Phenotypic evaluation of mast cells in bovine mammary tissue and mastitis in the context of fibrosis

Cansel Güzin Özgüden Akkoc, Ayşe Meriç Mutlu, Abdülkadir Keskin, Ezgi Yumuşak, Ahmet Akkoc

SUPPLEMENTARY FILE

Material and Methods

Tissue processing

The tissue samples were fixed in 4% paraformaldehyde solution (158127, Sigma Aldrich) at +4°C for 24 hours, and then a routine histological procedure was performed. After embedding in paraffin blocks, 4 µm serial sections were taken and mounted on polylysine-coated slides for histopathology, histochemistry, and immunohistochemistry. All slides were stained with Haematoxylin-Eosin (H&E) and examined by light microscope (CX41, Olympus Corporation, Tokyo, Japan) to reveal microscopic changes in mammary samples. Twenty samples were selected to represent each group (healthy control-acute mastitis-chronic mastitis) for further analyses.

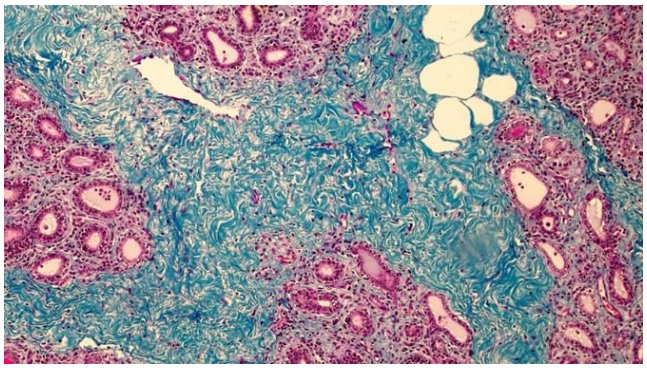
Quantitation of fibrosis

During the analyses, the saturation value in all photographs was set to 1% and then the intensity values of red, blue, and white colours were increased and decreased from the colour threshold

value menu to the desired level to select connective tissue or parenchyma areas. The pixel value of the connective tissue (blue colour) and parenchyma (red colour) areas were expressed as a percentage of the pixel value of the image. Lumens of alveoli, ducts, and blood vessels were not included in the calculation. The percentage values obtained were averaged and the results were expressed as the amount of connective tissue and fold increases for each group.

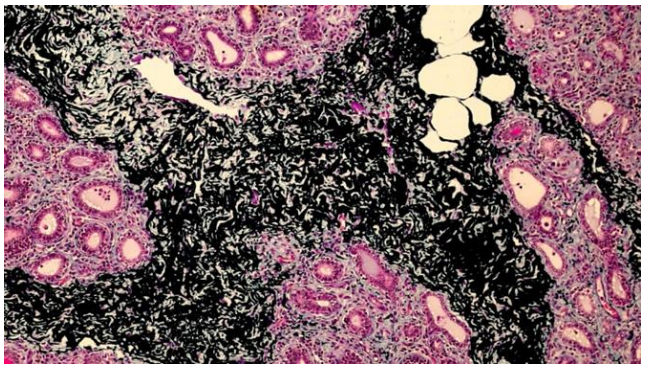
Image Analysis

Serial sections of healthy and mastitic tissues were stained with Masson's Trichrome staining and the amount of connective tissue in the samples was analysed. For this purpose, images were taken from 10 random areas of Masson's Trichrome stained tissues at x10 objective. To avoid subjective evaluation, the images were analysed with Olympus Stream Motion software by colour separation. With the software, blue (fibrotic), red (parenchyma), and white (extracellular areas) colour threshold values were adjusted one by one after 1% saturation in all images and colour measurements were made. The values obtained after measurement of parenchyma, connective tissue and the whole area are expressed as percentages. White coloured areas between alveolar lumens and other cells were not included in the calculation. The blue coloured areas obtained in this staining were determined as a percentage of the total colour areas together with the other colours. The group value was obtained by taking the average of these 10 areas.



