Exploring influenza A virus receptor distribution in the lactating mammary gland of domesticated livestock and in human breast tissue.

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ABSTRACT

The spread of the highly pathogenic avian influenza (HPAI) H5N1 virus among dairy cattle illustrates the adaptability of influenza A viruses (IAV) to infect non-traditional species. While IAV-specific sialic acid (SA) receptors have been identified in the mammary glands of dairy cattle, their presence in pigs, sheep, goats, and alpacas has not been studied until now. The zoonotic transmission of HPAI H5N1 to dairy and poultry farm workers during outbreaks raises public health concerns. This study employed lectin histochemistry to examine the mammary glands of livestock and humans. We found that these tissues were rich in SA α2,6-Gal receptors, followed by SA α2,3-Gal receptors, essential for IAV binding. Notably, the A(H5N1) clade 2.3.4.4b virus could bind to mammary tissue from both cattle and pigs. These findings highlight the potential for HPAI H5N1 to infect and spread within the mammary glands of production animals and humans.

Keywords: Sialic acid, α2,3-Gal, α2,6-Gal, H5N1, mammary gland, cattle, alpaca, sheep, goat, pig, human.

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INTRODUCTION

Since the first human case of highly pathogenic avian Influenza (HPAI) H5N1 was reported in 1997, there has been limited evidence to suggest human-to-human transmission. With spillover from birds to humans, there have been years in which the peak annual human cases worldwide reached <150 [1–4]. The case fatality ratios were close to 50%, raising serious public health concerns, albeit with low overall reported case numbers [2,4,5]. Interestingly, the current outbreak of HPAI H5N1 in dairy cattle and poultry in the U.S. has resulted in numerous spillovers into farm workers handling HPAI H5N1-affected dairy herds and poultry flocks. As of March 7, 2025, there have been 70 CDC-confirmed cases, with one reported death associated with HPAI H5N1 [6,7]. Influenza A viruses are known to replicate well in the respiratory and intestinal tract of animals and humans [8,9]. However, the continued HPAI H5N1 viral replication in the mammary gland of novel species like dairy cattle [10,11] with a combination of continued spillover into numerous naive mammalian species [12], raises the concern of HPAI H5N1 virus adaption to mammalian species with a possibility of continued mammal-to-mammal and even human-to-human spread.

To assess the current feasibility of HPAI H5N1 viral replication within the mammary glands of other exposed animals, this study aimed to delineate the spatial distribution of influenza A virus (IAV) specific sialic acids (SA) receptors in the mammary glands of various production animals: pigs, sheep, goats, cattle, and alpacas in comparison to human breast tissue. Due to recent spillovers from production animals into people, this study also examined human breast tissue.

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The mammary gland is a remarkable structure responsible for producing milk, a nutrient-rich fluid that nourishes. The importance of the mammary gland cannot be overstated, as it ensures species continuity and offspring health and development. It also contains antibodies that help to protect the young from infections [13,14]. Most humans depend on milk, or its byproducts, for this highly nutritious fluid that contains essential proteins, carbohydrates, fats, vitamins, and minerals [15]. The mammary gland's structure is complex and consists of a network of alveolar lobules interconnected by ducts and supported by connective, adipose, and smooth muscle tissues (Figure 1). The lobules are responsible for milk production, while the ducts transport the milk to the cisterns for suckling. The mammary gland undergoes significant hormonal and metabolic changes during pregnancy and lactation, adapting to the demands of milk production and the growth of the fetus [16]. The milk composition can vary among different species, reflecting the specific needs of their offspring [17].

However, the mammary gland is not immune to infections. Pathogens, such as bacteria, viruses, and fungi, can invade the mammary gland and cause mastitis, a painful and debilitating condition. Interestingly, retrograde infection is the most common route or method of infection, where microorganisms enter through the teat opening and into the canal and cistern [18]. In dairy cows, mastitis in animals can lead to decreased milk production, reduced quality, and even death in severe cases [19]. The infection can spread to other animals through contaminated equipment or by direct contact, posing a significant threat to the health of an entire herd [20]. The consequences of mastitis are far-reaching. In addition to causing pain and suffering for the affected animal, mastitis can have a negative impact on the economic viability of livestock

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production. This includes reduced milk production, increased veterinary costs, and the potential for culling infected animals, which can result in significant financial losses for farmers. Moreover, mastitis can lead to transmitting pathogens to humans through contaminated unpasteurized milk, posing a public health risk.

The recent discovery of HPAI H5N1 clade 2.3.4.4b virus in the mammary gland and milk of dairy cattle sets a new scientific precedent, emphasizing the adaptability of H5N1 HPAI viruses to non-avian species and its potential for cross-species transmission, including to humans. A key factor in the susceptibility to HPAI H5N1 infection is the presence of specific host cell receptors that the virus uses for entry [21]. Influenza A viruses utilize host SA for initial attachment and entry into cells [22]. Sialic acids are also potential receptors or co-receptors for other viral families such as *Coronaviridae* [23], *Paramyxoviridae*, *Flaviviridae* [24], *Picornaviridae* [25], *Reoviridae* [26], *Parvoviridae* [27], *Adenoviridae* [28], *Papillomaviridae* [29], *Polyomaviridae* [30], and *Caliciviridae* [31]. In addition, many bacteria, fungi, and parasites exploit sialic acids as growth substrates for immune evasion, host cell entry, and enhanced virulence, including those that cause mastitis, such as *E. coli*, *Streptococcus sps*., *Pasteurella sps*., *Candida sps*., *Aspergillus fumigatus*, *Leishmania* and *Trypanosomes* [32–36].

Also sialic acids are also one of the major components of milk and play an important role in human nutrition, particularly in brain development [37,38]. Cattle and goat milk predominantly contain sialic acid forms N-glycolylneuraminic acid (Neu5Gc) versus N-acetylneuraminic acid (Neu5Ac), and their content decreases with the progression of the lactation stage [39,40]. Also, sialic acid levels vary depending on the diet of the

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140 animal and have been shown to regulate the microbiota-gut-mammary axis during
141 mastitis of Holstein cows [41]. In addition, Neu5Ac was also one of the key metabolites
142 that was found to be elevated in metastatic breast tumors in humans [42]. Thus,
143 suggesting that SA levels may vary depending on the lactational stage, diet, and
144 underlying disease conditions.

145

146 Therefore, understanding the sialic acid interactions in a complex organ like a
147 mammary gland with frequent interactions with microbial and hormonal changes
148 during the lactational stage is important.

149

150 **MATERIALS AND METHODS**

151 ***Sample collection***

152 This study was approved by the Institutional Animal Care and Use Committee
153 (IACUC) of the College of Veterinary Medicine, Iowa State University, Ames, Iowa
154 (IACUC-24-090). In the case of the human mammary gland, consultation and
155 approvals from ISU and GMU [Institutional Review Board approval (GMU IRB#
156 478170 and 477703)] were obtained before using the archived tissue blocks. Three
157 animals of each species studied were procured from the Iowa State University
158 Research farms (sheep, goats, beef cattle) from approved vendors (alpacas), while
159 archived tissues for the rest (Table 1). Following euthanasia, formalin-fixed and
160 paraffin-embedded sections of the mammary gland from the area of the teat cistern,
161 gland cistern, interlobular (collecting) duct, and secretory alveoli (glandular
162 epithelium) were collected from these animals (Figure 2).

