

1 Comparative mammary gland postnatal development and tumourigenesis in the sheep,
2 cow, cat and rabbit: Exploring the menagerie

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12 **Abstract**

13 Sheep, cows, cats, and rabbits are kept by humans for agricultural purposes and as
14 companion animals. Much of the mammary research in these species has focussed on
15 mastitis in the case of ruminants and rabbits, and mammary tumourigenesis in cats
16 and rabbits. However, similarities with the human breast suggest that these species
17 may be currently underutilised as valuable comparative models of breast development
18 and disease. The mammary gland undergoes cyclical postnatal development that will
19 be considered here in the context of these non-traditional model species, with a focus
20 on the mammary microenvironment at different postnatal developmental stages. The
21 second part of this review will consider mammary tumour development. Ruminants
22 are thought to be relatively 'resistant' to mammary tumourigenesis, likely due to
23 multiple factors including functional properties of ruminant mammary
24 stem/progenitor cells, diet, and/or the fact that production animals undergo a first
25 parity soon after puberty. By contrast, unneutered female cats and rabbits have a
26 propensity to develop mammary neoplasms, and subsets of these may constitute
27 valuable comparative models of breast cancer.

28

29 **Keywords (up to 6):** cat; cow; mammary gland; rabbit; sheep; tumour

30

31 **1. Introduction**

32 Sheep, cows, cats and rabbits represent a spectrum of species that are kept by humans
33 for agricultural purposes and as companion animals. Much of the mammary research
34 in these species has focussed on mastitis in the case of ruminants and rabbits, and
35 mammary tumourigenesis in cats and rabbits. However, similarities with the human
36 breast suggest that these species may be valuable comparative models of breast
37 development and disease.

38

39 The mammary gland has a particularly fascinating cycle of postnatal development that
40 will be considered here in the context of the sheep, cow, cat and rabbit, with an
41 emphasis on the mammary microenvironment at different postnatal developmental
42 stages. The second part of this review will focus on mammary tumour development in
43 these species. In particular, the apparent 'resistance' of ruminants to mammary
44 tumourigenesis will be explored and contrasted with the propensity of unneutered
45 female cats and rabbits to develop mammary neoplasia. The value of mammary
46 carcinomas arising in cats and rabbits as comparative models of breast cancer will be
47 evaluated.

48

49 **2. Sheep**

50 The ovine mammary gland has two mammae each with one galactophore and ductal
51 system [1]. Supernumerary teats may be present, and larger supernumerary teats tend
52 to exhibit anatomical complexity including a teat canal and/or teat cistern in which
53 milk may accumulate, potentially predisposing to mastitis [2]. The mammary
54 parenchyma is characterised by the presence of terminal duct lobular units (TDLUs)
55 supported by collagenous intra- and interlobular stroma [1]. The collagenous stroma

56 of the ovine mammary gland is strikingly similar to the human breast and is in
57 contrast to the adipocyte-rich stroma of the mouse [3] (Fig. 1).

58

59 Sheep are polyoestrous seasonal breeders, with a cycle length of 14-20 days and the
60 occurrence of oestrus cycling controlled by decreasing photoperiod. Ovulation occurs
61 towards the end of oestrus. Gestation length is approximately 150 days [4].

62

63 **2.1. Ovine pre- and peri-pubertal mammary development**

64 A striking 20% of ovine mammary growth, based on dried fat-free tissue weights,
65 occurs between birth and the beginning of pregnancy [5]. Sheep reach puberty at 6 to
66 8 months old. Growth through the pre-pubertal period is not linear and is punctuated
67 by a phase of allometric mammary development, when the rate of growth of the
68 mammary gland exceeds that of the body as a whole. Delineation of the timing of the
69 period of allometric growth is likely affected by study design, including method of
70 determining growth rate, sheep breed [6], and body condition. One study suggests that
71 ovine mammary allometric growth occurs between 3 and 4 months of age [5].

72

73 Using DNA content of the mammary gland as a surrogate read-out for mammary
74 growth, other authors have demonstrated that the magnitude of mammary allometric
75 growth is perhaps counter-intuitively increased in lambs receiving rations designed to
76 give a low rate of weight gain compared to those receiving a higher plane of nutrition.
77 These data suggest that rations designed to produce high weight gain can result in a
78 decrease the rate of mammary allometric growth of pre-pubertal lambs [7].

79

80 During the pre-gestational phase of ovine mammary development, expression of
81 insulin-like growth factor-I mRNA, a likely mammary paracrine mitogen,
82 approximately mirrors the period of allometric growth [8]. Experiments in
83 ovariectomised lambs have demonstrated that the pre-pubertal period of allometric
84 growth is not dependent on ovarian hormones [9] and expression of insulin-like
85 growth factor-I mRNA is unaffected by ovariectomy [8]. However, when exogenous
86 oestrogen is administered to ovariectomised ewes, expression of insulin-like growth
87 factor-I mRNA in the mammary fat pad adjacent to the parenchyma is increased [8]
88 suggesting some degree of hormonal regulation. There is differential regulation of
89 expression of insulin-like growth factor-I and II. Expression of insulin-like growth
90 factor-II is again higher during the first period of postnatal growth, up to 23 weeks of
91 age, but parenchymal mRNA expression increases following ovariectomy and
92 expression in both the parenchyma and mammary fat pad adjacent to the parenchyma
93 is suppressed by administration of exogenous oestrogen [8].