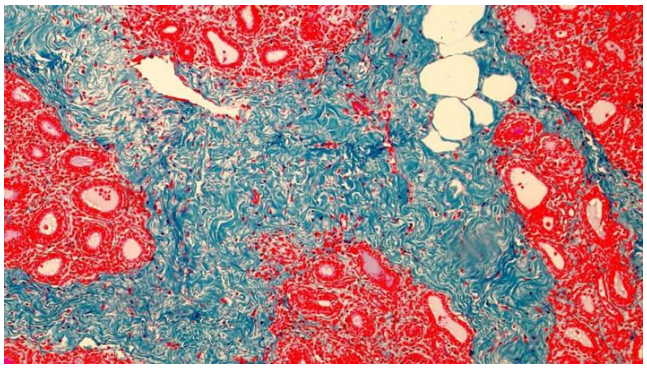
Supplementary Figure S1

Masson's Trichrome staining;
Fibrotic tissue: blue
Parenchyma: red
Acellular spaces: white



Supplementary Figure S2

Saturation and black colour selection for fibrotic tissue in the image.



Supplementary Figure S3

Saturation and red colour selection for parenchyma in the same image.

Immunophenotyping of mast cells

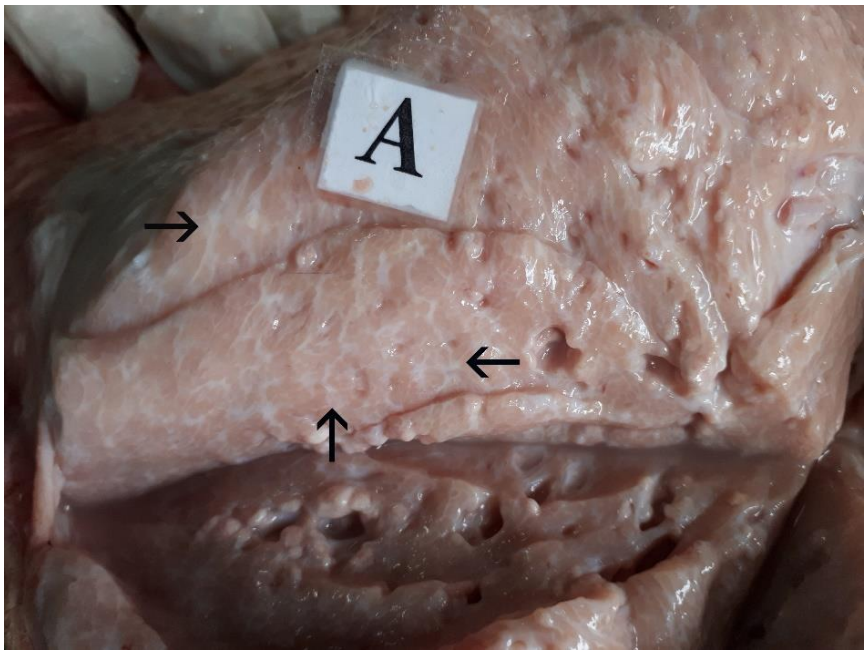
Briefly, all slides were deparaffinised in xylene, rehydrated with degraded ethanol, and washed with running water. Antigen retrieval was performed by immersing antigen retrieval solution (ab93678, Abcam, Cambridge, UK) in the microwave oven at 750 W for 10 min. After cooling, sections were washed with phosphate-buffered saline (PBS). Endogenous peroxidase was blocked by incubation of sections in peroxidase blocking reagent for 10 min at room temperature. The samples were incubated overnight at +4°C with primary monoclonal antibodies: anti-tryptase antibody (mouse anti-human tryptase antibody, 1:500, Sc-59587, Santa Cruz Biotechnology) and anti-chymase antibody (rabbit anti-human chymase antibody, 1:200, orb584539, Biorbyt Ltd, St Louis, MO, USA). After washing in PBS, tissue sections were incubated for 10 min at RT with a biotinylated secondary antibody (TP-015-HD, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, slides were washed with PBS and incubated for 10 min with streptavidin conjugated to horseradish peroxidase complex (TP-015-HD, Thermo Fisher Scientific, Waltham,

MA, USA). The colour visualization was visualized with 3,3-diaminobenzidine tetrahydrochloride. Sections were counterstained with Harris Haematoxylin Solution (HHS16, Sigma Aldrich), rinsed in tap water, dehydrated with grades of alcohol, cleared with xylene, cover-slipped, and evaluated under the light microscope. (CX41, Olympus Corporation, Tokyo, Japan).