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163 ***Lectin histochemistry***

164 Mammary tissues were characterized for sialic acids using lectin histochemistry assay
165 as previously described [43], for chromogenic and fluorescent staining. Briefly,
166 formalin-fixed, paraffin-embedded tissue sections of the mammary gland were
167 sectioned at 4 µm with placement on Superfrost® Plus slides (VWR International).
168 Slides were dried at 60°C for 20 minutes preceding deparaffinization and staining on
169 Roche Diagnostics' Ventana DISCOVERY ULTRA research platform. Heat retrieval
170 was accomplished via Cell Conditioning (CC1) solution at 100°C for 24 minutes
171 (Roche Diagnostics Corporation). Endogenous peroxidase was quenched for
172 chromogenic staining using Discovery Inhibitor (Roche Diagnostics Corporation).
173 Slides were then blocked with 1X Carbo-Free blocking solution for 32 minutes
174 (Vector Laboratories Inc.), followed by a Streptavidin/Biotin Blocking Kit (Vector
175 Laboratories Inc.) with a separate application each of 12 minutes. The sections were
176 incubated for 4 hours (fluorescent)/ 1 hour (chromogenic) at room temperature (RT)
177 with one of the three lectins (SNA, MAL-I, MAL-II) from Vector Laboratories at the
178 listed concentration in Table 2. For SA α2,6-Gal/GalNAc, slides were incubated with
179 biotinylated SNA for both fluorescent. A cocktail of fluorescein-labeled MAL-I
180 (specific for N-linked or O-linked glycans with SA α2,3-Galβ (1-4) GlcNAc) and
181 biotinylated MAL-II (specific for O-linked glycans with SA α2,3-Galβ (1-3) GalNAc)
182 were used for fluorescent staining. Biotinylated MAL-I was used separately for
183 chromogenic staining. Following lectin incubation, streptavidin conjugated with
184 Alexa Fluor 594 (ThermoFisher Scientific) was applied and incubated for two hours
185 at RT. In the case of chromogenic staining, streptavidin-HRP RTU (Vector
186 Laboratories Inc.) was incubated for 40 minutes on all three biotinylated lectins. The
187 chromogenic signal was further amplified with DISCOVERY Amplification anti-HQ

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188 Multimer (Roche Diagnostics Corporation). Fluorescent counterstaining was
189 performed with QD DAPI RUO (Roche Diagnostics Corporation) and mounted
190 manually with Prolong Gold antifade mountant without DAPI (ThermoFisher
191 Scientific). Chromogenic counterstaining was performed with hematoxylin and bluing
192 (Roche Diagnostics Corporation), coverslipped with Sakura Tissue-Tek Film.
193 Negative assay controls consisted of primary lectin or antibody omission. Positive
194 lectin assay controls were performed on porcine tissues as lectin labeling has been
195 previously established [44]. Slides were examined and imaged using a BX-53 Olympus
196 trinocular microscope equipped with an Olympus DP23 camera, Excelitas X-Cite
197 mini+ compact illumination system, and CellSyns Dimension software.

198 ***Virus preparation, inactivation, and labeling for virus histochemistry***

199 A reverse engineered A/bald eagle/Florida/W22-134-OP/2022 (H5N1; rgA/bald
200 eagle/FL/22) that contained an engineered low pathogenicity 2.3.4.4b H5 by removal
201 of the polybasic cleavage site, wild type N1, and 6 internal segments from A/Puerto
202 Rico/8/1943 (H1N1) was kindly provided by Richard Webby from St. Jude Children's
203 Research Hospital. Confluent layers of Madin-Darby canine kidney (MDCK) cells
204 were inoculated with LPAIV H5N1 2.3.4.4b rgA/bald eagle/FL/22. After 48 hours,
205 cell culture flasks went through two rounds of freeze-thaw cycles at -80°C. After the
206 second cycle, the supernatant was harvested and cleared by low-speed centrifugation.
207 The cleared supernatant was then centrifuged at 28,000 rpm in an SW32i rotor at 4°C
208 for 2 hours on a layer of 10% sucrose. Pellets were resuspended using PBS buffer and
209 measured for hemagglutination activity (HA) by 2-fold serial dilution with turkey red
210 blood cells. The virus was then incubated at room temperature with an equal volume
211 of 10% neutral buffered formalin for 1 hour. The inactivated virus was then dialyzed
212 against PBS. Inactivation was confirmed on MDCK cells, and an HA was performed

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213 to determine viral titer. The inactivated virus was labeled by mixing with an equal
214 volume of 0.1mg/ml fluorescein isothiocyanate (FITC) (Sigma-Aldrich) in 0.5 M
215 bicarbonate buffer (pH 9.5) for 1 hour 4°C with constant agitation. The labeled virus
216 was then dialyzed against PBS, and a final HA titer was determined.

217 ***Viral histochemistry***

218 Virus histochemical staining was performed on sections of lactating Holstein dairy
219 cattle and lactating cross-bred sows. Tissues were deparaffinized with xylene and
220 rehydrated through a series of graded alcohol solutions. A 3% hydrogen peroxide
221 solution (Fischer Bioreagents) was used to quench endogenous peroxidases. Slides
222 were then washed in TNT buffer (Tris (ThermoFisher Scientific), sodium chloride
223 solution (Sigma-Aldrich), and Tween20® (Sigma-Aldrich)). Slides were then
224 immersed in a blocking solution for 1 hour at room temperature (TNT buffer plus
225 blocking reagent, Akoya Biosciences). Slides were incubated overnight at 4°C in a
226 humidified chamber with FITC-labeled rgA/bald eagle/FL/22 (200 HA units per 50
227 µl). Tissues were then incubated in peroxidase-labeled rabbit anti-FITC antibody
228 (Agilent Technologies) followed by signal amplification through a biotin tyramide
229 amplification system (Akoya Biosciences). Finally, peroxidase was revealed with 3-
230 amino-9-ethyle-carbazole (AEC, ThermoFisher Scientific), and counterstaining was
231 performed using hematoxylin stain solution (Epredia) and ammonia hydroxide
232 solution (Sigma-Aldrich). A sialidase control was run on sow tissue sections. Sections
233 were pre-treated with 0.4 U/ml sialidase-A (Agilent Technologies) in 50 mM sodium
234 phosphate buffer (pH 6.0) overnight at 37°C and subjected to the protocol as outlined
235 above. Intestinal tissues from a chicken with and without the omission of the FITC-
236 labeled virus were used as positive and negative controls, respectively. Slides were

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237 examined and imaged using a BX43 Olympus microscope equipped with an Olympus
238 DP28 camera. Photomicrographs were captured using cellSens Standard.

239 **RESULTS**

240 The results were broadly classified into teat and gland cistern and interlobular duct
241 and secretory alveoli regions and summarized in Table 3.

242 ***Lectin labeling in dairy, beef, non-lactating cattle, sheep and goat***

243 In the ruminants (cattle, sheep, and goats), the epithelial lining of the teat (Figure 3,
244 Panel Ai-Av) and gland cistern (Figure 3, Panel Bi-Bv) showed multifocal to diffuse,
245 moderate positive labeling of SA α2-6 receptors (SNA) (Figure 3, Panel Ai-Av, Bi-
246 Bv). This labeling continued into the interlobular duct (Figure 3, Ci-Cv) and secretory
247 alveoli (Figure 3, Di-Dv) with mild to moderate intensity. There was a multifocal
248 labeling of MAL II on the epithelial lining of the teat (Figure 3, Ei-Ev) and gland
249 cistern (Figure 3, Fi-Fv). In the interlobular duct, MAL II labeling was rare,
250 multifocal, and minimal to mild in intensity (Figure 3, Gi-Gv), while on the surface of
251 secretory alveoli, the labeling was diffuse and moderate to marked in intensity (Figure
252 3, Hi-Hv). Interestingly, in nonlactating cattle, the intensity of MAL II labeling was
253 comparatively low from the gland cistern (Figure 3, Fiii), and it continued to decrease
254 in the interlobular duct and secretory alveoli (Figure 3, Giii and Hiii). Overall, the
255 apical labeling intensity was lower for MAL II than for SNA. The spatial distribution
256 of these receptors was apical on the epithelial cell surface and uniform across all
257 animals examined of each species.