94

95 Expression of another mammary mitogen, keratinocyte growth factor, has also been
96 assessed in ovine mammary tissue. The mammary stroma expresses two transcripts of
97 keratinocyte growth factor mRNA, whereas only one transcript is identifiable in the
98 mammary parenchyma, hinting at a possible paracrine mode of action for this factor
99 in ovine mammary development, and underlining the importance of the mammary fat
100 pad in participating in cross-talk with the epithelial compartment [1, 10]. Expression
101 of the neurotrophin growth factors, nerve growth factor and brain derived
102 neurotrophic factor, is higher in pre-pubertal mammary gland compared to later
103 developmental time points and may also contribute to local regulation of mammary
104 development [11].

105

106 **2.2. Ovine mammary development during gestation and lactation**

107 The udder volume of a pregnant ewe may increase exponentially over the last month
108 before lambing [12]. Based on assessment of dried fat-free tissue weights, 80% of
109 ovine mammary growth is associated with pregnancy, and mammary growth is
110 essentially complete by parturition [5]. As would be anticipated, this phase of growth
111 during pregnancy is accompanied by Ki67 expression in mammary epithelial cells,
112 with a Ki67 proliferation index of approximately 8% recorded in late gestation.
113 Interestingly, in ewes approximately 10 days prior to lambing, increased Ki-67
114 expression is observed in smaller diameter “immature” alveoli than in alveoli with a
115 large lumen and a correspondingly attenuated epithelium, suggesting that smaller
116 alveoli may contribute to a final pulse of mammary growth prior to parturition [13].

117

118 During this late gestational phase of rapid epithelial proliferation, the mammary
119 parenchymal area increases dramatically but the proportion of mammary gland area
120 occupied by parenchyma does not alter, allowing inference that the mammary fat pad
121 volume increases in parallel. In ewes with cleared contralateral fat pads, this increase
122 in fat pad volume is not observed in the contralateral cleared fat pad, again
123 highlighting interplay between the mammary epithelial compartment and the fat pad
124 [14].

125

126 Milk yield in sheep is proportional to number of offspring, and the placenta is thus
127 considered to be pivotal in the control of this dramatic phase of gestational mammary
128 development [1, 15]. Placental lactogen has been implicated in this regulation, with

129 concentration of placental lactogen correlating with udder size pre-partum and post-
130 partum milk yield [16].

131

132 Leptin is a hormone, classically associated with synthesis by adipocytes, that
133 influences energy balance and food intake. It is also expressed by the mammary gland
134 and may modulate cellular proliferation and differentiation, although many of the
135 studies demonstrating such activities have utilised *in vitro* systems, or
136 supraphysiological doses of leptin [17]. In the ovine mammary gland, leptin mRNA
137 levels are high at the beginning and end of gestation and are lower during mid-
138 pregnancy and lactation [18]. Whilst the role of leptin in the mammary gland is yet to
139 be fully elucidated, it seems likely to be involved in regulating mammary growth and
140 function during phases of development necessitating metabolic adaptation, such as
141 late pregnancy and early lactation [17].

142

143 A more recent study has focussed on the transcriptional changes occurring during late
144 gestation compared to early lactation using RNA-seq. In late pregnancy, key
145 biological processes include cell proliferation, beta-oxidation of fatty acids, and
146 translation, translation elongation, and translation initiation [19]. STAT5 genes
147 (STAT5A and STAT5B) are expressed more highly during late pregnancy than in
148 lactation [19]. This may reflect the role of STAT5 in mammary lobuloalveolar
149 development and upregulation of milk protein gene expression [20]. It may also point
150 to a relatively less important role of STAT5 in regulating milk protein gene
151 expression in sheep, similar to that suggested in cows [19, 21]. However, this finding
152 and associated implications must be interpreted with caution as STAT5 may be
153 regulated predominantly through protein activation rather than by transcription [22].

154 Of historical note, one of the original papers describing STAT5, then termed milk
155 protein binding factor, discovered it to be highly expressed in the mammary glands of
156 sheep [23].

157

158 RNA seq also identifies changes in the expression of epigenetic regulators,
159 particularly chromatin remodellers, between pregnancy and lactation. This suggests
160 that epigenetic regulation may contribute to coordination of the considerable
161 transcriptional changes required when the mammary gland undergoes transition from
162 a pregnancy ‘growth and development’ phase to a lactational ‘synthesis and secretion’
163 phenotype [19].

164

165 **2.3. Ovine post-lactational mammary gland involution**

166 As in other species, ovine post-lactational mammary gland involution may be
167 initiated, in both experimental and natural settings, by either abrupt or gradual
168 cessation of lactation [24]. Ultrasonography allows visualisation of a transient
169 increase in gland cistern volume following abrupt weaning. At approximately one
170 week of involution, milk clots are appreciable within the gland cistern, and these
171 accumulations are likely gradually resorbed, leading to a reduction in gland cistern
172 volume as involution progresses [25].

173

174 In rodent models involution is a bipartite process comprising waves of epithelial cell
175 death, and subsequent removal of these dead cells, coupled with stromal remodeling
176 [26]. During ovine involution, there is evidence of apoptosis [13, 27]. Although
177 apoptosis has long been appreciated as one cell death mechanism occurring during
178 mammary involution [28], more recent studies in mice have revealed that murine

179 involution can proceed in the absence of executioner caspases 3 and 6, and that Stat3-
180 regulated lysosomal-mediated cell death is a critical cell death pathway [29-31]. The
181 contribution of lysosomal-mediated cell death to ovine post-lactational mammary
182 regression is yet to be established.