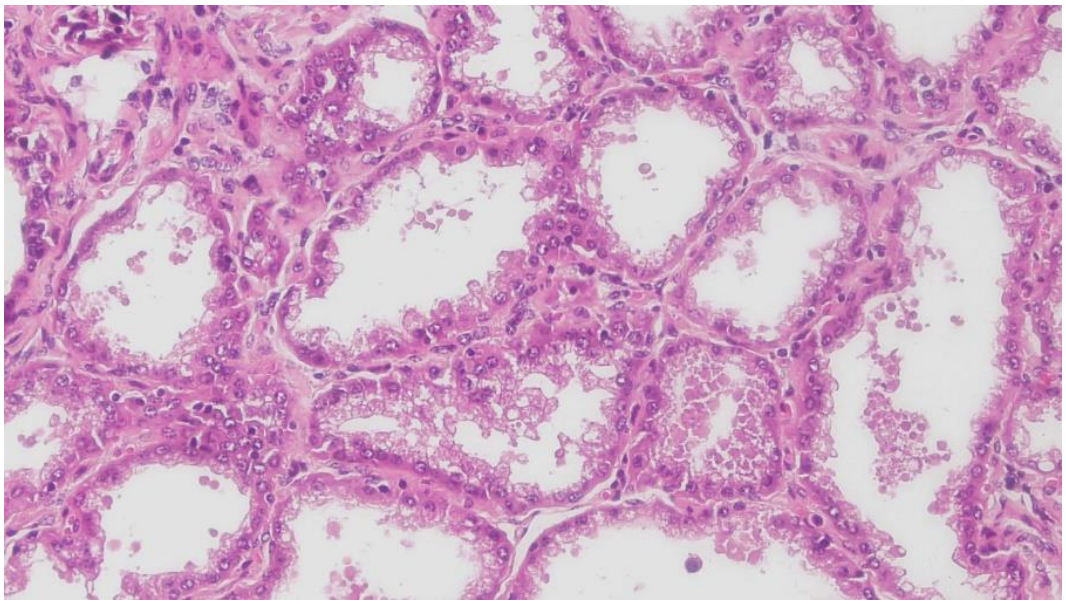
Quantification of MC_T, MC_C and MC_{TC} phenotype density was evaluated in ten randomly selected areas at X200 magnification. The number of MC_T and MC_C were expressed as mean numbers and fold increases for each group. For demonstrating MC_{TC} cells, MC_T and MC_C cells were immunohistochemically detected in serial sections. Images taken from the same fields in both slides were compared and cells found to be positive in both images were considered MC_{TC} cells.

