointestinal transit. *Journal of Dairy Science* **103**, 42–51.
- Donovan SM, Monaco MH, Drnevich J, Kvistgaard AS, Hernell O and Lönnnerdal B** (2014) Bovine osteopontin modifies the intestinal transcriptome of formula-fed infant rhesus monkeys to be more similar to those that were breastfed. *The Journal of Nutrition* **144**, 1910–1919.
- Dudemaine PL, Thibault C, Alain K and Bissonnette N** (2014) Genetic variations in the SPP1 promoter affect gene expression and the level of osteopontin secretion into bovine milk. *Animal Genetics* **45**, 629–640.
- Gebreyesus G, Lund MS, Buitenhuis B, Bovenhuis H, Poulsen NA and Jans LG** (2017) Modeling heterogeneous (co)variances from adjacent-SNP groups improves genomic prediction for milk protein composition traits. *Genetics, Selection, Evolution: GSE* **49**, 89.
- Khatib H, Zaitoun I, Wiebelhaus-Finger J, Chang YM and Rosa GJM** (2007) The association of bovine PPARGC1A and OPN genes with milk composition in two independent Holstein cattle populations. *Journal of Dairy Science* **90**, 2966–2970.
- Leonard S, Khatib H, Schutzkus V, Chang YM and Maltecca C** (2005) Effects of the osteopontin gene variants on milk production traits in dairy cattle. *Journal of Dairy Science* **88**, 4083–4086.
- Lok ZSY and Lyle AN** (2019) Osteopontin in vascular disease. *Arteriosclerosis, Thrombosis and Vascular Biology* **39**, 613–622.
- Lönnnerdal B, Kvistgaard AS, Peerson JM, Donovan SM and Peng Y-M** (2015) Growth, nutrition and cytokine response of breast-fed infants and infants fed formula with added bovine osteopontin. *Journal of Pediatric Gastroenterology and Nutrition* **62**, 650–657.
- Madsen P and Jensen J** (2013) A user's guide to DMU. *A Package for Analysing Multivariate Mixed Models Version 6*, 1–33.
- Poulsen NA, Gustavsson F, Glantz M, Paulsson M, Larsen LB and Larsen MK** (2012) The influence of feed and herd on fatty acid composition in 3 dairy breeds (Danish Holstein, Danish Jersey and Swedish Red). *Journal of Dairy Science* **95**, 6362–6371.
- Schack L, Lange A, Kelsen J, Agnholt J, Christensen B, Petersen TE and Sørensen ES** (2009a) Considerable variation in the concentration of osteopontin in human milk, bovine milk, and infant formulas. *Journal of Dairy Science* **92**, 5378–5385.
- Schack L, Stapulionis R, Christensen B, Kofod-Olsen E, Skov Sørensen UB, Vorup-Jensen T, Sørensen ES and Höllsberg P** (2009b) Osteopontin enhances phagocytosis through a novel osteopontin receptor, the alphaXbeta2 integrin. *Journal of Immunology* **182**, 6943–6950.
- Sheehy PA, Riley LG, Raadsma HW, Williamson P and Wynn PC** (2009) A functional genomics approach to evaluate candidate genes located in a QTL interval for milk production traits on BTA6. *Animal Genetics* **40**, 492–498.
- Sørensen ES and Petersen TE** (1993) Purification and characterization of three proteins isolated from the proteose peptone fraction of bovine milk. *The Journal of Dairy Research* **60**, 189–197.
- Sørensen ES, Højrup P and Petersen TE** (1995) Posttranslational modifications of bovine osteopontin: identification of twenty-eight phosphorylation and three O-glycosylation sites. *Protein Science: A Publication of the Protein Society* **4**, 2040–2049.
- Strucken EM, Laurenson YCSM and Brockmann GA** (2015) Go with the flow—biology and genetics of the lactation cycle. *Frontiers in Genetics* **6**, 118.
- VanRaden PM** (2008) Efficient methods to compute genomic predictions. *Journal of Dairy Science* **91**, 4414–4423.
- Wilmink JBM** (1987) Adjustment of test-day milk, fat and protein yield for age, season and stage of lactation. *Livestock Production Science* **16**, 335–348.
- Yamniuk AP, Burling H and Vogel HJ** (2009) Thermodynamic characterization of the interactions between the immunoregulatory proteins osteopontin and lactoferrin. *Molecular Immunology* **46**, 2395–2402.