183

184 Efferocytosis, the removal of dead, damaged, or superfluous cells by neighbouring
185 cells or professional phagocytes [32], is an important facet of involution. During
186 murine mammary involution, epithelial cells contribute to efferocytosis [33] and we
187 and others have documented the importance of immune cells, particularly
188 macrophages, in the murine mammary microenvironment [34-40]. Similarly,
189 mammary epithelial cells and macrophages are effectors of efferocytosis during ovine
190 mammary involution [41-44].

191

192 During involution, ovine basal mammary epithelial cells, or myoepithelial cells,
193 appear to undergo morphological changes similar to those observed in the mouse,
194 with shrinkage and re-direction of the cellular processes [38, 42]. There is also
195 ultrasonographic evidence of stromal remodeling in the involuting ovine mammary
196 gland [25].

197

198 In an agricultural context, ovine mammary involution may be initiated as an abrupt or
199 gradual process, according to the production system. It is important to note that when
200 lambs being raised for meat production are removed synchronously from dams at the
201 production stage termed 'weaning', a variable degree of natural weaning will have
202 already occurred. As a result, ovine mammary tissue sampled at the production stage
203 termed 'weaning' frequently exhibits variable, but often profound, levels of

204 regression (author's unpublished results) (Fig. 1). In experimental mice, cell death
205 also occurs during involution induced by natural weaning although there is a slower
206 onset of this process compared to during involution induced by forced synchronous
207 litter removal [45].

208

209 **3. Cattle**

210 The bovine mammary gland has four inguinal mammae each with one galactophore
211 and ductal system [26]. The arrangement of TDLUs and the mammary stroma is
212 broadly similar to the sheep and thus also to the human breast [1, 3]. In the bovine
213 mammary gland some authors favour the term terminal ductal units to the descriptor
214 TDLUs [46].

215

216 Cattle are polyoestrous and have an oestrous cycle of 18-24 days. Ovulation occurs
217 approximately 10-12 hours after the end of oestrus. Gestation length varies according
218 to breed but is frequently in the region of 279-290 days [4].

219

220 **3.1. Bovine pre- and peri-pubertal mammary development**

221 Pre-gestational mammary development in heifers partially shapes future lactation
222 potential. The reader is directed to a recent review examining the importance of this
223 developmental phase in the context of future productivity [47]. This section will focus
224 firstly on comparison to ovine pre-gestational development and secondly on recent
225 observations regarding the mammary microenvironment during this phase.

226

227 In contrast to the lamb, ovariectomy in the heifer perturbs mammary growth [48]. In
228 dairy heifers undergoing ovariectomy, mammary epithelial progesterone receptor

229 expression is considerably reduced and the intensity of oestrogen receptor expression
230 is decreased. There is reduced Ki67 expression in both the mammary epithelial cells
231 and the stroma [49].

232

233 There is clear recognition of the importance of immune cells in coordinating
234 mammary development in mice [37, 38, 50-55]. Compared to laboratory rodents,
235 there is very little known about immune cell regulation of mammary development in
236 the cow. However, localization of immune cells in female calves up to 42 days old
237 hints at a similarly important role in this species, with significantly more
238 macrophages, mast cells, and eosinophils present in ‘near stroma’ within 100-150
239 microns of mammary epithelium, than in the more distant ‘far stroma’ [56].

240

241 In addition to immune cells, myoepithelial cells are also attracting increasing attention
242 as cellular players that may impact development of the pre-pubertal bovine mammary
243 gland [47]. In 100 day old calves, more numerous myoepithelial cells are detectable in
244 individuals that have been ovariectomised than in intact heifers. In the former it is
245 suggested that oestrogen may negatively regulate myoepithelial differentiation and
246 that, in the absence of oestrogen, more abundant myoepithelial cells may constrain
247 luminal epithelial proliferation [57]. However, in calves receiving tamoxifen, in
248 which there is an ensuing 50% reduction in mammary growth, fewer myoepithelial
249 cells are noted [58]. It seems likely that the role of myoepithelial cells in the
250 developing mammary gland is currently under-appreciated and this is likely to be a
251 focus of future research efforts [47].

252

253 **3.2. Bovine mammary development during gestation and lactation**

254 The majority of mammary growth is achieved during the final trimester of pregnancy.
255 As in the sheep, placental lactogen (see also Section 2.2) is considered likely to be
256 important in regulating this growth [59]. To a lesser degree, prolactin is also likely
257 important [1]. When quantification of mammary DNA is used as a surrogate read-out
258 of udder size, it correlates poorly with milk yield in lactating dairy cows [59].

259

260 Historically it has been noted that in early lactation in the murine mammary gland
261 cells active in DNA synthesis do not undergo mitosis. Thus it was postulated that
262 there is an amplification of genomic DNA in order to increase the number of gene
263 copies to support the high rate of RNA and protein synthesis in lactating epithelial
264 cells [60-62]. A more recent study has revealed that approximately 40% of the
265 luminal alveolar epithelial cells in the bovine mammary gland during late gestation
266 are binucleated. It has been suggested that these may increase milk production
267 capacity [63] or represent a downstream sequel to the DNA synthesis required to
268 sustain lactation [62] (Fig. 2).

