Pregnancy Obstructs Involution Stage II of the Mammary Gland in Cows: General Biological Implications

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Background

Repeated research findings over the last 4 decades show that involution of mammary glands in dairy cows did not regress to same extend as that noticed in other mammalian species.

Methodology/Principal Findings

We took an advantage of a rare event in the normal modern dairy farming: A cow that was false-positively identified as being pregnant was "dried up" (i.e., induced into involution) conventionally about 60 before her expected parturition. This cow was culled, and samples of her mammary gland tissue were examined for gross histology. In this study we demonstrate for the first time that modern dairy cow may undergo extensive obliteration of the lobular-alveolar structure, as expected in involution stage II.

Conclusions/Significance

We conclude that lack of histological evidence for the appearance of involution stage II in the vast majority of modern cow's population is related to the peculiar modern dairy husbandry, in which dairy cows are induced into involution still pregnant. Because retardation of involution stage II in pregnant mammals is most likely a general physiological phenomena, it might occurs in other mammals, particularly in lactating humans. Thus, based on basic comparative physiology considerations, we suggest that concurrent lactation and pregnancy should be considered as an independent risk factor for breast cancer.

Introduction

Mammary glands of adult's mammals undergo cycles of avolution (proliferation), lactation, and involution (regression) in coordination with the reproductive cycle [1]. Abrupt weaning or abrupt cessation of milk removal induces the rapid and acute onset of involution in the unmilked mammary gland at any stage of lactation [2-6]. In most mammals under natural conditions the weaning process is gradual and the active stage of involution starts after the mammary gland already naturally produces low amount of milk, relative to the amount produced at peak lactation. In nature, mammals are not often pregnant while still nursing their infant [1-3].

Modern farming in the Western dairy cows industry lead to a situation which considerably departs from the general dogma in most mammals: First, virtually all cows in a given herd are pregnant during most of the lactation period, because those who fail to conceive at the start of the lactation (~80 days post partum out of 300 hundred day of lactation cycle) were culled. Secondly, cows are induced into involution (also known as "dry off") by abrupt cessation of milking, usually about 60 days before the expected parturition [2-3]. Because of intensive selection for high milk yield and increase in milk yield persistency during the last 4 to 6 decades [7], dairy cows are dried off while producing considerable amounts of milk: 20, 40 and sometimes even 50 L day^{-1}. Such a practice results in the accumulation of milk in the udder, which leads to udder engorgement, milk leakage, increased risk to acquire bacterial infection [2], and frequently causes noticeable agony to the cow, which might scream loudly for several days [8]. The length of the dry off period in modern dairy farming is a compromise between the farmer's wish to maximize milk production and the need for a period sufficiently long to accomplish involution and avolution of the glands and thus to prevent a decline in milk production in the following lactation [2-3]. However, the physiological processes underlying involution/avolution cycle are not fully ascertained in the dairy cows and the optimal length of the dry period is under renewed considerations [2].

After the induction of the acute phase of involution, mammary regression proceeds through at least two stages of morphogenetic alterations [1], which are reflected in respective phenotypic reformation of the amount and composition of mammary secretion, which are well characterized in dairy ruminants [9-11]. Stage I of involution involves the widespread apoptosis of alveolar epithelial cells, which reverse the dramatic expansion of this compartment during the previous cycle of avolution [1-6]. The first phenotypic sign for induction of stage I is the disruption of the tight junctions between epithelial cells, which is reflected in mark increase in the concentration of plasma electrolytes, sodium and chlorine, and mark inflow of leukocytes into the mammary gland [9-11]. The secretion of components of the innate immune system such as immunoglobulins [9-11], lactoferrin [9-11], albumin [12], and many other soluble components of the innate immune system [13-14] is also invoked at this stage

During the second stage of involution the lobular-alveolar structure of the gland is obliterated by proteinases that degrade the basement membrane and extra-cellular matrix, resulting in regression of the gland to morphological picture that resembles the pre-adult stage [1]. Stage II is therefore associated with irreversible loss in the capacity of the gland to produce and secrete milk [1, 5-6]. Mammary secretion in cows at this stage become scant, watery, turbid (serum-like) and rich in leukocytes [9-11]. In comparison to stage I, stage II consists with increase in the proportion of lymphocytes and macrophage and reduction in the proportion of polymorphonuclear cells [2].