258 ***Lectin labeling in alpacas, pigs, and humans***

259 Overall, the alpaca showed a similar SNA/MAL II distribution pattern to ruminants,
260 but the labeling intensity along the epithelial lining of teat cistern, gland cistern,

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261 interlobular ducts, and secretory alveoli (Figure 4, Ai-Di and Ei-Hi), was
262 comparatively more. This labeling on the epithelial lining is multifocal (teat cistern)
263 to diffuse (all other tissues), intense, and apical from teat cistern to secretory alveoli.
264 Both SNA and MAL II labeling was observed in all anatomic regions examined in the
265 closely related monogastric species pigs and humans. In pigs, the epithelial lining of
266 the teat cistern, gland cistern, interlobular ducts, and secretory alveoli displayed
267 diffuse apical labeling of moderate to marked intensity for both SNA and MAL II
268 (Figure 4, Aii – Hii). In humans, teat and gland cistern are absent hence, no images
269 were captured (Figure 4, Aiii, Biii, Eiii, and Fiii). However, there was multifocal to
270 diffuse apical epithelial labeling in the interlobular duct and secretory alveoli of
271 moderate intensity for both SNA and MAL II (Figure 4, Ciii and Diii, Giii, and Hiii)
272 in four human specimens tested.

273 ***MAL I lectin labeling in ruminants and non-ruminants***

274 The labeling of MAL I, which is specific for N-linked or O-linked glycans with SA
275 α2,3-Galβ (1-4) GlcNAc) varied across tissue regions of mammary glands and species
276 evaluated. In the teat and gland cistern of dairy and beef cattle, sheep, and goat, the
277 labeling was multifocal to diffuse and moderately positive compared to occasional
278 positive cells along the epithelial lining in non-lactating dairy cattle (Figure 5, Ai-Av
279 and Bi -Bv). The labeling along the epithelial lining of the interlobular duct and
280 secretory alveoli was weak positive with occasional focal labeling among all
281 ruminants, again with non-lactating dairy cattle showing poor or no labeling (Figure
282 5, Ci-Cv and Di -Dv).

283 In alpaca and pig, the teat and gland cistern MAL I labeling was multifocal (Figure 5,
284 Ei- Eii and Fi-Fii). Alpacas had multifocal MAL I labeling in interlobular ducts, while
285 pigs had continuous apical labeling (Figure 5, Gi and Gii). Like ruminants, MAL I

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286 labeling along the epithelial lining of the secretory alveoli was weak or had no
287 labeling in alpacas and pigs (Figure 5, Hi and Hii). Meanwhile, human samples
288 showed continuous apical labeling of MAL I in the secretory alveoli (Figure 5, Hiii).

289 All samples were further tested using chromogenic labeling to confirm the MAL I
290 labeling. This was done because the labeling for MAL I seemed to be less than for
291 SNA and MAL II. Therefore, we wanted an additional assay to confirm our MAL I
292 IFA findings. In lactating dairy cattle, multifocal labeling was seen in the teat cistern
293 (Figure 6, Ai), with minimal to no labeling in other examined areas (Figure 6, Bi-Di).
294 In beef cattle, there was multifocal to diffuse labeling in the teat cistern, gland cistern,
295 and interlobular duct (Figure 6 Aii-Cii). No labeling was seen in the secretory alveoli
296 (Figure 6m Dii). In non-lactating cattle, minimal to no labeling was noted across all
297 anatomic locations examined (Figure 6, Aiii-Diii). For sheep and goats, there was
298 multifocal to diffuse labeling in the teat cistern, gland cistern, and interlobular duct
299 (Figure 6m Aiv-Civ, Av-Cv), but no labeling in the secretory alveoli in either species
300 Figure 6 Div and Dv). In alpacas, there was multifocal to diffuse labeling for MAL I
301 in all examined areas (Figure 6, Ei-Hi). Conversely, pigs only had MAL I labeling in
302 the teat cistern (Figure 6, Eii). The only examined tissue for humans in this case, the
303 interlobular duct, had diffuse apical labeling (Figure 6, Giii).

304 ***Virus histochemistry – ruminant (dairy cattle) and non-ruminant (pig)***

305 Viral receptors, as detected by virus histochemistry, were identified at the apical
306 epithelial surface in the interlobular ducts and secretory alveoli of both lactating
307 Holstein cows and crossbred sows. The distribution of receptors and labeling intensity
308 in the interlobular ducts of lactating Holstein cows varied within individual animals
309 (Figures 7A and 7B). Labeled cells of interlobular ducts commonly had a basilar
310 nucleus and apical cytoplasm. Cells with minimal to no labeling were commonly

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311 attenuated or not organized as a stratified cuboidal epithelial layer (Figure 7A and
312 7B). Intra-animal variation was also present in secretory alveoli, with some alveoli
313 having circumferential intense labeling adjacent to secretory alveoli with no to
314 minimal labeling to more consistent labeling of one or more epithelial cells and
315 luminal secretions of adjacent alveoli (Figure 7C and 7D). In crossbred sows, viral
316 receptors were commonly identified on many of the epithelial cells lining interlobular
317 ducts, one or more epithelial cells lining secretory alveoli, and luminal secretions of
318 adjacent alveoli (Figure 7E and 7F).

319 **DISCUSSION**

320 As HPAI H5N1 continues to enter farms in the U.S., infecting several animals and
321 causing human spillover events [7,45], this study aims to assess the risk of HPAI
322 infecting the mammary glands of other domestic species and humans. There is a
323 potential health risk for farm workers, and there are concerns that the virus could
324 appear in other milk-associated products, such as goat milk or cheese, similar to the
325 presence of HPAI H5N1 viral RNA in dairy products [46]. In addition, many backyard
326 farms throughout the U.S. practice mixed animal rearing, and a potential virus entry
327 into these farms may allow for interspecies transmission, as evidenced in Oregon,
328 USA [47], with the potential to mutate or reassort in these animal species. Influenza A
329 virus infections are well known to affect the respiratory system, and it is the primary
330 site of viral replication and subsequent pathology. But in March of 2024, the detection
331 of HPAI H5N1 in the mammary glands of cows was indeed surprising. Identification
332 of high levels of virus RNA in the milk and the mammary gland epithelial cells of
333 infected dairy cattle suggested the mammary gland as a predilection site for HPAI
334 H5N1 clade 2.3.4.4b replication in this species [43,48]. These observations have further
335 been confirmed through experimental studies [10,11]. In these challenge studies, cows

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336 inoculated with HPAI H5N1 clade 2.3.4.4b viruses in the udder showed viral mastitis
337 with epithelial necrosis within the secretory alveoli. Sequelae included exposure of
338 the basal laminae and myoepithelial cells or replacement of epithelial cells with
339 fibrous connective tissue [10,11].

340

341 One of the factors contributing to virus attachment and replication is SA, which is
342 abundantly expressed in cow mammary gland epithelium, in particular, O-linked
343 glycans with SA α2,3-Galβ (1-3) GalNAc, which has more affinity towards IAVs of
344 avian origin [43]. To the current dairy cattle outbreak, the relative abundance of both the
345 avian and mammalian type influenza receptors on the surface of secretory epithelial
346 cells in the mammary glands of dairy cattle most likely allowed for viral binding and
347 entry of HPAI H5N1 2.3.4.4b into this novel anatomic area [43]. Therefore, comparing
348 the spatial distribution of these SA receptors in the mammary glands of ruminants and
349 non-ruminant species, including humans, can help assess viral attachment, entry, and
350 risk of replication in this tissue. Pathogens usually gain entry to the mammary gland
351 through the teat canal and spread through the ducts or can be systemic and attach to
352 the host cells with appropriate receptors. In this study, we looked at the SA along the
353 epithelial lining of the mammary gland, starting from the teat canal to the alveoli
354 lobules, where milk secretion occurs (Figure 1).

355

356 Although several studies have looked at the distribution of SAs in humans, pigs,
357 cattle, sheep, goat, and camel respiratory tracts [49,50], to our knowledge, this is the first
358 study to compare these receptors in the mammary glands across these species. In
359 alpaca, testis, and placental epithelial cells, both SA α2,3-Gal and SA α2,6-Gal were

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360 present [51]. In addition, sialic acid receptors are associated with triggering early host
361 immune responses such as cell death signaling (necrosis/apoptosis) [52]. Therefore, it is
362 of utmost priority to evaluate the predisposing factors responsible for this new affinity
363 of IAV towards mammary gland epithelium and the consequences it may have for
364 mammalian species, including humans.