269

270 During lactation, there is concomitant epithelial cell death and the proportion of
271 epithelial cells is therefore highest at 90 days lactation and then declines [64].

272 Interestingly, when once daily milking is carried out unilaterally in dairy cows, with
273 the other half of the udder milked twice a day, epithelial proliferation decreases and
274 cell death increases in the half that is milked once a day. These observations, together
275 with related transcriptional changes, suggest that local factors and milk build-up
276 regulate the decrease in milk yield associated with once daily milking [65]. Such
277 experiments echo those in mice, where unilateral teat sealing has been utilised to

278 study the first stage of post-lactational regression and has demonstrated that cell death
279 is induced by local factors, presumably associated with accumulation of milk [66].

280

281 **3.3. Bovine post-lactational mammary gland involution**

282 Although some of the comments relating to ovine post-lactational mammary
283 regression (Section 2.3) are applicable to cows, there are specific features of bovine
284 involution that are noteworthy. Several different methods of reducing milking and
285 modulating nutritional intake may be employed to induce involution [67]. Many cows
286 are already in late pregnancy at the point of involution or ‘drying off’, and these
287 animals therefore exhibit what we have previously termed a ‘parallel pregnancy’
288 involution signature [26]. In mice undergoing forced involution, the degree of cell
289 death is reduced when there is concurrent pregnancy [68]. However, there is earlier
290 onset of cell death in mice that undergo natural weaning whilst pregnant [45]. Overall,
291 the process of involution in mice that are concurrently pregnant differs notably from
292 the progression of involution in non-pregnant mice in terms of tissue histo-
293 morphology, cell death dynamics, and gene expression patterns [45]. Similarly one of
294 the key features of the bovine mammary microenvironment in cows with a ‘parallel
295 pregnancy’ involution signature is that there is dual evidence of cell death and cell
296 renewal, and the importance of the latter is underlined by the suggestion that dairy
297 cows undergo ‘regenerative involution’ [46, 69, 70].

298

299 Through use of murine models, post-lactational regression has been demonstrated to
300 be a two-stage process. The first stage is reversible and even though there is extensive
301 cell death, suckling of pups can successfully recommence if the offspring are returned
302 during this phase. The second stage, from approximately 48 hours onwards, is

303 irreversible and comprises further cell death and tissue remodelling [20].
304 Interestingly, experiments in cows subject to an abrupt discontinuation of milking at
305 mid lactation, demonstrate that after seven days of non-milking, milking can be
306 reinitiated with almost comparable milk yield and composition to that observed prior
307 to induction of involution [71]. Involution appears to be partially reversible following
308 11 days of involution [72] suggesting that this time point may be approaching the
309 transition to irreversibility.

310

311 As already described (Section 2.3) Stat3 activation is fundamental to the progression
312 of murine mammary involution [29-31] and the available evidence supports a similar
313 role in bovine involution. As in other species, the pattern of bovine involution is
314 somewhat heterogeneous, and phosphorylated STAT3 (pSTAT3) is detectable in the
315 bovine mammary gland within 36 hours of abrupt cessation of milking at mid
316 lactation in samples with low level of milk protein expression [73]. Robust expression
317 of pSTAT3 is detectable by 72 hours of involution [74].

318

319 **4. Domestic cats**

320 Cats generally have four pairs of mammae, each with multiple ductal trees [75]. The
321 feline mammary gland can be considered to have terminal duct lobular units [76].

322

323 Cats are seasonally polyoestrous long day seasonal breeders, with a cycle length of
324 14-21 days. Cats are induced ovulators, with ovulation occurring 24-48 hours after
325 breeding or equivalent stimulus. Gestation length is approximately 65 days [4].

326

327 **4.1. Feline pre- and peri-pubertal mammary development**

328 Compared to farm animals, there has been less focus on pre- and peri-pubertal
329 mammary development in cats. At puberty, oestrogen stimulates mammary epithelial
330 proliferation [75]. Progesterone, increasing in levels during dioestrus and later
331 pregnancy, likely elicits mammary production of growth hormone, and concomitant
332 synthesis of insulin-like growth factors, that have also been implicated in the process
333 of feline mammogenesis [75, 77, 78]. Correspondingly, feline mammary tissue
334 exhibits progesterone receptor and oestrogen receptor expression [79] [80-82].
335 Histological changes associated with the oestrus cycle have been described in the
336 mammary gland of dogs and are likely to also occur in the mammary gland of cats
337 although it should be noted that, unlike dogs, cats are induced ovulators [75, 83, 84].

338

339 **4.2. Feline mammary development during gestation and lactation**

340 To the author's knowledge, there is very little species-specific information regarding
341 the development of the mammary gland during pregnancy and lactation in the cat.
342 Interestingly, the whey protein beta-lactoglobulin that is found in the milk of many
343 species except humans, rodents, and lagomorphs, exists in three forms in the cat and
344 the cat possesses at least two distinct beta-lactoglobulin genes, compared to the cow
345 that has one [85] [86].