In comparison to rodents in which involution is completed within less than a week [1, 5-6], involution in cows is complete only after 21–30 days after drying-off and never rich the histological features seen at stage II in rodents and other mammals [15-18 and see ref. 2 for the most recent review]. Reduction in milk secretion and initiation of extensive apoptosis of the epithelial cells is considerably delayed in comparison to rodents [2]. However, the most distinguishing feature between cows and rodents and other mammals is the maintenance of intact lobular-alveolar structure throughout the 60-days prepartum period after milking has been terminated [2, 15-18]. Consistent with the continued presence of intact alveoli, milk production in the dairy cow was partially restored by mammary secretion removal after 11 days of milk stasis [19]. For comparison, it would need only 4 days of milk stasis to rich a complete involution and irreversibility in the capacity to secrete milk in mouse [1, 5-6].

The rate of mammary gland involution is affected by systemic hormones, local factors, pregnancy and stage of lactation [1-6]. Involution stage II can be inhibited by systemic glucocorticoids and progesterone [20-21] and by pregnancy [22].

Thus, an interesting question which arise from the above introduction is: does the involution process, particularly stage II, is fundamentally different in cows in comparison to rodents or other mammals or merely reflect the selection for high milk yield, the artificial habitual situations involved with modern dairy farming and the fact that most research in this aspect was carried with modern cows?

The present study is concerned with the clarification this question and evaluation of the general biological implication of simultaneous involution and pregnancy in cows and humans.

Results

The histological data presented in figure 1 and 2 differed dramatically from those of cows induced into "dry-off" pregnant two days before their butcher (Figure 3 and 4). In fact, it would be difficult to differentiate between the histology of these glands from the histology of lactating glands. This indicates that during the first 2 days of "dry off", the mammary gland parenchyma undergoes minimal changes, which is consistent with previous reports [2]. Thus, in figures 3 and 4 we show that the "abnormal" features of dairy cows involution are the predominant situation when they are induced into dry off pregnant, as shown many times in the pass (15-18].

In contrast, in figures 1 and 2 we shows unequivocally that the mammary gland of dairy bovine may undergoes involution stage II. The main indications that the gland undergoes involution stage II, were reduction in lobular size, irregular outline and collapse of the alveoli lumen and marked increased interlobular collagen rich fibrous stroma (red arrows in Figure 1) and fat (blue arrow in Figure 1). Higher magnification shows attenuation of the alveolar cuboidal epithelial cells (blue arrows in Figure 2) and the decreased luminal size of alveoli (red arrow in Figure 2). These mark histological features are also reflected by marked morphological differences in the shapes of the udders of a cow induced into involution pregnant versus the test case cow, which was non-pregnant at dry off (supplementary figure 1 vs., supplementary figure 2).

Discussion

Results interpretation

The results of the present communication have shown for the first time (to the best of our knowledge) that the mammary gland of dairy cow may undergo extensive obliteration of the lobular-alveolar structure, as expected in involution stage II.

There are two possible explanations to the phenomenon described in this communication. First; this cow differs genetically from the vast majority of cows in the world. However, the probability of such a possibility is quite remote because this cow already underwent two typical lactation cycles without being noticed for being unusual and in view of the fact that for being differed from the rest of the cows she had to be mutated in a major gene without any phenotypic physiological defect.

Alternatively, pregnancy, at the 3rd trimester, obstructs involution stage II in cows. Thus, when cows are induced into involution non-pregnant, even if yielding a lot of milk, which is the common situation in modern dairy cow husbandry, they will go through involution stage I and II as most other mammals. This conclusion is independently supported by data from the literature.