365

366 The current study showed that the mammary tissues from ruminants (cattle, sheep,
367 and goat) and non-ruminants (alpaca, pig, and human) labeled SA α2,3-Gal and SA
368 α2,6-Gal receptors along the mammary epithelium, suggesting that both mammalian
369 and avian IAVs have potential to bind. The SA α2,3-Gal spatial distribution identified
370 using MAL-I specific for N-linked or O-linked glycans with SA α2,3-Galβ (1-4)
371 GlcNAc and MAL-II specific for O-linked glycans with SA α2,3-Galβ (1-3) GalNAc
372 lectin labeling, was particularly interesting because of poor MAL I labeling along the
373 epithelium (teat and gland cistern) of non-lactating cattle (Figure 5, Aiii and Biii)
374 compared to lactating cattle. A recent study in *ex vivo* explant cultures of bovine
375 mammary gland showed enhanced HPAI H5N1 (A/dairycattle/Texas/24-008749-
376 001/2024) viral replication in the teat and gland cistern compared to chicken H5N1
377 belonging to the same 2.3.4.4b clade [53]. Studies in mice showed selective blocking of
378 SA α2,3-Gal associated with MAL I showed a reduction in viral replication and
379 transmission efficiency in contact mice [54]. Therefore, it is possible that N-linked or O-
380 linked glycans with SA α2,3-Galβ (1-4) GlcNAc (MAL I) may play an important role
381 in viral attachment and replication. In addition, there is also variability in lectin
382 binding studies in the mammary gland of cattle both from the studies performed by
383 our team and others [43,53,55]. Further infectious and glycan biology studies are needed

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384 to confirm the role of MAL I during HPAI H5N1 viral replication in lactating dairy
385 cattle.

386

387 This study also compared the spatial distribution of SAs in human breast tissue and
388 showed the presence of SA α2,3-Gal and SA α2,6-Gal receptors, which agrees with
389 recent findings [56]. Their study further demonstrated that the proteins from HPAI
390 H5N1 (/Texas/37/2024) bound to human mammary gland epithelium but human
391 H1N1 and H3N2 do not bind to this tissue. The potential for HPAI H5N1 to bind to
392 this tissue and possible replication could lead to viral mastitis, complications due to
393 unknown etiology, breastfeeding complications, inflammation, pain, and discomfort,
394 compromising both maternal and neonatal health. Hence, there is an immediate
395 urgency to understand the consequences of HPAI H5N1 in human breast tissues.

396

397 The LPAIV H5N1 2.3.4.4b rgA/bald eagle/FL/22 was able to bind the apical surface
398 of a majority of interlobular duct epithelium and a subset of epithelial cells ling
399 secretory alveoli using virus histochemistry studies. Both structures appeared to have
400 more consistent viral binding in the sow mammary gland than the cow mammary
401 gland, suggesting that while not all cells are similarly susceptible to viral attachment,
402 the sow mammary gland may contain a higher percentage of susceptible cells. The
403 virus binding pattern partially agrees with multifocal labeling SA α2,3-Gal receptors
404 (Table 3; Fig 3-6). However, further co-localization studies are required to determine
405 the specificity. Pigs are susceptible to HPAI H5N1 2.3.4.4b virus strains, but whether
406 the virus can successfully travel toward the oronasal cavity of the sow or a suckling
407 piglet to the mammary gland is unknown [57]. Transmission of 2009 H1N1 IAV-

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408 inoculated kits to the dam's mammary gland has occurred [58]. The presence of H5
409 receptors on the sow mammary gland suggests that attachment and replication may
410 also be a possibility in the sow mammary gland. Endemic swine-adapted H1 and H3
411 IAV infection is not uncommon in suckling piglets. Yet, associated mastitis in sows
412 has not been documented, either due to the inability of H1 or H3 IAV to cause
413 mastitis or due to a combination of low prevalence, mild disease, and/or difficulty
414 identifying mastitis in sows as mammary glands are not mechanically milked and not
415 routinely evaluated without overt clinical illness. Endemic swine H1 and H3 virus
416 histochemistry on sow mammary tissue is beyond the scope of this study.

417

418 While virus histochemistry may provide a more accurate evaluation of the presence
419 and distribution of viral receptors than lectins or recombinant HA proteins, it has
420 limitations. The duration of tissue fixation, tissue autolysis, and the type of fixative
421 may alter results. In this study, livestock tissues were collected shortly after
422 euthanasia and formalin-fixed for less than 48 hours. Additionally, the LPAIV H5N1
423 2.3.4.4b rgA/bald eagle/FL/22 virus was cultivated in MDCK cells. While the impact
424 of variable virion shapes and sizes on AIV binding to receptors is not yet completely
425 known, a virus originating from cell culture rather than a clinical specimen could
426 impact virus binding and interpretation [59]. In addition, the current study also showed
427 intra-animal variability in the distribution of viral receptors based on virus
428 histochemistry in lactating Holstein dairy cattle, suggesting that not all cells are
429 equally susceptible to viral attachment and, therefore, infection. This variable in
430 susceptibility is also supported by the finding that not all alveoli in inoculated animals
431 are infected or affected by IAV and, at some level, may also contribute to the

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432 variability in the clinical disease reported from animals in the field and lesion severity
433 of inoculated animals [10].

434

435 Manifestation of clinical disease is multifactorial, and while this study evaluated more
436 Holstein dairy cattle than prior publications, it did not actively evaluate Holstein dairy
437 cattle at different stages of lactation or known pathologic conditions that may
438 contribute to differential glycan expression [39,40]. The observation that epithelial cells
439 of probable different maturation vary in labeling by virus histochemistry supports
440 investigating differential glycan expression across different pathologic and
441 physiological states to elucidate host factors that could influence susceptibility.

442 **CONCLUSION**

443 Based on these findings, we suggest IAVs have the potential to bind to the mammary
444 glands of cattle, sheep, goats, alpacas, pigs, and humans. Further investigation into the
445 possible sustained IAV replication in the mammary glands of these species, both in
446 experimental and natural environments, needs to be performed. Meanwhile, routine
447 milk or milk product testing from these species, together with continued surveillance
448 of the human agricultural interface, will aid in containing the virus spread,
449 establishing mitigation strategies, and understanding evolutionary changes in IAV and
450 their interactions with different species.

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59. Partlow EA, Jaeggi-Wong A, Planitzer SD, Berg N, Li Z, Ivanovic T. Influenza A virus rapidly adapts particle shape to environmental pressures. Nature Microbiology 2025 10:3 [Internet]. Nature Publishing Group; 2025 Feb 10 [cited 2025 Mar 3];10(3):784–794. Available from: https://www.nature.com/articles/s41564-025-01925-9

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Table 1. Samples collected

	Breed	**Age**
Dairy Cattle	Holstein Friesian	multiparous
Beef Cattle	Simmetal/Angus cross	5-9 years old
Non-lactating heifer calves	Holstein/Angus cross	14-16 weeks
Sheep	Polypay composite	2-6 lactations
Goat	American alpine	2-6 freshenings/lactations
Alpaca	Brown and Light fawn	2-8 cria/lactations
Pig	Large White and Yorkshire cross	parity 2 or greater
Human*	De-identified	De-identified

* one normal control breast tissue slide from a commercial vendor (Mopec, Madison Heights, MI, USA; Cat no. SC936) and three formalin-fixed paraffin-embedded (FFPE) archived breast tissues collected with informed consent from George Mason University provided de-identified.

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Table 2. Lectin histochemistry and Viral binding assay reagents.

	Conjugate	Working Concentration	Manufacturer	Product number
Lectin Staining				
Maackia amurensis -I	Fluorescein	20 µg/ml	Vector Laboratories	FL-1311-2
Maackia amurensis -I	Biotinylated	20 µg/ml	Vector Laboratories	B-1315-2
Maackia amurensis -II	Biotinylated	4 µg/ml	Vector Laboratories	B-1265-1
Sambucus nigra	Biotinylated	20 µg/ml	Vector Laboratories	B-1305-2
Streptavidin	Alexa Fluor-594	2 µg/ml	ThermoFisher Scientific	S11227
Streptavidin	Horseradish Peroxidase (HRP) - R.T.U	1mg/ml	Vector Laboratories	SA-5704-100
Virus histochemistry				
Fluorescein Isothiocyanate isomer (FITC)	none	0.1 mg/ml	Sigma-Aldrich	3326-32-7
Polyclonal Rabbit Anti-FITC antibody	HRP	20 µg/ml	Agilent Technologies	P5100
Biotin tyramide Amplification System	Streptavidin conjugated-HRP	10 µg/ml	Akokya Biosciences	NEL700A001KT
3-amino-9-ethyle-carbazol (AEC) Substrate Chromogen	none	Stock	ThermoFisher Scientific	001122

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Table 3. Sialic acid distribution in the mammary glands of ruminants, monogastric, and pseudo-ruminant/camelid.