346

347 **4.3. Feline post-lactational mammary gland involution**

348 There is a relative paucity of information regarding the specific features of post-
349 lactational mammary regression in the cat. However, patterns of canine mammary
350 involution have been described, and potentially similar morphological changes may
351 arise in the cat. Historically post-lactational alveolar regression has been suggested to
352 commence from ten days post partum, with complete regression achieved by 40 days

353 post partum [87] but more recently authors have described morphological evidence of
354 canine involution from day 56 of lactation, with almost complete involution achieved
355 by the end of the third month of lactation. In the latter study, the authors suggested a
356 likelihood for involution to start slightly earlier in cranial mammae that are less
357 preferred for sucking by pups [88].

358

359 **5. Rabbits**

360 The rabbit usually has four pairs of mammae, with one thoracic pair, two abdominal
361 pairs and one inguinal pair [26]. There are six or seven ductal systems per mamma
362 [89]. The rabbit mammary gland has a terminal duct lobular unit structure [90].

363

364 Wild rabbits are seasonally polyoestrous long day seasonal breeders, with a suggested
365 cycle length of 16-18 days. However, the oestrus cycle is less well-defined in some
366 domestic rabbits. Rabbits are induced ovulators, with ovulation occurring 10-13 hours
367 after breeding or equivalent stimulus. Gestation length is approximately 31-32 days
368 [91].

369

370 **5.1. Leporine pre- and peri-pubertal mammary development**

371 Consistent with use of rabbits as a laboratory model, much of the work concerning
372 pre- and peri-pubertal mammary development in rabbits has focussed on the impact of
373 diet. Consumption of an obesogenic diet between 8-13 weeks of age significantly
374 alters the composition of the mammary gland, when assessed at gestation day 8 of a
375 subsequent pregnancy. There is increased deposition of adipose tissue, and a relative
376 reduction in the percentage of the gland composed of connective tissue and
377 epithelium. Interestingly, there is also less epithelial proliferative activity, as

378 measured by Ki67 immunofluorescence [92]. By contrast, when an obesogenic diet is
379 administered from prior to puberty to mid-pregnancy, the proportion of epithelium is
380 significantly higher at mid pregnancy than in control animals [93]. Extending the
381 period of abnormal diet by raising newborn rabbits on lactating dams fed an
382 obesogenic diet and then maintaining such a diet for the offspring from the onset of
383 puberty and throughout early pregnancy also results in abnormal mammary tissue
384 when sampled at day 8 of pregnancy. Rabbits maintained on this regimen exhibit
385 ectatic mammary ducts containing proteinaceous material and disorganisation of
386 mammary alveolar structures [94].

387

388 **5.2. Leporine mammary development during gestation and lactation**

389 In contrast to the sheep, where the majority of mammary growth occurs during
390 gestation, and growth is essentially completed by parturition, historical analyses of
391 mammary gland DNA content in New Zealand white rabbits indicate that 67% of
392 growth occurs during gestation and 33% in lactation [95]. Gestation length in the
393 rabbit is 31-32 days, and the majority of pregnancy-associated mammary gland
394 growth occurs between gestation days 16 and 26 [95]. Growth dynamics are likely
395 influenced by breed and the experimental methodologies employed. By measuring
396 mammary gland weight as a percentage of body weight in Dutch Belted rabbits, other
397 authors have suggested that approximately 30-40% of total mammary gland growth is
398 attained during pregnancy with the remaining 60-70% occurring in lactation [96].
399 Thus, a notable degree of mammary growth occurs during both pregnancy and
400 lactation in the rabbit although the projected relative contributions of each
401 developmental stage may vary between studies. Corroborating this assertion, we have
402 demonstrated multifocally abundant Ki67 expression in luminal mammary epithelial

403 cells during both pregnancy and lactation in the rabbit [89]. This is in contrast to the
404 mouse in which the majority of epithelial proliferation occurs in association with
405 pregnancy rather than lactation [97, 98].

406

407 In contrast to the sheep where leptin expression is higher in early and late pregnancy
408 than in lactation [18] (see Section 2.2), in the rabbit mammary gland, leptin exhibits
409 low levels of mRNA expression during pregnancy, but increases significantly
410 between days 3 and 16 of lactation in luminal epithelial cells. It has been suggested to
411 have autocrine or paracrine regulatory functions [99].

412

413 The presence and potential significance of binucleated mammary epithelial cells in
414 the udder of the cow has already been discussed (see Section 3.2). Similarly, we have
415 observed binucleated luminal epithelial cells in both mammary alveoli and sinus-like
416 dilatations of the mammary milk ducts during lactation in the rabbit [89].

417

418 Limited studies have examined the mammary microenvironment in rabbits during
419 pregnancy and lactation. We have identified a population of CD3-positive T
420 lymphocytes that are present both in stromal clusters and also in intraepithelial foci,
421 suggesting exocytosis into the milk, and implying a contribution to the mucosal
422 immune system of the gland in this species [89]. We have also observed macrophages
423 in the rabbit mammary gland, particularly in association with impaired mammary
424 development tentatively linked with abdominal ectopic pregnancy [100]. Rabbits are
425 notable for being a species in which ectopic pregnancy is relatively frequently
426 reported [101] and we and others have observed cases of retarded mammary

427 development in rabbits that have a concurrent abdominal ectopic pregnancy [100,
428 102].

429

430 **5.3. Leporine post-lactational mammary gland involution**

431 By involution day 14 in the rabbit, mammary gland weight expressed as a percentage
432 of body weight is 0.5% and is similar to that recorded in the virgin animal.