Results

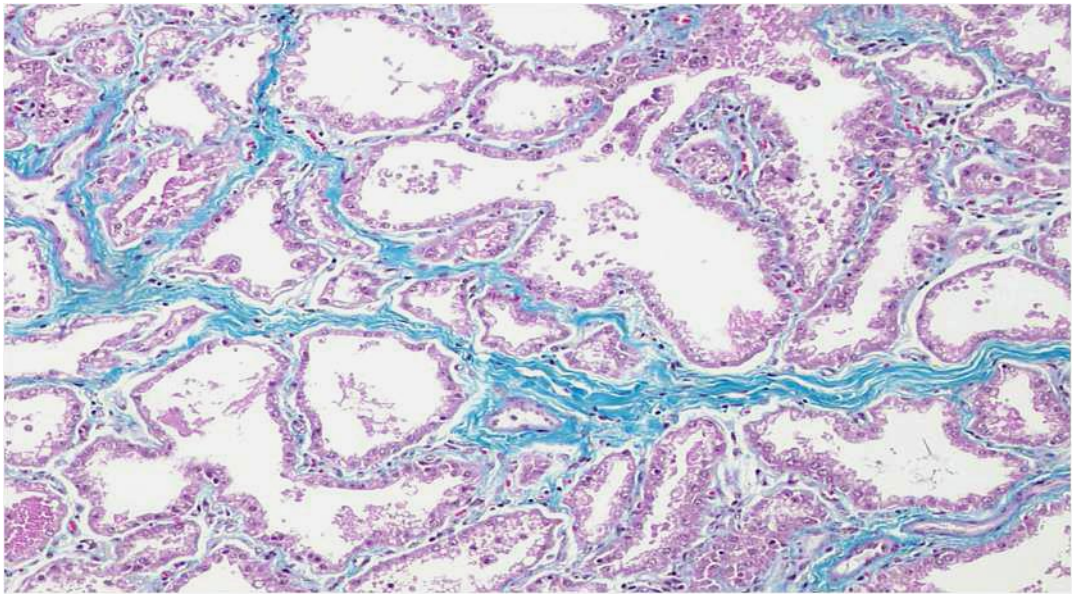
Figures and Legends



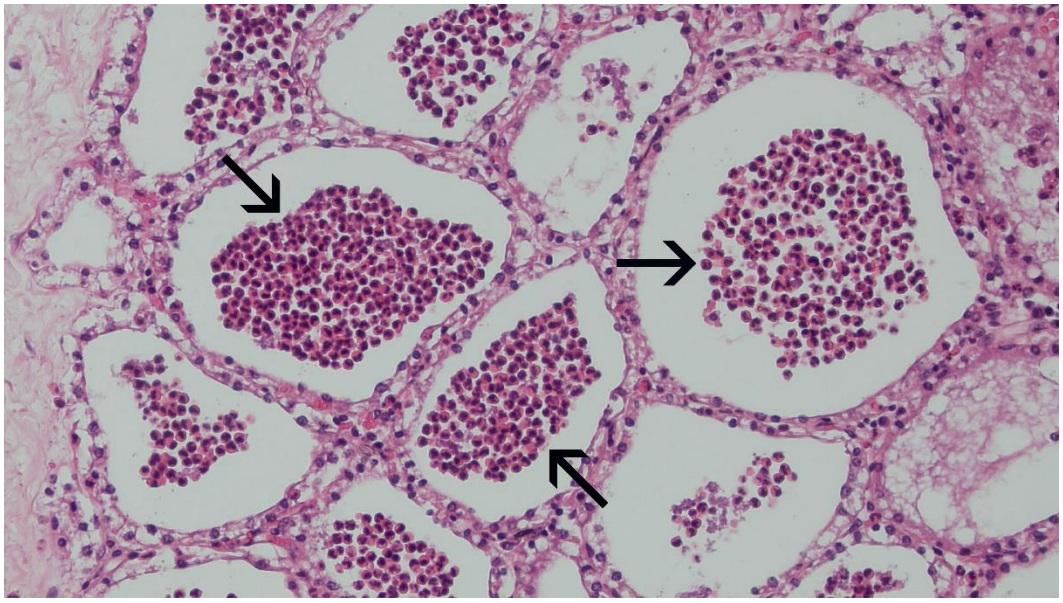
Supplementary Figure S4 Chronic mastitis with fibrosis, the thickening (arrows) of interstitial tissue in the cross section of mammary tissue.



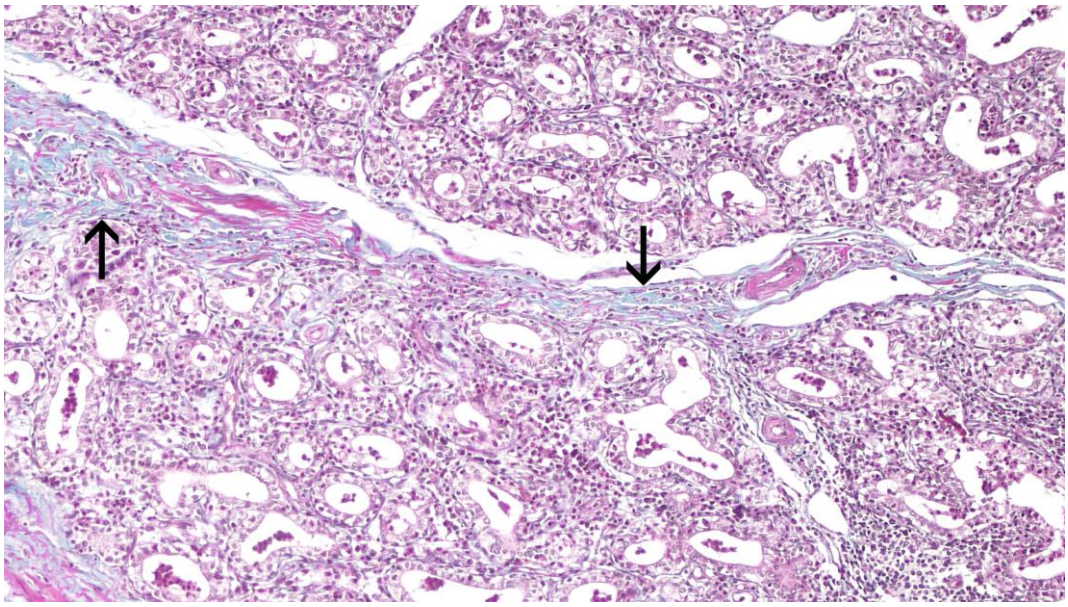
Supplementary Figure S5A Tissue section from healthy, lactating mammary tissue. H&E staining, X200 magnification.



