NOVEL MILK PROTEIN BIOMARKERS FOR MASTITIS IN DAIRY ANIMALS AND THEIR IMPLEMENTATION IN DIAGNOSTIC TOOLS

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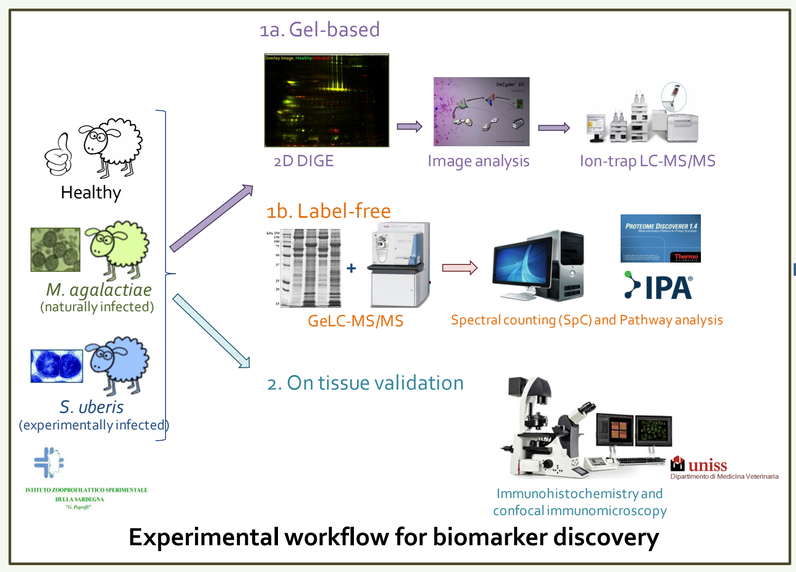
1. Introduction

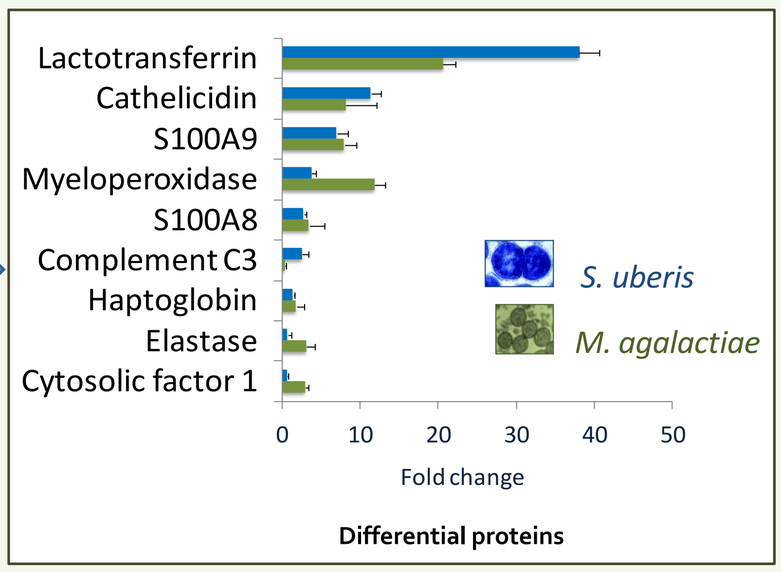
The milk somatic cell count (SCC) is a routine parameter used for monitoring udder health and milk quality in cows, and it is currently implemented also in ewes and goats. Nevertheless, the question of which thresholds to use in small ruminants is still strongly debated. In fact, adding to intramammary infections, a number of other factors impact on small ruminant SCCs more significantly than in cows. Therefore, for an earlier and sensitive monitoring of udder health, as well as for a better definition of thresholds, SCCs would benefit of the integration with additional markers. Several biomarkers were suggested as potential indicators of IMI, such as haptoglobin, serum amyloid A, lactoferrin, and others. We propose the presence of cathelicidins as a reliable and pathogen-independent signal of an inflammatory process of the mammary gland.

2. Biomarker Discovery and validation

Milk from healthy sheep, from sheep naturally infected by *M. agalactiae*, and from sheep experimentally infected with *S. uberis* was subjected to 2D-DIGE-MS followed by ESI-Q-TOF-MS/MS, and in parallel by SDS-PAGE followed by band cutting, digestion, and LTQ-Orbitrap Velos, and then by label-free quantitation and Ingenuity Pathway Analysis for characterization of differential proteins. The cellular origin of the markers of interest was investigated and validated by immunohistochemistry (IHC) on infected mammary tissues. Among the proteins with the highest fold change, cathelicidin shows the lowest variation between intramammary infections by the two infectious agents. Thus, the antimicrobial peptide was chosen for validation in large cohorts of milk samples from different farmed animals. Cathelicidins expression was determined by means of a specific and sensitive sandwich ELISA developed in-house.