433 Unfortunately the timing of induction of involution, if forced involution was initiated,
434 is unclear from these experiments [96]. In rabbits in which synchronous involution is
435 initiated by removal of the young at day 14 of lactation (day 0 involution), prolactin
436 receptor numbers decrease from 10 days of involution [103].

437

438 In other systems, rabbit mammary gland involution may proceed by means of natural
439 weaning (see Section 2.3). In this scenario, milk yield reduces from the fourth week
440 although does may continue to lactate for a further 2-4 weeks. It is noteworthy that
441 does may have a 'parallel pregnancy and lactation signature' at this time. Milk yield
442 peaks more rapidly and then drops more precipitously in female rabbits that are bred
443 soon after parturition than those in which breeding is delayed until 35-56 days after
444 parturition [91].

445

446 **6. Comparative mammary tumourigenesis in the sheep, cow, cat, and rabbit**

447 There are striking species differences in susceptibility to development of mammary
448 tumours. Mammary tumours are the third most common tumour type in cats [104,
449 105] (Fig. 3). In a recent survey 11% of neoplasms detected in European shorthaired
450 cats were of mammary origin, and of these, 97.3% were malignant [106]. This is in
451 concordance with a previous study suggesting that these tumours carry a poor

452 prognosis, with a malignant:benign ratio of 9:1 [107]. Classification of feline
453 mammary tumours [75, 108] and prognostic markers [109, 110] are considered
454 elsewhere and will not be described further here. Not dissimilar to cats, a recent
455 investigation in rabbits found mammary adenocarcinoma to be the second most
456 frequently diagnosed neoplasm [111].

457

458 By contrast, mammary tumours in sheep and cows are rare, even in populations of
459 animals attaining advanced age [112, 113]. Quantification of contemporary incidence
460 of mammary neoplasia in ruminants is challenging due to the scarcity of cases,
461 frequently limited to single case reports [113-117]. However, a large study of autopsy
462 and biopsy specimens from goats revealed interesting data. This survey of 1146
463 caprine specimens revealed that 100 goats that were presented with neoplasia
464 (totalling 102 neoplasms), and of these, 7 submissions (approximately 0.6% of all
465 submissions), comprised mammary neoplasia [118].

466

467 The purpose of this section is illuminate interesting facets of mammary
468 tumourigenesis in the species in question. The following sections will examine
469 potential reasons why mammary tumours are rare in sheep and cows (Section 6.1),
470 and the utility of feline and leporine mammary tumours as models of human breast
471 cancer (Sections 6.2 and 6.3.1 respectively).

472

473 **6.1. Mammary tumourigenesis in sheep and cows: a rare event**

474 As already stated (Section 6), mammary tumours in sheep and cows are rare.
475 Interestingly, reports in sheep document the occurrence of benign mammary
476 adenomas [115] and fibroadenomas [117], and low grade carcinomas known to have

477 been present for some time, suggesting slow clinical progression [113]. By contrast,
478 in cows mammary carcinomas metastatic to draining lymph nodes have been recorded
479 [114, 116] and historically reviewed [119] and in some cases, more distant metastatic
480 spread has also been noted [116, 119]. Primary mammary epithelial neoplasms in the
481 sheep and cow therefore cannot be assumed to have the same clinical behaviour and a
482 more malignant phenotype is tentatively suggested for a subset of bovine mammary
483 neoplasms.

484

485 The rarity of mammary neoplasms in ruminants raises the interesting broader question
486 regarding the reasons for variable susceptibility to cancer development in different
487 mammals. Peto's Paradox encompasses the notion that large and long-lived species
488 do not develop more neoplasms although dogma suggests that these mammals would
489 accrue a greater number of somatic mutational 'hits' and thus develop more tumours
490 [120]. Whilst mechanisms such as the evolution of multiple copies of the TP53 gene
491 in the elephant [121] partially account for this paradox, it remains poorly understood.

492

493 On possible explanation for the differential susceptibility of the mammary gland to
494 neoplastic transformation in different species is that the properties of mammary
495 stem/progenitor cells (MaS/PCs) vary in different species. Differences in MaS/PCs
496 between species may impact tumourigenesis because long-lived, self-renewing
497 MaS/PCs are considered to be a potential cell of origin for mammary neoplasia [122].

498

499 Bovine MaS/PCs have been historically described as "morphologically distinct pale-
500 staining cells" [123, 124]. When bromodeoxyuridine was administered to prepubertal
501 heifers, these cells accounted for approximately 40% of the bromodeoxyuridine

502 positive population in spite of comprising only 10% of the epithelial population [123].
503 Subsequently, a number of groups have used various techniques to attempt
504 identification and isolation of bovine MaS/PCs (reviewed in [125, 126]). More
505 recently, investigators have propagated non-adherent cells derived from bovine
506 primary mammary epithelial cell cultures as mammospheres and after 11 days of
507 selection have cultured cells derived from the mammospheres, a fraction anticipated
508 to be enriched in MaS/PCs [127]. At present the functional properties of ruminant
509 MaS/PCs that may confer reduced tumourigenic potential to the ruminant mammary
510 gland remain undetermined. There may also be other factors also contributing to the
511 apparent reduced tumour incidence in these species.