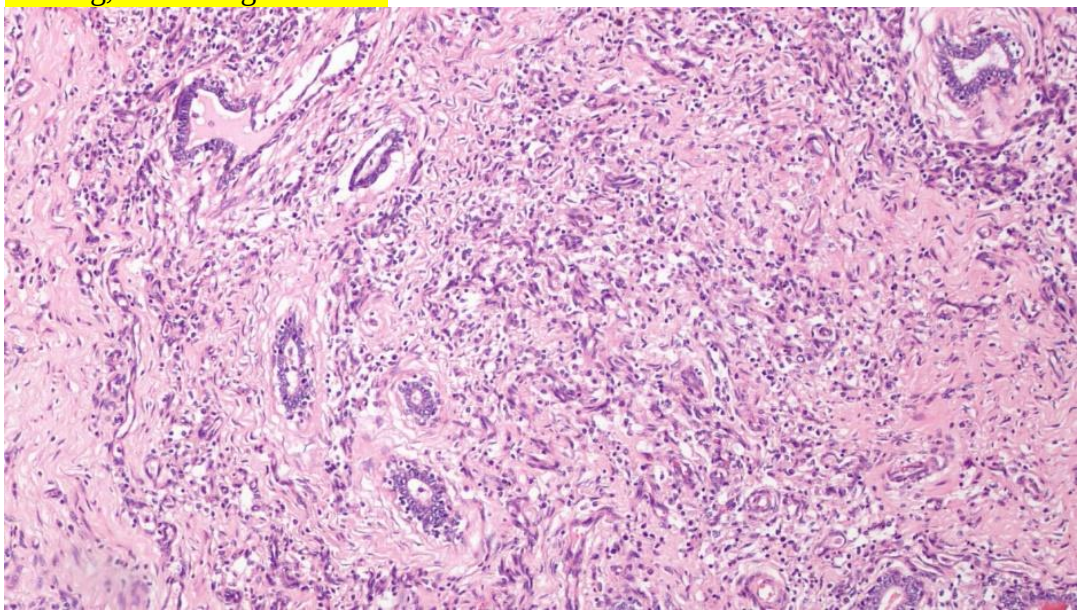
Supplementary Figure S5B Tissue section from healthy, lactating mammary tissue. The collagen fibers are demonstrated in blue colour. Masson's Trichrome staining, X100 magnification.



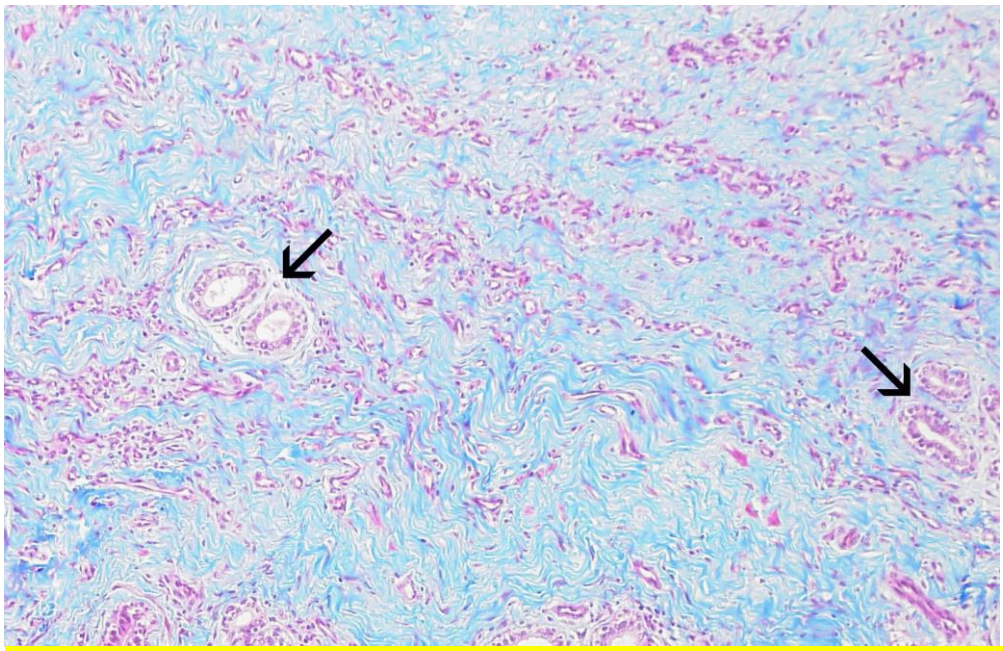
Supplementary Figure S6A Acute suppurative mastitis, there is severe infiltration of neutrophils (arrows) within the alveoli, H&E staining, X200 magnification.