In sheep induced into involution by weaning 5 d after parturition, the mammary glands were completely involuted after 30 d [23]. Thus, this experiment has clearly showed that in non-pregnant ruminant specie, weaning was associated with complete involution. Similarly, pregnancy retarded mammary gland involution in mice. The mechanism for retarding or obstructing involution during pregnancy appears to be hormonal. After weaning, injections of glucorticoids, progesterone, and prolactin inhibit mammary involution in mice (21), and similar results have been observed following grafting progesterone and deoxycorticosterone implants in the mammary gland (22). Conversely, reduction of circulating concentrations of prolactin by bromocriptine accelerated mammary involution [3]. Accelerating the rate of involution in dairy cows to 3 days, as in mice is possible by treating the cows with casein hydrolyzate [10]. However, this treatment was not associated with obliteration of the lobular-alveolar structure (unpublished results). Thus, all in all, the present results support a hypothesis stating that incurrent pregnancy retard or obstruct involution stage II.

General biological implications

In the wild, concurrent pregnancy and lactation only occur when nutritional conditions are favorable. If conditions are poor, rebreeding will be delayed and lactation will continue, at an energetically-sustainable level, for much longer than its ‘normal’ duration. In this way the twin energetic burdens of pregnancy and lactation are separated, and extremes are avoided [24]. In at least two mammalian species these natural interrelationships among pregnancy, lactation and nutritional state appeared to have been broken up. The first example as described above and by Knight [24] is the modern dairy cows; in which aggressive trait selection enforce the cows to be concurrently pregnant and lactating for most of their adulthood life. However, it would be difficult to separate the long term of such selection on the cow's health, because the life expectancy of modern cows in a given herd is very short, between two to three lactations cycles. (i.e. years). The life of humans in modern Western societies is characterized with abundance of available food, and the challenge is usually to avoid gaining weight, or loosing overweight, rather than facing with nutritional deficiency. It is also well known the breast-feeding is a "poor contraceptive". Thus, in humans concurrent pregnancy and lactation is expected to be more common than in other mammalian species.

In women, pregnancy reduces breast cancer risk, but the reduction is relatively small and occurs only in women who had their first child at an early age (25-28). The susceptibility for breast cancer increases under conditions that reduce mammary epithelial differentiation, increase the expression of genes regulating cell proliferation, and down-regulate genes that improve DNA damage repair or induce apoptosis or differentiation [25]. Recently, evidence were presented and reviewed, which suggest that oxidative damage to the mammary gland epithelial cells is among the causes for the declining phase of lactation in mammals, even in animals that are continued to be milked or suckled [29]. This highlights the physiological role of involution stage II as a mean to replace oxidative-damaged tissue with newly synthesized intact tissue.

All of the above-considerations suggest that retardation or obstruction of involution stage II is a risk factor for acquiring breast cancer. Thus, it is remarkable that we could not trace even a single example in the literature where concurrent lactation and pregnancy have been examined as an independent risk factor for breast cancer. Looking for such a possibility is perhaps the most important proposition that may be deriving from this communication.

Materials and Methods

In this study we took an advantage of a rare event in the normal modern dairy farming. A cow which was false-positively identified as being pregnant was "dried up" conventionally about 60 before her expected parturition. However, after elapsing of more than 60 day without the cow giving birth, it was figured out that the cow was never pregnant. Thus, the mammary glands in this cow were induced into involution while the cow was non-pregnant. We were attracted by the short and compact udder in this cow, which resembled more the udder morphology of virgin heifer rather than the udder of involuted grown pregnant cow (supplementation 1 vs. supplementation 2). The histology of the parenchyma of the mammary gland in this cow was examined in order to find out whether the lobular-alveolar structure was completely obliterated, as expected in involution stage II.