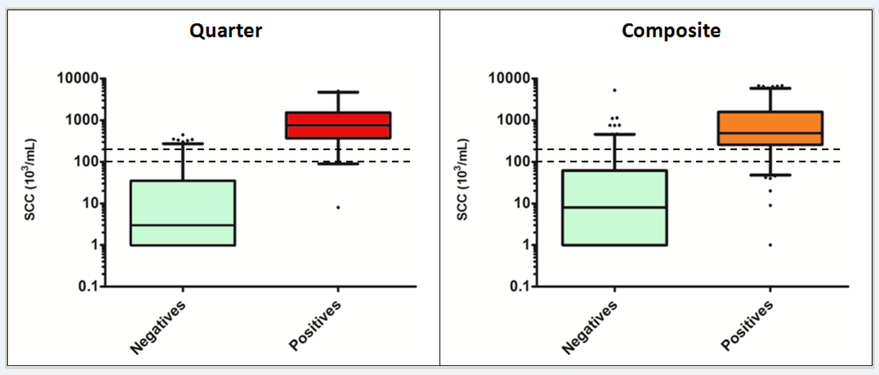
Experimental workflow for biomarker discovery



Differential proteins

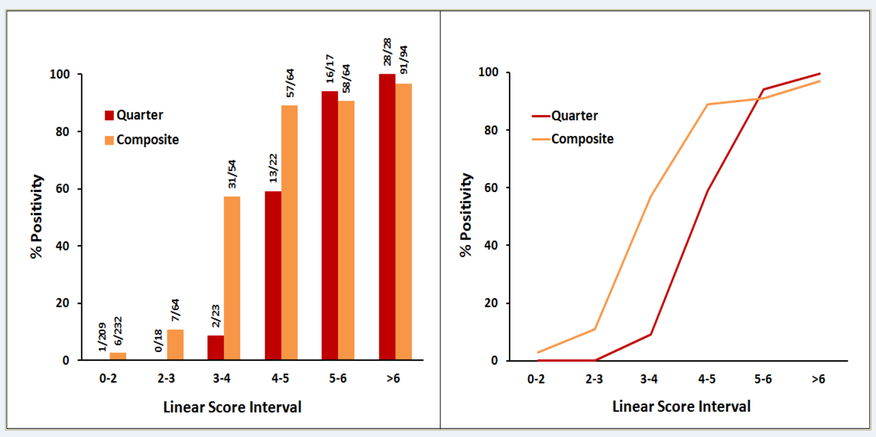
3a. Cathelicidins in cow's milk

Positivity to cathelicidins was assessed in a panel of 317 quarter and 572 composite milk samples from 652 lactating cows.



1. Box-and-whiskers plots indicating the cathelicidin status in milk samples according to SCC. *Distribution of cathelicidin negative and positive samples according to the SCC in quarter (left), and composite samples (right). The dashed lines indicate the 100,000 cells/mL (lower line) and 200,000 cells/mL (upper line) threshold values. Boxes indicate values falling within the 25th and 75th percentiles, with the central line indicating the median value. Whiskers indicate values falling within the 2.5th and 97.5th percentiles, and individual dots indicate values falling outside the whiskers.*

The cathelicidin negative and positive sample groups were significantly separated both in quarters and composites, as illustrated in Fig.1. A better separation of negatives and positives was observed in quarters when compared to composite samples. In fact, positivity to cathelicidin was seen at lower SCC in composite samples, and it was distributed around a lower median value when compared to quarter samples, probably due to a dilution effect.

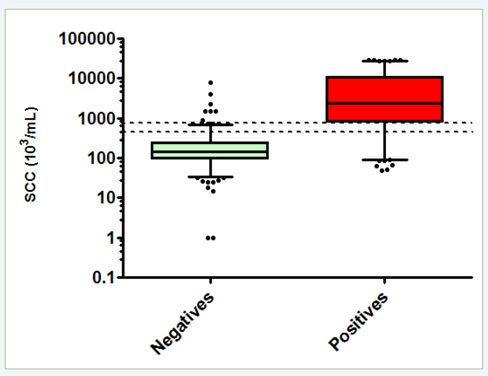


2. Intraclass frequency distribution of cathelicidin positivity by increasing linear score (LS). *Left: percent positivity of milk to cathelicidins in quarter (red) and in composite samples (orange) according to frequency classes of 1 LS unit. The number of positive samples against the total number of samples in the class is indicated over the bar. Right: overlay of the intraclass percent positivity trend in quarter and in composite samples.*

The distribution of cathelicidin positivity was evaluated in terms of linear score (LS) frequency classes by converting logarithmically the SCC to a linear score from 0-9 (Fig. 2). Cathelicidin positivity was clearly evident at the LS interval 3-4 and 4-5 in quarters and composites, respectively.