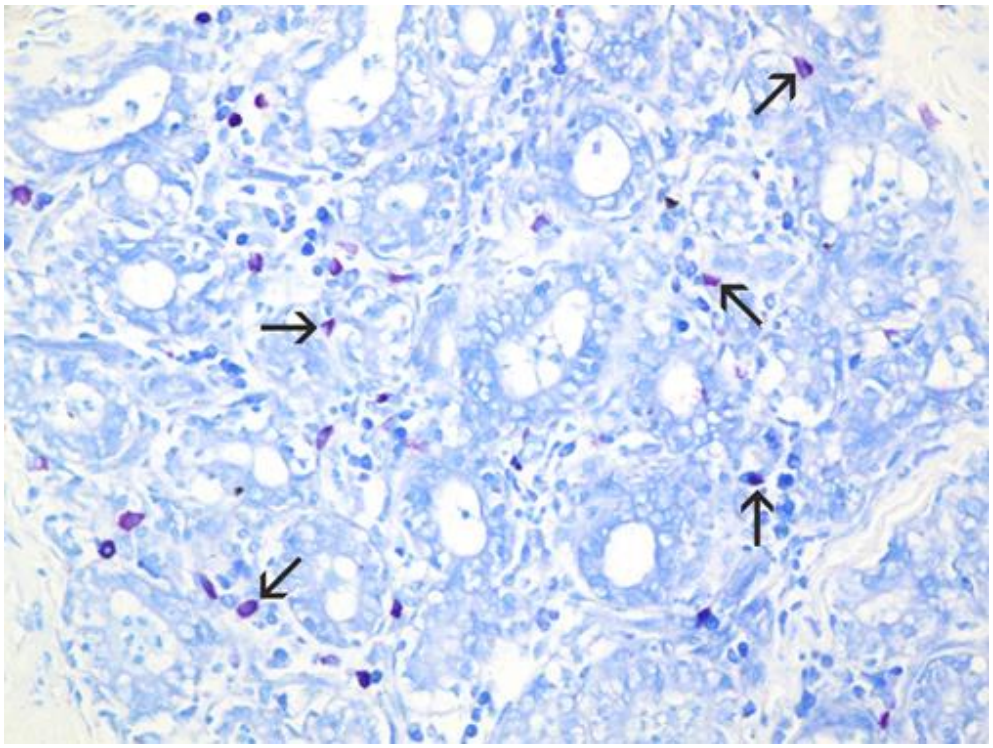
Supplementary Figure S6B Small amount of blue coloured collagen fibers (arrows) in mammary gland with acute mastitis, Masson's Trichrome staining staining, X100 magnification.