Mammary gland regions	**MAL I (SA α2,3-Galβ (1-4) GlcNAc)**	**MAL II (SA α2,3-Galβ (1-3) GalNAc)**	**SNA (SA α2,6-Gal/GalNAc)**
Ruminants (Goat/Sheep/Dairy/ Beef cattle)			
Teat Cistern	+ (apical; m/f)	+ (apical; m/f)	+ (apical; d)
Gland Cistern	+ (apical; m/f)	+ (apical; m/f)	+ (apical; d)
Interlobular (Collecting) Duct	+ (apical; * m/f)	+ (apical; * m/f)	+ (apical; d)
Secretory Alveoli (glandular epithelium)	+/– (apical; * m/f)	+ (apical;(m/f) * to d)	+ (apical; d)
Non-ruminant monogastric (Pig/Human)			
Teat Cistern	+ (apical; m/f)/DNE	+ (apical; d)/DNE	+ (apical; d)/DNE
Gland Cistern	+ (apical; m/f)/DNE	+ (apical; d)/DNE	+ (apical; d)/DNE
Interlobular (Collecting) Duct	+ (apical; m/f)/DNE	+ (apical; d)/ + apical; m/f	+ (apical; d)/+apical; m/f
Secretory Alveoli (glandular epithelium)	+/– (apical; m/f)/ + apical; d	+ (apical; d)/+ apical; m/f	+ (apical; d)/+ apical m/f
Pseudo-ruminant/camelid (Alpaca)			
Teat Cistern	+ (apical; m/f to d)	+ (apical; m/f)	+ (apical; d)
Gland Cistern	+ (apical; m/f to d)	+ (apical; d)	+ (apical; d)
Interlobular (Collecting) Duct	+ (apical; m/f to d)	+ (apical; d)	+ (apical; d)
Secretory Alveoli (glandular epithelium)	+ (apical; m/f to d)	+ (apical; d)	+ (apical; d)

830 Data representative of n = 3 for all animal species; n= 4 for humans. + denotes positive labeling; – no or minimal labeling; apical means labeling

831 lectins on the epithelial lining; m/f = multifocal labeling; d=diffuse labeling; * denotes non-lactating cattle; DNE = did not examined.

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832 **FIGURE LEGENDS**

833 **Figure 1: Illustration of sagittal section anatomy of the mammary gland of a cow.**

834 Inset image showing the microscopic anatomy of alveolar lobules with secretory
835 epithelial cells, myoepithelial cells, and milk secretions.

836

837 **Figure 2: Cross-sectional gross anatomy of the mammary gland of a cow.**

838 Lines indicate representative areas where samples were collected for histological
839 studies.

840

841 **Figure 3: Distribution of sialic acids in the mammary gland of ruminant species.**

842 The mammary glands of dairy cattle, beef cattle, nonlactating cattle, sheep, and goats
843 showing *Sambucus nigra lectin* (SNA, purple pseudocolor, Alexa Fluor 594) and
844 *Maackia amurensis lectin-II* (MAL-II, red pseudocolor, Alexa Fluor 594), using
845 fluorescent labeling.

846 The labeling described is in reference to the epithelial surface of each image. In
847 images Ai-Cv, there is apical, multifocal to diffuse SNA labeling in the teat cistern,
848 gland cistern, and interlobular duct of ruminants. In Di - Dv, there is positive, diffuse,
849 apical labeling for SNA of the epithelial surface of secretory alveoli.

850 In images Ei-Fv, there is multifocal labeling of MAL II on the epithelial lining of the
851 teat and gland cistern. In Gi-Gv, MAL II labeling was rare, multifocal, and minimal to
852 mild, with the exception of the nonlactating cow in Giii, which showed minimal
853 appreciable labeling for MAL II. In Hi-Hv, there was diffuse MAL II labeling of the
854 epithelial surface of secretory alveoli, again with the exception of the nonlactating

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855 cow in Hiii, which also showed minimal appreciable labeling for MAL II. However,
856 the apical labeling intensity was less for MAL II than for SNA labeling. Arrows
857 indicating the labeling on the epithelial lining. Scale bar =50 µm.

858

859 **Figure 4: Distribution of sialic acids in the mammary gland of non-ruminant species.**

860 The mammary glands of alpaca, pig, and human showing *Sambucus nigra lectin*
861 (SNA, purple pseudocolor, Alexa Fluor 594) and *Maackia amurensis lectin-II* (MAL-
862 II, red pseudocolor, Alexa Fluor 594), using fluorescent labeling.

863 In micrographs, Ai-Di, the alpaca, showed intense diffuse labeling along the epithelial
864 lining of the teat cistern, gland cistern, interlobular duct, and secretory alveoli for
865 SNA. Apical, multifocal, intense labeling was seen in the teat cistern for alpacas (Ei),
866 and labeling for MAL II was diffuse, intense, and apical for MAL II on the gland
867 cistern, interlobular ducts, and secretory alveoli (Fi, Gi, Hi). In Aii – Hii, images of
868 pigs showed diffuse, apical labeling of moderate to marked intensity for SNA and
869 MAL II in all anatomic regions examined. In humans, Ciii, Diii, Giii, and Hiii, a
870 multifocal to diffuse apical epithelial labeling for both SNA and MAL II was
871 observed in sections of the interlobular duct and secretory alveoli. Teat and gland
872 cistern are uncommon in the human breast, hence no images, Aiii, Biii, Eiii and Fiii.
873 Arrows indicating the labeling on the epithelial lining. Scale bar =50 µm.

874

875 **Figure 5: Distribution of sialic acids in the mammary gland of ruminant and non-**
876 **ruminant species, MAL I specific images using IFA.**

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877 The mammary glands of dairy cattle, beef cattle, nonlactating cattle, sheep, goats,
878 alpacas, pigs, and humans showing *Maackia amurensis lectin-I* (MAL-I, green
879 pseudocolor, Alexa Fluor 594), using fluorescent labeling.

880 Micrographs Ai-Cv, showing apical, multifocal MAL I labeling along the epithelial
881 lining of the teat and gland cistern, as well as the interlobular duct of ruminants. The
882 labeling in nonlactating cattle teat and gland cistern, Aiii, and Biii, was relatively low
883 than in other species, including the interlobular duct (Ciii). In the secretory alveoli of
884 all ruminant species, Di - Dv, there is minimal to no apical labeling for MAL I on the
885 epithelial surface. Ei-Gii, there is apical, multifocal MAL I labeling in the teat cistern,
886 gland cistern, and interlobular ducts of alpacas and pigs. In the secretory alveoli of
887 alpacas and pigs, Hi and Hii, there is apical, multifocal, minimal labeling for MAL I.
888 There is apical, diffuse labeling for MAL I in human secretory alveoli (Hiii). Arrows
889 indicating the labeling on the epithelial lining. Scale bar =50 µm.