The test-case cow was 3 years old animal that was dried at day 279 of her 2nd lactation. The cow milk yield until this day was 9930 L and her milk yield at dry off was 30 L/day. These figures are typical to many Israeli Holstein cows. The somatic cell count (i.e., total count of leukocytes and epithelial cells) were on average 50000 cells/ml, indicating that her udders were free of bacterial infection. The cow was sacrificed in abattoir 96 days after she was induced into dry off.

At slaughter, the udder was removed quickly and pieces of mammary tissue parenchyma (~ 3 cm^3) were dissected from each of three zones of the gland; upper, middle and lower (the later located just above the gland cistern). Tissue samples were placed immediately in fixative and processed as described before [23]. The samples were analyzed for gross histology as described before [23]. Tissue from the mammary parenchyma of glands of 3 "normal" cows, induced into "dry off" pregnant 2 days before their scarifying, were taken to illustrate the "abnormal" features of involution in cow induced into dry off pregnant. The mammary gland tissue samples from these cows were analyzed for gross histology in the same manner.

Figure legends

Figure 1.
Gross histology of the mammary gland of the test cow at x 10 magnification. There is reduction in lobular size, when compared to figure 4 Alveoli have irregular outline and there is collapse of the lumen. There is marked increased interlobular collagen rich fibrous stroma (red arrows) and fat (blue arrow) (10x).

Figure 2:
Gross histology of the mammary gland of the test cow at x 40 magnification. Note the attenuation of the alveolar cuboidal epithelial cells (blue arrows) and the decreased luminal size of alveoli (red arrow).

Figure 3:
Gross histology of the mammary gland of "normal" cow (pregnant, two days after dry off) at x 10 magnification.. Alveoli are distended. In most alveoli lumen there is evidence that proteinaceous material was secreted to the alveoli lumen (blue arrow). Interlobular stroma space is slight (red arrow) and interlobular fat is not detectable.

Figure 4:
Gross histology of the mammary gland of "normal" cow (pregnant, two days after dry off) at x 40 magnification.. Note the normal cellular height, rich in cytoplasmic vacuolation of the alveolar epithelial cells in comparison to the situation in the test cow (figures 1 and 2)

Supporting Information

Figure s1.
A picture of the udders of test-case cow that was taken shortly before her culling.

Figure s2
A typical picture of the udders of a cow after being induced into dries off for ~3 weeks

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Author's contribution

GL and NS set in motion this study; UM carried out the pictures of the supplementary data, A-MA carried out the histology analysis; NS wrote the first version of the manuscript and GL and UM contributed to its final format.

References cited

1. **Lund LR Romer J Thomasset N Solberg H Pyke C Bissell MJ Dano K Werb Z**. (1996). Two distinct phases of apoptosis in mammary gland involution: Proteinase-independent and -dependent pathways. Develop 122: 181-193.
2. **Capuco AV, Akers RM**. (1999). Mammary involution in dairy animals. J Mam Gland Biol Neoplasma 4:137-144, 1999.
3. **Wilde CJ Knight CH Flint DJ**. (1999). Control of milk secretion and apoptosis during mammary involution. J. Mammary Gland Biol. Neoplasia 4: 129-136.
4. **Quarrie LH Addey CVP Wilde CJ**. (1996). Programmed cell death during mammary involution induced by weaning, litter removal and milk stasis. J. Cell. Physiol. 168: 59-569.
5. **Li M Liu X Robinson G Bar-Peled U Wagner K-U Young WS Hennighausen L Furth PA**. (1997). Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. Proc. Natl. Acad. Sci. U.S.A. 94: 3425-3430.
6. **Marti A Feng ZW Altermatt HJ Jaggi R**. (1997). Milk accumulation triggers apoptosis of mammary epithelial cells. Eur. J. Cell. Biol. 73: 158-165.
7. **Kadzere CT Murphy MR Silanikove N Maltz E**. (2002) Heat stress in high producing dairy cows: a review. Livestock Prod. Sci., 77: 59-91.
8. **Leitner G Jacoby S Maltz E Silanikove N**. (2007). Casein hydrolyzate intrammary treatment improves the comfort behavior of cows induced into dry-of. Livestock Sci110: 292-297.
9. **Shamay A shapiro F Mabjeesh SJ Silanikove N**. (2002). Casein-derived phosphopeptides disrupt tight junction integrity, and precipitously dry up milk secretion in goats. Life Sci.. 70: 2707-2719.
10. **Shamay A Shapiro F Leitner G Silanikove N**. (2003). Infusions of casein hydrolyzates into the mammary gland disrupt tight junction integrity and induce involution in cows. J. Dairy Sci. 86:1250-1258.