512

513 For women under 20 years old, having a full term pregnancy is associated with a
514 relative 'protective' effect against breast cancer equivalent to an approximately 50%
515 risk reduction [128]. It may be postulated that production animals undergoing a first
516 parity soon after puberty, and having multiple lactation cycles, may be subject to a
517 similar decrease in mammary tumour risk associated with the effect of a full term
518 pregnancy. This risk reduction may, for example, be the result of changes to the
519 MaS/PCs that occur in association with late pregnancy or parturition. However, other
520 authors have noted that the low incidence of mammary neoplasia appears to extend to
521 ruminants maintained in zoos and other environments where they are not used for
522 breeding, which would argue against this hypothesis [113].

523

524 A further consideration may be dietary: ruminants eat a high fibre, low fat diet
525 whereas small domestic carnivores like cats have a diet more similar in composition
526 to that of humans. Thus small domestic carnivores consume a diet that is not only

527 higher in fat but may also lead to accumulation of more dietary carcinogens through
528 the process of biomagnification [113, 129].

529

530 At present, the reasons for the rarity of mammary tumours in ruminants remain poorly
531 understood. It is possible that this phenomenon may be multifactorial and that all the
532 considerations described above are contributory factors.

533

534 **6.2. Feline mammary tumours as models for human breast cancer**

535 There has been longstanding recognition of the potential benefits of using feline
536 mammary tumours as models for particular breast cancer subtypes [130] and several
537 recent studies have further underlined this concept. Importantly, surrogate definitions
538 of the intrinsic subtypes of human breast cancer can be generated by
539 immunohistochemical measurements of oestrogen receptor, progesterone receptor,
540 Ki-67 and HER2 [131]. When this St. Gallen International Expert Consensus Panel
541 immunohistochemical classification of breast cancer subtypes was applied to feline
542 mammary carcinomas, the most common feline subtype was the luminal B-
543 like/HER2-negative subtype (almost 30% of cases). The second most common feline
544 subtype was the luminal B-like/HER2-positive group (approximately 20% of cases).
545 Subtypes were associated with differing clinical and histological features, and
546 survival times, and these associations frequently appeared to parallel those of human
547 breast cancer. For example, the longest survival time was recorded with the luminal A
548 subtype [132].

549

550 The breast cancer intrinsic basal-like subtype and its triple-negative surrogate
551 definition are not exact synonyms and show approximately 80% overlap [131]. Thus,

552 some triple-negative breast cancers are not basal-like and are in fact one of the other
553 intrinsic subtypes [133]. Similarly, in one study only 57% of feline triple-negative
554 carcinomas were basal-like [132]. However, it is likely that the genuinely basal-like
555 subgroup of the triple-negative feline mammary carcinomas may be a useful model
556 for triple-negative breast cancers in women [134]. Interestingly, a recent study has
557 demonstrated that the presence of large numbers of regulatory T cells, identified on
558 the basis of FoxP3 expression, is a negative prognostic indicator in feline triple-
559 negative mammary carcinomas [135]. The authors therefore suggested that these
560 tumours may be a useful model for the basal-like immune-suppressed subset of the
561 triple-negative basal-like breast cancer group. This will require further molecular
562 subtyping using a marker panel reflecting the accepted immunohistochemical profile
563 of the basal-like immune-suppressed subset [136].

564

565 Any consideration of a potential tumour model requires a balanced assessment of the
566 advantages and limitations. The cat is an attractive model, in part because cats
567 frequently share the same environment as their owners [137], in some cases exhibit
568 similar co-morbidities such as those associated with advanced age or obesity, and
569 develop mammary tumours spontaneously. However, many pet cats undergo
570 ovariectomy at a young age, and this important difference needs to be
571 considered in any evaluation of feline mammary tumours as a comparative model
572 [109]. In addition, in contrast to the case in women, cats may frequently be presented
573 with multiple mammary tumours and this causes difficulty in interpretation of results
574 and comparison with breast cancer [132].

575

576 **6.3. Rabbit mammary tumours**

577 Mammary tumours are regularly diagnosed in pet rabbits, particularly older non-
578 neutered females [138]. In one study examining rabbit surgical biopsy submissions to
579 a German veterinary diagnostic laboratory, mammary tumours comprised
580 approximately 20% of the submitted specimens [139]. The majority of these
581 neoplasms are carcinomas, with smaller numbers of benign neoplasms reported [90,
582 138, 139]. Recently, the presence of tumour infiltrating lymphocytes has been
583 described in pet rabbit mammary carcinomas, with higher numbers of stromal
584 lymphocytes present in central foci in neoplasms with a lower mitotic count and lower
585 histological grade [140].

586

587 There are several facets of mammary tumourigenesis in rabbits that merit special
588 mention. Several authors have presented evidence of the potential, in some but not all
589 cases, for a continuum of tumour development from benign lesions, through *in situ*
590 carcinomas, to overt carcinomas. Such evidence includes historic descriptions of a
591 progression of tumour development from a benign neoplasm to an invasive carcinoma
592 [141], the observation that benign neoplasms tend to be smaller and observed in
593 younger animals [139], and the description of non-neoplastic entrapped myoepithelial
594 cells within rabbit mammary carcinomas [142].

595

596 Interestingly, several morphological features of mammary carcinomas appear to be
597 potentially more common in rabbits than in cats. Some tumours exhibit a notable
598 degree of squamous differentiation leading them to be classified as adenosquamous,
599 and squamous differentiation is also prominent in some ductal carcinomas [90, 139].