890

891 **Figure 6: Distribution of sialic acids in the mammary gland of ruminant and non-**
892 **ruminant species, MAL I specific images using chromogenic staining.**

893 The mammary glands of lactating dairy cattle, beef cattle, non-lactating cattle, sheep,
894 and goats with *Maackia amurensis lectin-I* (MAL-I, brown chromogen), using lectin
895 histochemistry. There is similar multifocal, apical labeling for MAL I, with no
896 significant apical labeling observed in Aiii, Bi, Biii, Ci, and Ciii. Similarly, to the IFA
897 technique, there is minimal labeling observed in secretory alveoli, Di-Dv. The most
898 intense apical labeling was observed in the alpaca mammary gland, with strong apical
899 labeling in the teat cistern, moderate labeling in the gland cistern, scant labeling in the
900 interlobular duct, and moderate labeling within the secretory alveoli (Ei, Fi, Gi, and
901 Hi). Faint apical labeling was observed in the teat cistern of the pig (Eii). No

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902 significant labeling was detected in the gland cistern, interlobular duct, or secretory
903 alveoli (Fii, Gii, Hii). Strong apical labeling was present within the interlobular duct
904 of human breast tissue (Giii). Image panel from one representative animal. Scale bar
905 =200 µm.

906

907 **Figure 7. Distribution of LPAIV H5N1 2.3.4.4b rgA/bald eagle/FL/22 2.3.4.4b labeling by**
908 **virus histochemistry (red) in the mammary gland of lactating Holstein cows and cross-**
909 **bred sows.**

910 Interlobular ducts in the same tissue section of a Holstein dairy cow with minimal labeling (A;
911 arrow) and intense locally extensive labeling (B; brace). Cells with minimal to no labeling were
912 commonly attenuated (A; arrowhead) or not organized as a stratified cuboidal epithelial layer
913 (B; arrowhead). C) Nearly circumferential intense labeling of two secretory alveoli (circled)
914 adjacent to secretory alveoli with no to minimal labeling of the apical epithelial surface in a
915 Holstein dairy cow (asterisk;). D) More consistent labeling of the apical surface of one or more
916 epithelial cells (arrows) and luminal secretions of adjacent alveoli in a Holstein dairy cow. E)
917 Interlobular duct of a crossbred sow with apical labeling of a single epithelial cell (arrow) or row
918 of epithelial cells (brace). F) Consistent labeling of the apical surface of one or more epithelial
919 cells (arrows) and luminal secretions of adjacent alveoli in a crossbred sow. Scale bar = 20 µm
920 (A, B, and E); 50 µm (C, D, and F).

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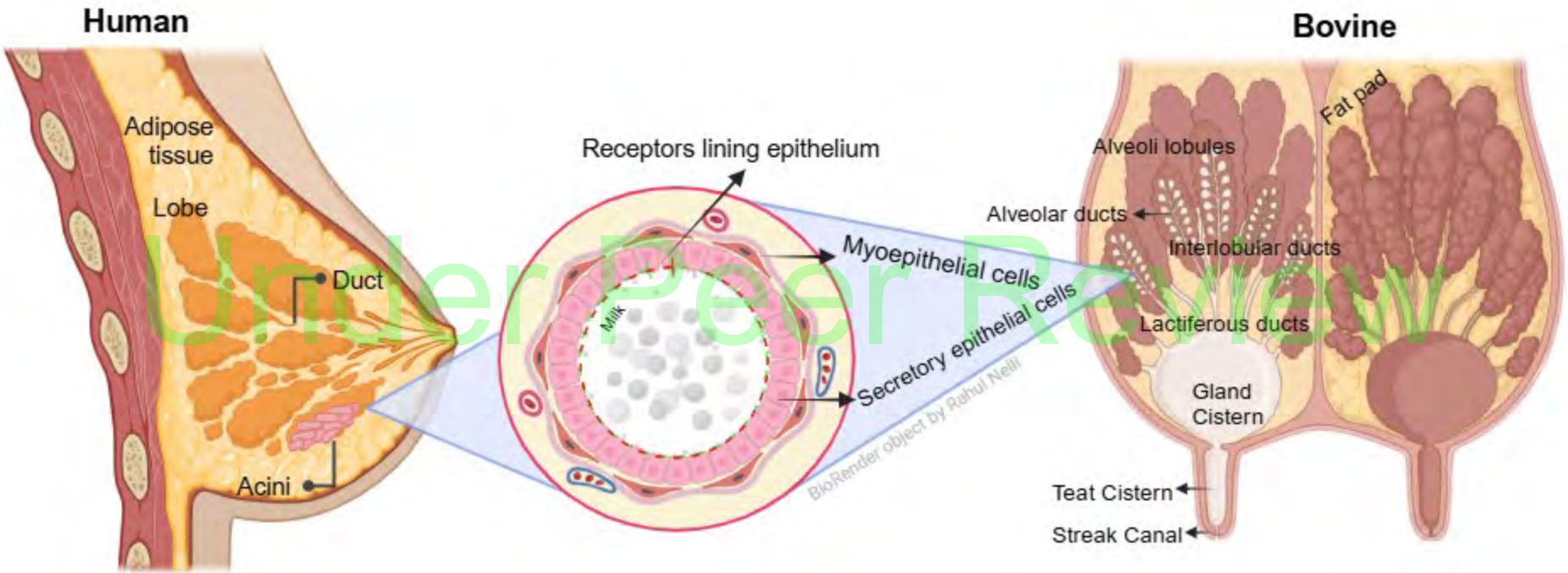

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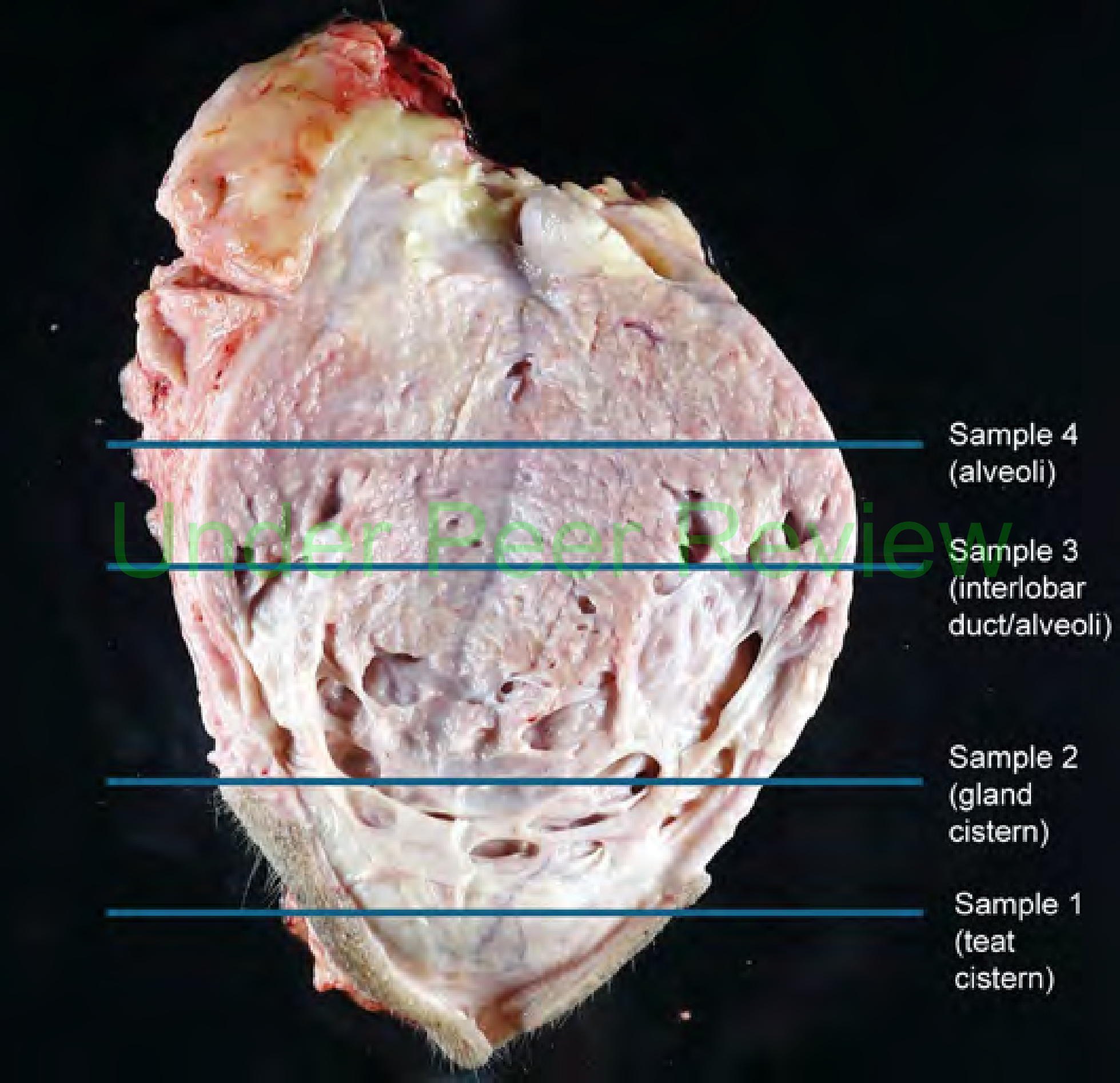