11. **Silanikove N Shapiro F Shamay A Leitner G**. (2005). Role of xanthine oxidase, lactoperoxidase, and NO in the innate immune system of mammary secretion during active involution in dairy cows: manipulation with casein hydrolyzates. Free Radicals Biol. Med., 38: 1139-1151.
12. **Shamay A Homans R Fuerman Y Levine I Barash H Silanikove N Mabjeesh, SJ**. (2005). Expression of albumin in nonhepatic tissues and its synthesis by the bovine mammary gland. J. Dairy Sci. 88:569-576.
13. **Clarkson RW Wayland MT Lee J Freeman T Watson CJ**: (2004). Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. Breast Cancer Res 6:R92-R109.
14. **Stein T Morris JS Davies CR Weber-Hall SJ Duffy MA Heath VJ Bell AK Ferrier RK Sandilands GP Gusterson BA**: (2004). Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. Breast Cancer Res 6:R75-R91.
15. Swanson **EW** Pardue **FE** Longmire **DB**. (1967). Effect of gestation and dry period on deoxyribonucleic acid and alveolar characteristics of bovine mammary glands. J. Dairy Sci. 50: 1288.
16. Holst BD Hurley WL Nelson DR (1987)**.** Involution of the bovine mammary-gland - histological and ultrastructural-changes. J. Dairy Sci. 70:935-944.
17. Sordillo LM Nickerson SC. (1988). Morphologic changes in the bovine mammary-gland during involution and lactogenesis. Am. J. Vet. Res. 49: 1112-1120.
18. **Capuco AV Akers RM Smith JJ** (1997). Mammary growth in Holstein cows during the dry period: Quantification of nucleic acids and histology. J. Dairy Sci. 80: 477-487.
19. **Noble MS Hurley WL**. (1999). Effects of secretion removal on bovine mammary gland function following an extended milk stasis. J. Dairy Sci 82:1723-1730.

20. **Ossowski L Beigel D Reich E**. (1979). Mammary plasminogen activator: correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. Cell:16:929-940.

21. Feng Z Marti A Jehn B Altermatt HJ Chicaiza G Jaggi R. (1995). Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. J Cell Biol:131:1095-1103.[Abstract]

22. **Capuco AV Lib M Long E Ren S Hrusk KS Schorr K Furth PA**. (2002). Pregnancy Retards Mammary Involution: Effects on Apoptosis and Proliferation of the Mammary Epithelium after Forced Weaning of Mice. Biolo Reprod 66:1471-1476.
23. **Tatarczuch L Philip C Lee, CS**. (1977). Involution of sheep mammary gland. J. Anat. 190: 405-416.
24. **Knight CH**. (2004).Lactation and gestation in dairy cows: flexibility avoids nutritional extremes. Proc Nutr Soc 60: 527-537.

25. **Pike MC Krailo MD Henderson BE Casagrande JT Hoel DG**. (1983). " Hormonal" risk factors, "breast tissue age" and the age-incidence of breast cancer. Nature: 303: 767-70.
26. **Ewertz M Duffy SW Adami HO Kvale G Lund E Meirik O Mellemgaard A Soini I Tulinius H**. (1990). Age at first birth, parity and risk of breast cancer: a meta-analysis of 8 studies from the Nordic countries. Int J Cancer 46 597-603.
27. **Bain C Willett W Rosner B Speizer FE Belanger C Hennekens CH** (1981). 1 Early age at first birth and decreased risk of breast cancer. Am J Epidemiol 114:705–709.
28. **MacMahon B Cole P Lin TM Lowe CR Mirra AP Ravnihar B Salber EJ Valaoras VG Yuasa S.** (1970). Age at first birth and breast cancer. Bull World Health Org 43:209-221.
29. **Hadsell D George J Torres D**. (2007). The Declining Phase of lactation: Peripheral or central, programmed or pathological? J. Mammary Gland Biol. Neoplasia 12: 59-70.