3b. Cathelicidins in ewe's milk

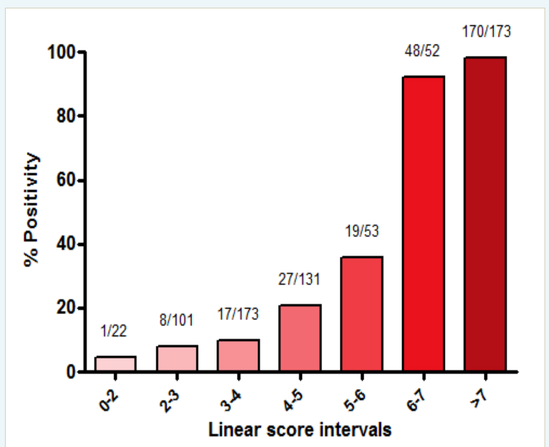
Expression of cathelicidins was also tested on 705 single udder halves milk samples of Sarda sheep, from 3 flocks, in Sardinia, Italy.



3. Box-and-whiskers plot of SCC in ewe's milk samples. *ELISA negative and ELISA positive SCC distribution. Dotted lines indicates 400,000 cells/mL (lower line) and 800,000 cells/mL (upper line) threshold values .*

As shown in Fig.3, the SCC in ewes has a larger overlap of ELISA negatives and positives, compared to cow's milk samples. Specifically, the negative group in ewes has a higher median value (144,000 cells/mL) than SCC in cattle (3,000 cells/mL in quarters and 8,000 cells/mL in composites).

ELISA positivity to cathelicidins suggest the presence of an inflammatory process inside the udder.
Fig.4 indicates that samples with an inflammatory event are more widely distributed along the linear scores than the cow milk samples.

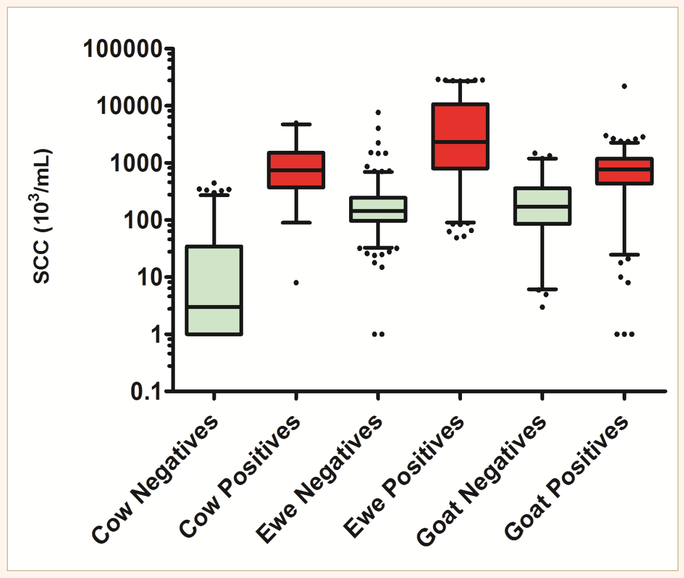


4. Percentage of intraclass positivity in udder-half milk samples. *Bars show the intraclass positivity to cathelicidins according to linear score intervals.*

Above linear score 6 (≥800,000 cells/mL) almost 97% of the samples (218/225) are positive to cathelicidins, while between linear score 5 and 6 (400,000-799,000 cells/mL) only 36% (19/53) are positive, indicating that at this interval it is difficult to correctly identify samples belonging from udder halves with inflammation or from healthy udder halves.
Even though some SCC thresholds were proposed in the literature, ranging from 400,000 to 1,500,000 cells/mL, there is no universally accepted threshold of SCC to discriminate infected from non infected udder halves in ewes. In fact, several factors can influence the number of somatic cell in milk in ewes, such as number of parity, breed, genetics and stage of lactation, paving the way to a wrong diagnosis of mastitis. This indicates that the integration of cathelicidin measurement might provide a useful support to SCC in detecting IMI in sheep.

3c. Cathelicidins in goat milk

The presence of the biomarker in goat milk is currently under investigation. 436 milk samples from a single flock were studied for the expression of cathelicidins in udder halves. Preliminary results suggest a transitional clustering of the distribution of goat milk samples between cattle and sheep.
As highlighted in Fig.5, the distribution of ELISA negative goat samples is closely related to the same group from ewes. Positive samples distribution, instead, has a similar trend to the cattle milk samples distribution. Nevertheless, only post-partum samples have been examined to date.



5. Box-and-whiskers plot comparison of SCC in milk samples. *ELISA negative and ELISA positive SCC distribution in all tested samples.*

4.Conclusions

The molecules identified in the proteomic discovery study hold promise as novel mastitis markers, and open valuable perspectives for the development of diagnostic tools enabling a better monitoring of udder health and milk quality. In addition, these novel markers can support the cross-validation of SCCs for monitoring small ruminant mastitis, as well as a better evaluation of SCC levels, dynamics, and reliability in these dairy animals.
Furthermore, the expanded parameters to be monitored should improve the early detection of both clinical and, especially, sub-clinical mastitis with significant economical and animal health implications.

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