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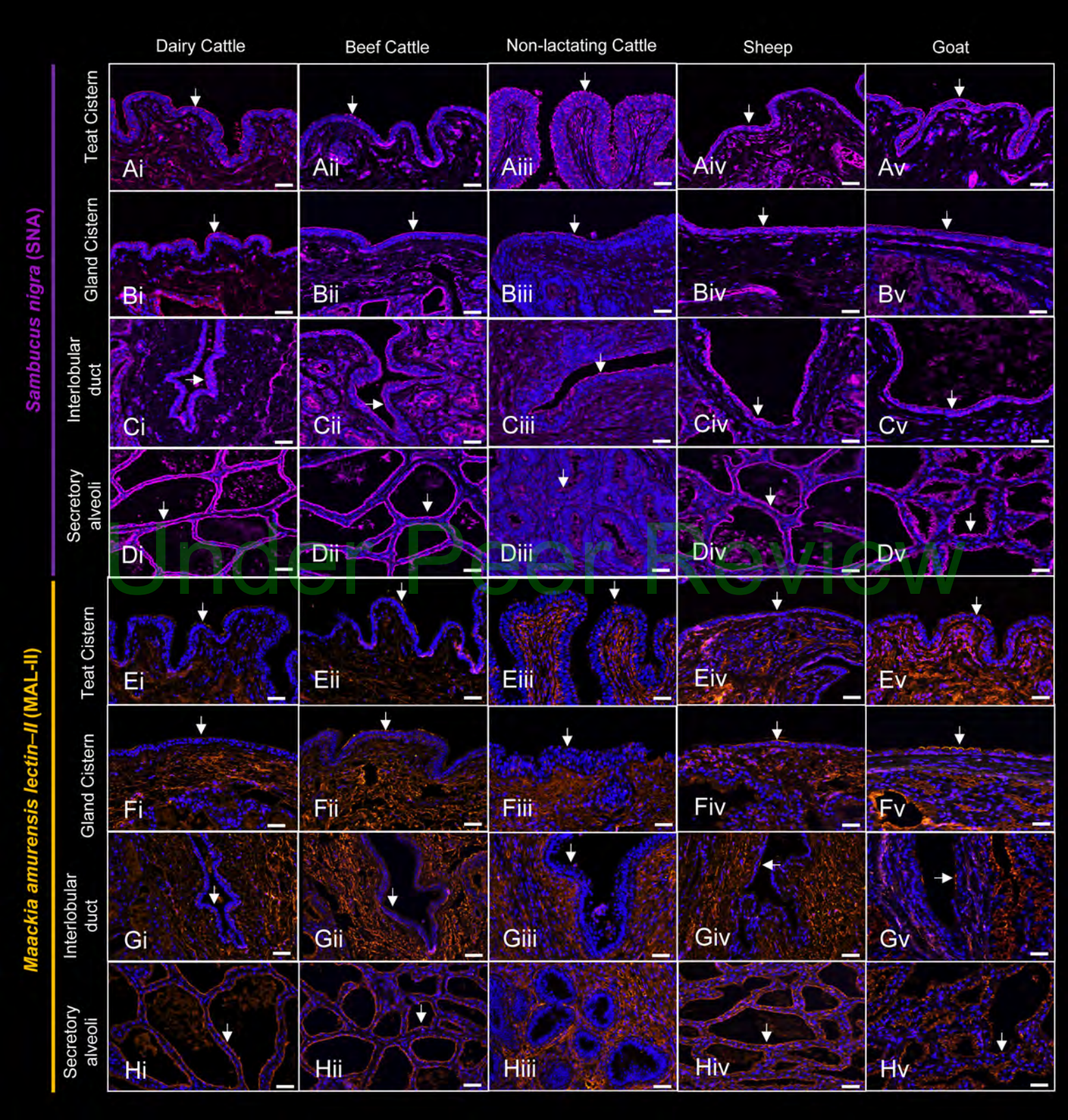

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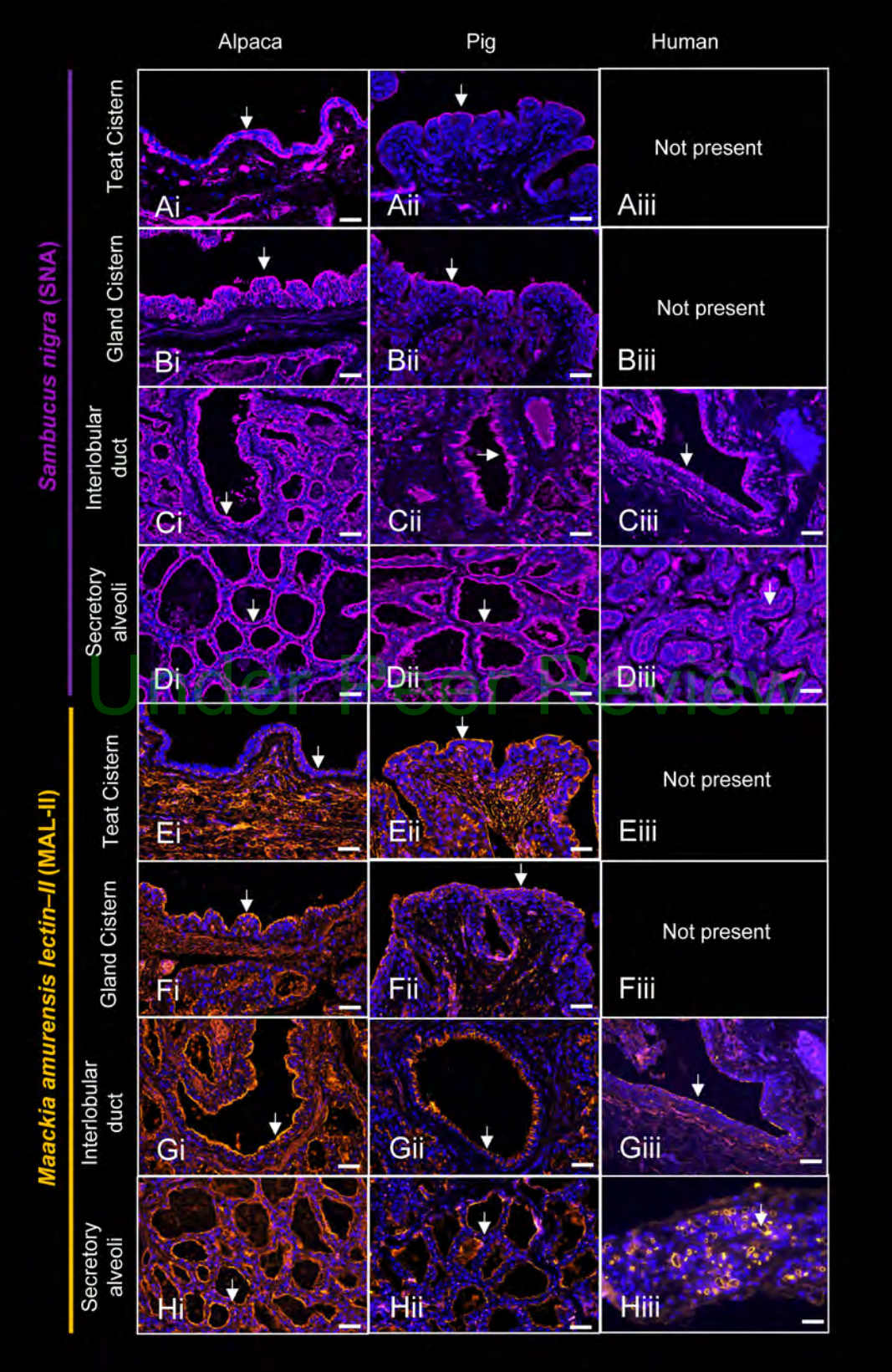