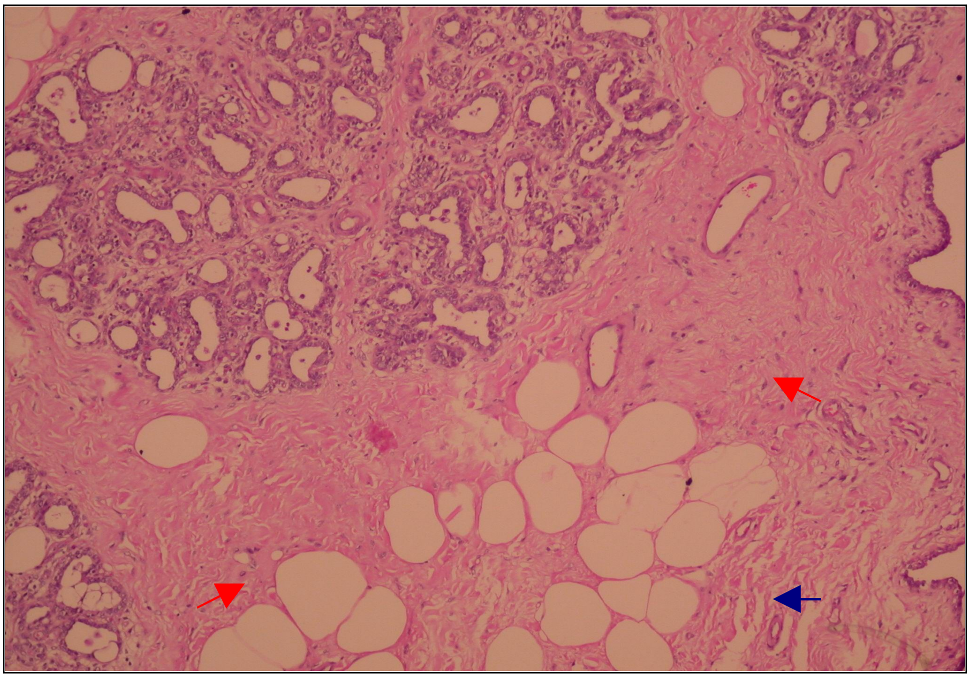


Figure 1