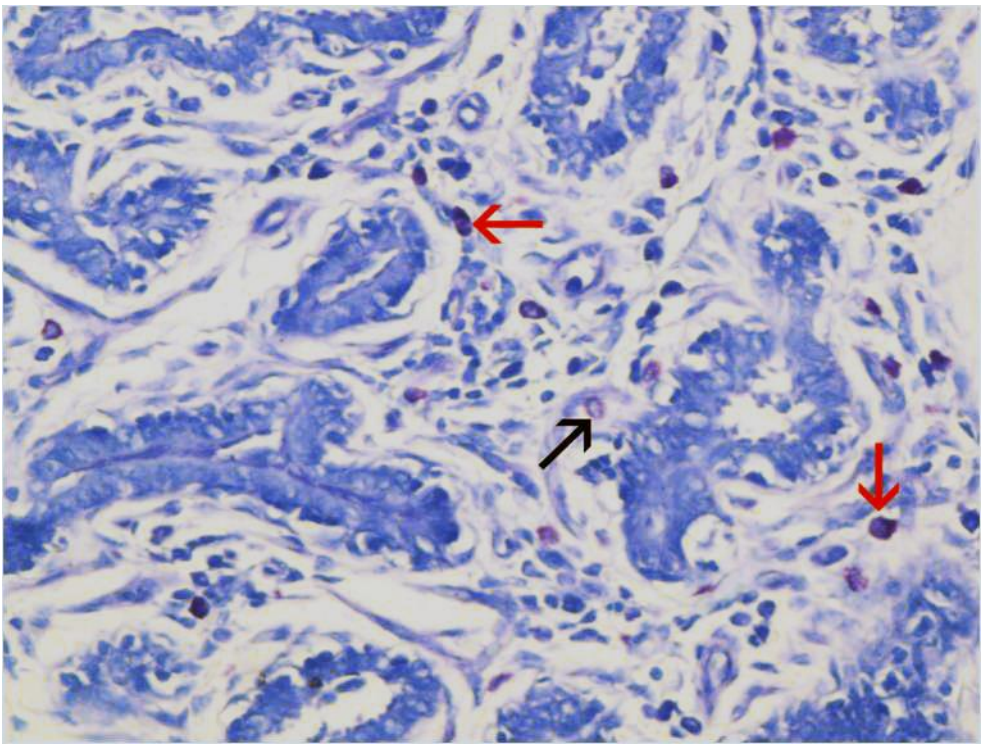
Supplementary Figure S7A Chronic mastitis with fibrosis, there is severe infiltration of mononuclear cells in the interstitial spaces, an increased amount of extracellular matrix dissecting parenchyma. H&E staining, X100 magnification.



Supplementary Figure S7B Severe, chronic mastitis with fibrosis, an increased amount of blue coloured collagen fibers with alveoli remnants (arrows). Masson's Trichrome staining staining, X100 magnification.



Supplementary Figure S8A Demonstration of mast cells in a mammary tissue with chronic mastitis. Mast cells (arrows) are clearly visible around the alveolar structures and within the interstitial spaces. Toluidine blue staining, X200 magnification.



Supplementary Figure S8B Representative image of granulated (red arrows) and degranulated mast cells (ghost cells) (black arrow) are visible in the mammary tissue. Toluidine blue staining, X200 magnification.

SupplementaryTable S1 Total, granulated (intact) and degranulated mast cell numbers in healthy, acute, and chronic mastitic samples.

Samples	Granulated (intact) mast cell numbers (fold increase)	Degranulated mast cell numbers (fold increase)	Total mast cell number (foldincrease)
Healthy	3.18 (1)	0.96 (1)	4.14 (1)
Acute mastitic	7.61 (2.39)	2.44 (5.54)	10.05 (2.42)
Chronic mastitic	25.87 (8.13)	6.87 (7.15)	32.7 (7.9)

Supplementary Table S2 *p* values obtained from statistical analyses of mast cell numbers in healthy, acute, and chronic mastitic samples.

	P values		
	Healthy vs acute mastitic samples	Acute mastitic vs chronic mastitic samples	Healthy vs chronic mastitic samples
Granulated (intanct) mast cell number	<0.001*	<0.001*	<0.001*
Degranulated (active) mast cell number	0.001*	<0.001*	<0.001*
Total mast cell number	<0.001*	<0.001*	<0.001*

(*) indicates a statistically significant difference.

Supplementary Table S3 Average numbers of MC phenotypes in healthy, acute, and chronic mastitis samples.

	MC_T (fold increase)	MC_C (fold increase)	MC_{TC} (fold increase)
Healthy	1.8 (1)	1.0 (1)	1.4 (1)
Acute mastitic	4.4 (2.4)	2.6 (2.6)	2 (1.4)
Chronic mastitic	19 (10.5)	7.8 (7.8)	5.8 (4.1)

Supplementary Table S4 *p* values obtained from statistical analyses of mast cell phenotypes in healthy, acute, and chronic mastitic samples.

	***p* values**		
	Healthy vs acute mastitic samples	**Acute mastitic vs chronic mastitic samples**	**Healthy vs chronic mastitic samples**
MC_T	0.123	<0.001*	<0.001*
MC_C	0.015*	0.001*	<0.001*
MC_{TC}	0.548	0.008*	0.008*

(*) indicates a statistically significant difference.

Supplementary Table S5 *Pearson correlation coefficient (r) values* obtained from the comparison of fibrosis with Mast cells.

	***r* values**		
	Total Mast Cell	**Granulated Mast Cell**	**Degranulated Mast Cell**
Fibrosis	0.76	0.69	0.55