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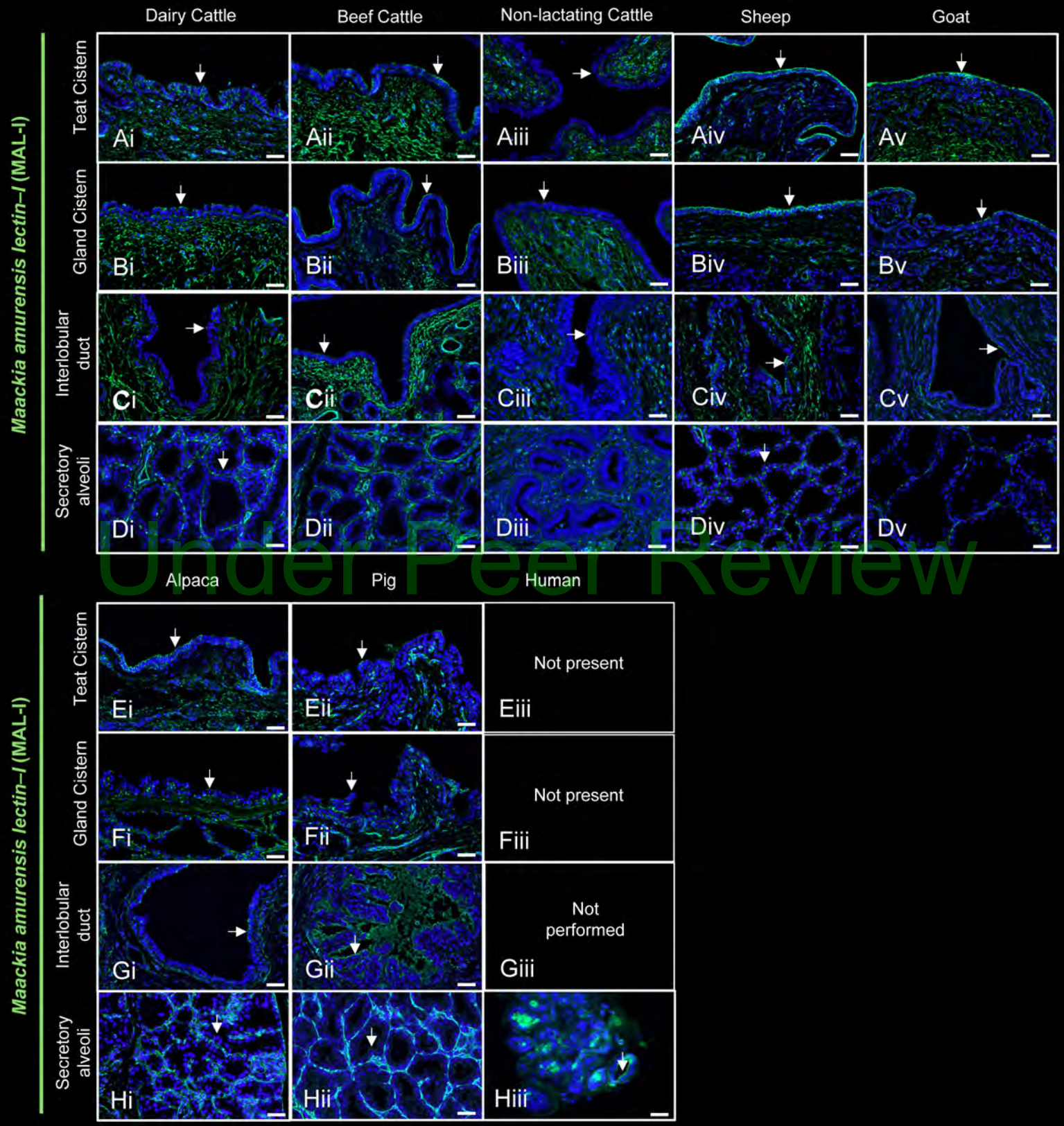

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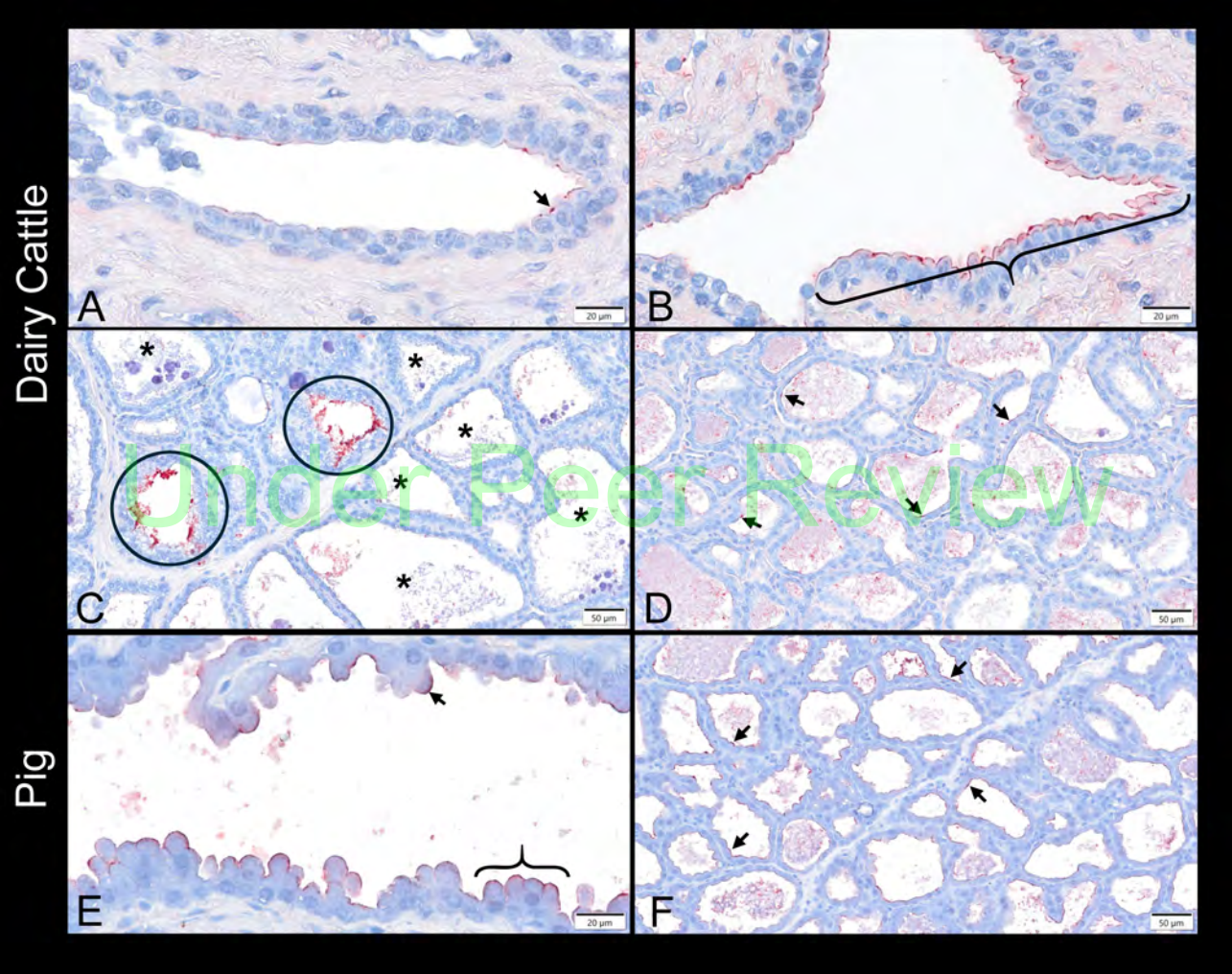

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