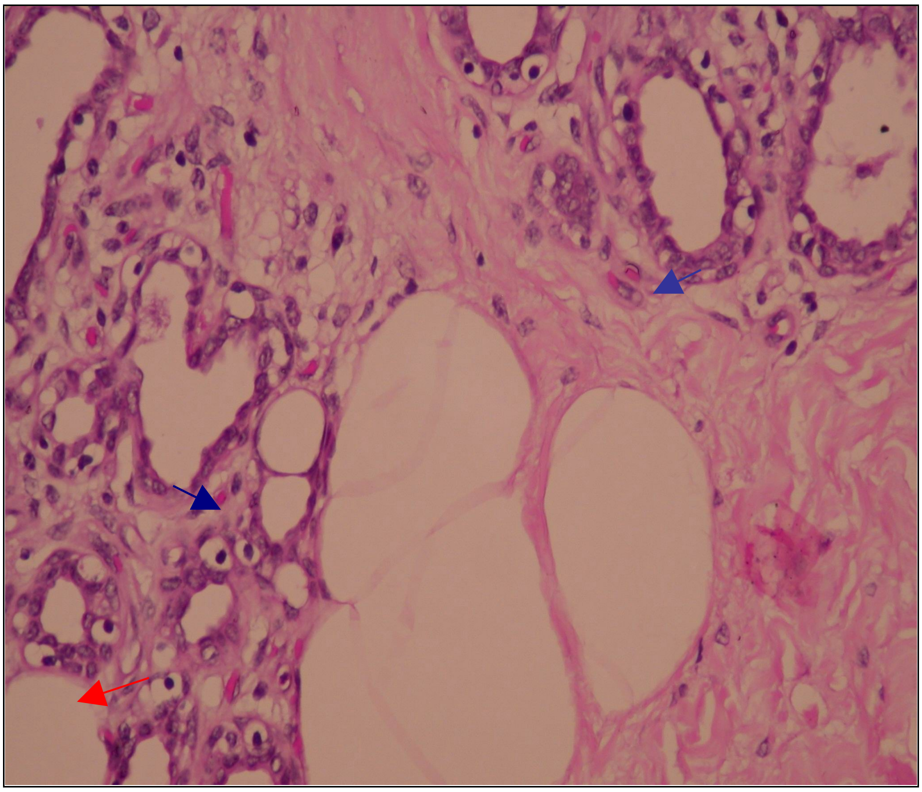
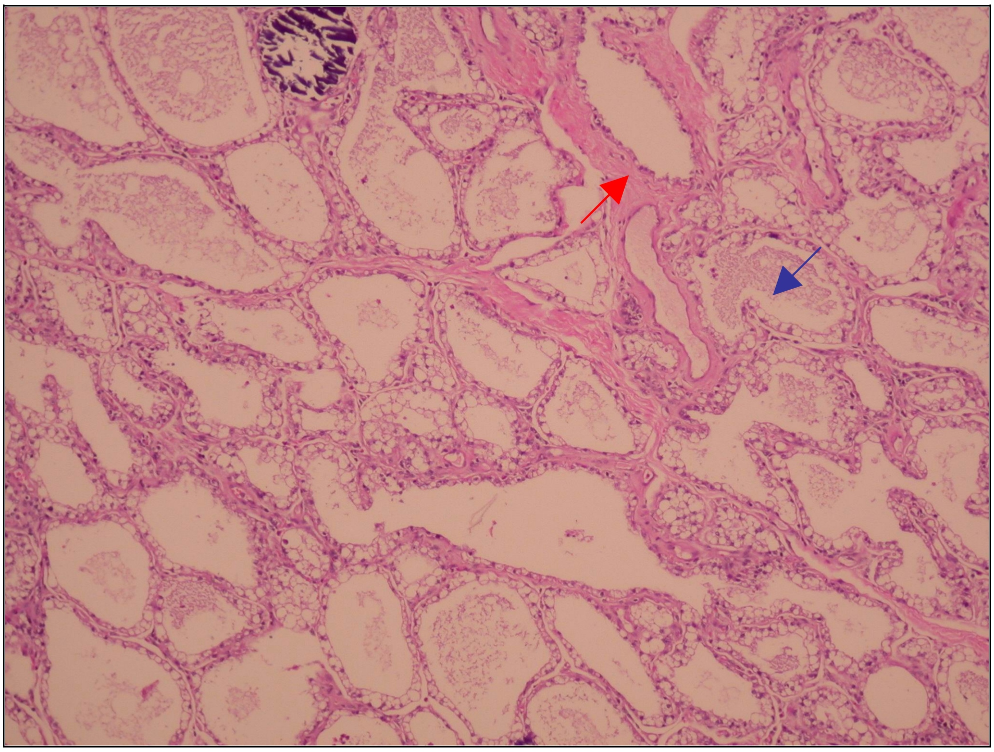