600 A vacuolated lipid rich mammary carcinoma variant is also described, and is

601 postulated to reflect the high lipid content of rabbit milk [90, 139, 143, 144].

602

603 Rabbit mammary dysplasia or neoplasia may be influenced by hormones, particularly
604 prolactin and oestrogen [138]. Prolactin-secreting pituitary adenomas have been
605 reported in association with cystic mammary dysplasia in nine New Zealand white
606 rabbit does [145] and, in a separate report, with mammary hyperplasia, dysplasia, and
607 a cystic mammary adenocarcinoma [146]. Oestrogen receptor alpha and progesterone
608 receptor expression have been documented in some benign rabbit mammary lesions,
609 but it has been suggested that the majority of carcinomas do not exhibit expression of
610 these steroid hormone receptors [143]. However, this data could be consistent with a
611 role for steroid hormones acting as growth promoters, impacting early stages of rabbit
612 mammary tumourigenesis. Mammary tumours may also be diagnosed in rabbits that
613 are presented with uterine hyperplasia or adenocarcinoma, the latter a common
614 tumour in this species [138, 144].

615

616 **6.3.1. Rabbit mammary tumours as a model for human breast cancer**

617 We have documented the presence of sinus-like dilatations in the milk ducts of rabbits
618 subjacent to the teat. Given the similarities to the human breast, including the
619 presence of multiple galactophores per mamma, we have previously suggested that
620 the rabbit mammary gland may represent a useful alternative model for the breast
621 [89]. In parallel, other investigators have asserted that the rabbit may represent a
622 model for administration of mammary intraductal treatments [147] and that rabbit
623 mammary tumours may constitute a useful model for particular breast cancer subtypes
624 [144]. There is therefore a compelling need for further research into the biology of the
625 rabbit mammary gland, and particularly into the molecular characterization of rabbit
626 mammary tumours [144]. The use of spontaneously occurring mammary tumours

627 arising in pet rabbits as an additional breast cancer model is a potentially attractive
628 prospect that should not be overlooked. In this regard the case of mammary tumours
629 arising in house rabbits is particularly notable as these pets share a similar
630 environment to their owners.

631

632 **7. Conclusions and future perspectives**

633 Studying mammary gland biology in species such as the sheep, cow, cat and rabbit is
634 of dual interest. Firstly, mammary pathology arising in these species may severely
635 compromise animal welfare or constitute a cause for euthanasia. Concomitantly, in
636 farm animals mastitis may markedly affect productivity, with ensuing economic
637 implications. There are therefore compelling reasons to focus on mammary pathology
638 in these species. A further example of these species intrinsic drivers for research into
639 mammary gland biology would be the recognition that mammary involution or the
640 ‘dry period’ in dairy cows constitutes a period of milk accumulation within the udder
641 and thus a phase of increased risk of development of intramammary infections. One
642 focus of current research is whether acceleration of involution might be beneficial in
643 this species, balancing the need to reduce mastitis risk with the importance of the non-
644 milking period as a time of cellular renewal [148].

645

646 Beyond these species intrinsic drivers for mammary research, comparative mammary
647 biology promotes a wider awareness of similarities and differences between species in
648 terms of mammary development, microenvironment, and tumourigenesis. Such an
649 appreciation underpins the One Health narrative in which the study of non-traditional
650 model species increases our understanding of mammary development and disease in
651 humans and animals. As has been noted elsewhere, laboratory rodents provide

652 extremely tractable models, but there are important species differences between the
653 rodent mammary gland and the human breast [1, 3, 26]. The study of non-traditional
654 model species, particularly in the context of mammary development and
655 spontaneously occurring natural disease, may provide new insights that are
656 translatable to the human breast and are of benefit to both humans and animals.

657

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674

675

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1109 **Figure legends**

1110 **Figure 1. The ovine mammary postnatal developmental cycle.** Histological
1111 sections of mammary gland from a neonatal lamb (A), ewe in early lactation (B), ewe
1112 at point of lamb removal following a degree of natural weaning (C), and ewe 4 weeks
1113 post lamb removal (D). Note the clear demarcation between intra- and interlobular
1114 stroma in the neonatal lamb (arrowheads). A notable degree of post-lactational
1115 regression has occurred at the point of weaning, reflecting natural weaning in an

1116 agricultural system. Mineralised concretions (corpora amylacea) (asterisks) are a
1117 common feature of the ruminant mammary gland. Haematoxylin and eosin stain.
1118 Scale bar indicates 200 microns.

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1120 **Figure 2. Binucleated cells are present in the bovine mammary gland in late**
1121 **gestation.** Immunofluorescence staining for E-cadherin (magenta), α -smooth muscle
1122 actin (α -SMA; cyan), and DNA (DAPI; gold) in a bovine mammary gland at 250 days
1123 gestation (bovine gestation length is approximately 283 days). A likely binucleated
1124 luminal epithelial cell is indicated (arrow). Scale bar indicates 50 microns.

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1126 **Figure 3. Feline tubulopapillary carcinoma.** Immunofluorescence staining for E-
1127 cadherin (magenta), IBA1 (macrophages; cyan) (arrows), and DNA (DAPI; gold) in a
1128 feline tubulopapillary carcinoma. Arrowhead indicates papillary projections of
1129 neoplastic cells. Scale bar indicates 50 microns.

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