Figure 2



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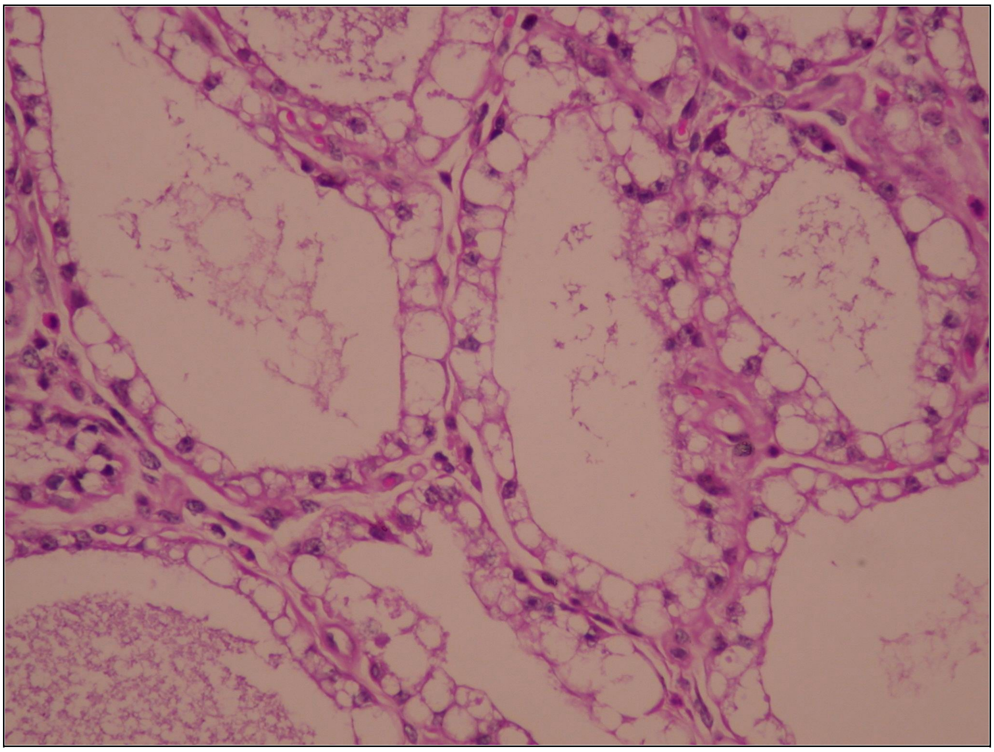

Figure 4

Fig s1

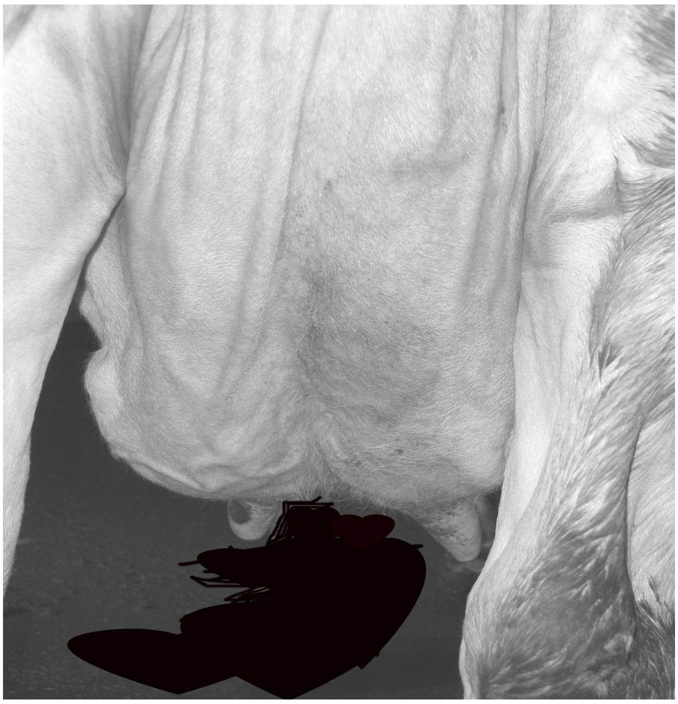

Fig s2